British Pharmacopoeia 2022

Volume V

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The monographs of the Tenth Edition of the European Pharmacopoeia (2019), as amended by Supplements 10.1 to 10.5, published by the Council of Europe are reproduced either in this edition of the British Pharmacopoeia or in the associated edition of the British Pharmacopoeia (Veterinary).

See General Notices

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Monographs of the European Pharmacopoeia are distinguished by a chaplet of stars against the title. The term European Pharmacopoeia, used without qualification, means the Tenth Edition of the European Pharmacopoeia comprising, unless otherwise stated, the main volume, published in 2019, as amended by any subsequent supplements and revisions.

Patents In this Pharmacopoeia certain drugs and preparations have been included notwithstanding the existence of actual or potential patent rights. In so far as such substances are protected by Letters Patent their inclusion in this Pharmacopoeia neither conveys, nor implies, licence to manufacture.

Effective dates New and revised monographs of national origin enter into force on 1 January 2022. The monographs are brought into effect under regulation 320(2) of the Human Medicines Regulations 2012.

Monographs of the European Pharmacopoeia have previously been published by the European Directorate for the Quality of Medicines & HealthCare, in accordance with the Convention on the Elaboration of a European Pharmacopoeia, and have been brought into effect under the Human Medicines Regulations 2012, as amended, and the Veterinary Medicines Regulations 2013, as amended.

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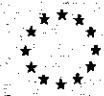
Part I

The British Pharmacopoeia comprises the entire text within this publication. The word 'official' is used in the Pharmacopoeia to signify 'of the Pharmacopoeia'. It applies to any title, substance, preparation, method or statement included in the general notices, monographs and appendices of the Pharmacopoeia. The abbreviation for British Pharmacopoeia is BP.

European Pharmacopoeia

Monographs of the European Pharmacopoeia are reproduced in this edition of the British Pharmacopoeia by incorporation of the text published under the direction of the Council of Europe (Partial Agreement) in accordance with the Convention on the Elaboration of a European Pharmacopoeia (Treaty Series No. 32 (1974) CMND 5763) as amended by the Protocol to the Convention (Treaty Series No. MISC16 (1990) CMND 1133). They are included for the convenience of users of the British Pharmacopoeia. In cases of doubt or dispute reference should be made to the Council of Europe text.



Monographs of the European Pharmacopoeia are distinguished by a chaplet of stars against the title and by reference to the European Pharmacopoeia monograph number included immediately below the title in italics. The beginning and end of text from the European Pharmacopoeia are denoted by means of horizontal lines with the symbol '*Ph Eur*' ranged left and right, respectively.

The general provisions of the European Pharmacopoeia relating to different types of dosage form are included in the appropriate general monograph in that section of the British Pharmacopoeia entitled Monographs: Formulated Preparations. These general provisions apply to all dosage forms of the type defined, whether or not an individual monograph is included in the British Pharmacopoeia. In addition, the provisions of the European Pharmacopoeia General Monograph for Pharmaceutical Preparations apply to all dosage forms, whether or not an individual monograph is included in the British Pharmacopoeia.

Texts of the European Pharmacopoeia are governed by the General Notices of the European Pharmacopoeia. These are reproduced as Part III of these notices.

Part II

The following general notices apply to the statements made in the monographs of the British Pharmacopoeia other than those reproduced from the European Pharmacopoeia and to the statements made in the Appendices of the British Pharmacopoeia other than when a method, test or other matter described in an appendix is invoked in a monograph reproduced from the European Pharmacopoeia.

Official Standards The requirements stated in the monographs of the Pharmacopoeia apply to articles that are intended for medicinal use but not necessarily to articles that may be sold under the same name for other purposes. An article intended for medicinal use that is described by means of an official title must comply with the requirements of the relevant monograph. A formulated preparation must comply throughout its assigned shelf-life (period of validity). The subject of any other monograph must comply throughout its period of use.

A monograph is to be construed in accordance with any general monograph or notice or any appendix, note or other explanatory material that is contained in this edition and that is applicable to that monograph. All statements contained in the monographs, except where a specific general notice indicates otherwise and with the exceptions given below, constitute standards for the official articles. An article is not of pharmacopoeial quality unless it complies with all of the requirements stated. This does not imply that a manufacturer is obliged to perform all the tests in a monograph in order to assess compliance with the Pharmacopoeia before release of a product. The manufacturer may assure himself that a product is of pharmacopoeial quality by other means, for example, from data derived from validation studies of the manufacturing process, from in-process controls or from a combination of the two. Parametric release in appropriate circumstances is thus not precluded by the need to comply with the Pharmacopoeia. The general notice on Assays and Tests indicates that analytical methods other than those described in the Pharmacopoeia may be employed for routine purposes.

Requirements in monographs have been framed to provide appropriate limitation of potential impurities rather than to provide against all possible impurities. Material found to contain an impurity not detectable by means of the prescribed tests is not of pharmacopoeial quality if the nature or amount of the impurity found is incompatible with good pharmaceutical practice.

The status of any statement given under the headings Definition, Production, Characteristics, Storage, Labelling or Action and use is defined within the general notice relating to the relevant heading. In addition to any exceptions indicated by one of the general notices referred to above, the following parts of a monograph do not constitute standards: (a) a graphic or molecular formula given at the beginning of a monograph; (b) a molecular weight; (c) a Chemical Abstracts Service Registry Number; (d) any information given at the end of a monograph concerning impurities known to be limited by that monograph; (e) information in any annex to a

monograph. Any statement containing the word 'should' constitutes non-mandatory advice or recommendation.

The expression 'unless otherwise justified and authorised' means that the requirement in question has to be met, unless a competent authority authorises a modification or exemption where justified in a particular case. The term 'competent authority' means the national, supranational or international body or organisation vested with the authority for making decisions concerning the issue in question. It may, for example, be a licensing authority or an official control laboratory. For a formulated preparation that is the subject of monograph in the British Pharmacopoeia any justified and authorised modification to, or exemption from, the requirements of the relevant general monograph of the European Pharmacopoeia is stated in the individual monograph. For example, the general monograph for Tablets requires that Uncoated Tablets, except for chewable tablets, disintegrate within 15 minutes; for Calcium Lactate Tablets a time of 30 minutes is permitted.

Many of the general monographs for formulated preparations include statements and requirements additional to those of the European Pharmacopoeia that are applicable to the individual monographs of the British Pharmacopoeia. Such statements and requirements apply to all monographs for that dosage form included in the Pharmacopoeia unless otherwise indicated in the individual monograph.

Where a monograph on a biological substance or preparation refers to a strain, a test, a method, a substance, etc., using the qualifications 'suitable' or 'appropriate' without further definition in the text, the choice of such strain, test, method, substance, etc., is made in accordance with any international agreements or national regulations affecting the subject concerned.

Definition of Terms

Where the term 'about' is included in a monograph or test it should be taken to mean approximately (fairly correct or accurate; near to the actual value).

Where the term 'corresponds' is included in a monograph or test it should be taken to mean similar or equivalent in character or quantity.

Where the term 'similar' is included in a monograph or test it should be taken to mean alike though not necessarily identical.

Further qualifiers (such as numerical acceptance criteria) for the above terms are not included in the BP. The acceptance criteria for any individual case is set based on the range of results obtained from known reference samples, the level of precision of the equipment or apparatus used and the level of accuracy required for the particular application. The user should determine the variability seen in his/her own laboratory and set in-house acceptance criteria that he/she judges to be appropriate based on the local operating conditions.

Expression of Standards

Where the standard for the content of a substance described in a monograph is expressed in terms of the chemical formula for that substance an upper limit exceeding 100% may be stated. Such an upper limit applies to the result of the assay calculated in terms of the equivalent content of the specified chemical formula. For example, the statement 'contains not less than 99.0% and not more than 101.0% of $C_{20}H_{24}N_2O_2$,HCl' implies that the result of the assay is not less than 99.0% and not more than 101.0%, calculated in terms of the equivalent content of $C_{20}H_{24}N_2O_2$,HCl.

Where the result of an assay or test is required to be calculated with reference to the dried, anhydrous or ignited substance, the substance free from a specified solvent or to the peptide content, the determination of loss on drying, water content, loss on ignition, content of the specified solvent or peptide content is carried out by the method prescribed in the relevant test in the monograph.

Temperature The Celsius thermometric scale is used in expressing temperatures.

Weights and Measures The metric system of weights and measures is employed; SI Units have generally been adopted. Metric measures are required to have been graduated at 20° and all measurements involved in the analytical operations of the Pharmacopoeia are intended, unless otherwise stated, to be made at that temperature. Graduated glass apparatus used in analytical operations should comply with Class A requirements of the appropriate International Standard issued by the International Organization for Standardization. The abbreviation for litre is 'L' throughout the Pharmacopoeia.

Atomic Weights The atomic weights adopted are the values given in the Table of Relative Atomic Weights 2001 published by the International Union of Pure and Applied Chemistry (Appendix XXV).

Constant Weight The term 'constant weight', used in relation to the process of drying or the process of ignition, means that two consecutive weighings do not differ by more than 0.5 mg, the second weighing being made after an additional period of drying or ignition under the specified conditions appropriate to the nature and quantity of the residue (1 hour is usually suitable).

Expression of Concentrations The term 'per cent' or more usually the symbol '%' is used with one of four different meanings in the expression of concentrations according to circumstances. In order that the meaning to be attached to the expression in each instance is clear, the following notation is used:

Per cent w/w (% w/w) (percentage weight in weight) expresses the number of grams of solute in 100 g of product.

Per cent w/v (% w/v) (percentage weight in volume) expresses the number of grams of solute in 100 mL of product.

Per cent v/v (% v/v) (percentage volume in volume) expresses the number of millilitres of solute in 100 mL of product.

Per cent v/w (% v/w) (percentage volume in weight) expresses the number of millilitres of solute in 100 g of product.

Usually the strength of solutions of solids in liquids is expressed as percentage weight in volume, of liquids in liquids as percentage volume in volume and of gases in liquids as percentage weight in weight.

When the concentration of a solution is expressed as parts per million (ppm), it means weight in weight, unless otherwise specified.

When the concentration of a solution is expressed as parts of dissolved substance in parts of the solution, it means parts by weight (g) of a solid in parts by volume (mL) of the final solution; or parts by volume (mL) of a liquid in parts by volume (mL) of the final solution; or parts by weight (g) of a gas in parts by weight (g) of the final solution.

When the concentration of a solution is expressed in molarity designated by the symbol M preceded by a number, it denotes the number of moles of

the stated solute contained in sufficient Purified Water (unless otherwise stated) to produce 1 litre of solution.

Water Bath The term 'water bath' means a bath of boiling water, unless water at some other temperature is indicated in the text. An alternative form of heating may be employed providing that the required temperature is approximately maintained but not exceeded.

Reagents The reagents required for the assays and tests of the Pharmacopoeia are defined in appendices. The descriptions set out in the appendices do not imply that the materials are suitable for use in medicine.

Indicators Indicators, the colours of which change over approximately the same range of pH, may be substituted for one another but in the event of doubt or dispute as to the equivalence of indicators for a particular purpose, the indicator specified in the text is alone authoritative.

The quantity of an indicator solution appropriate for use in acid-base titrations described in assays or tests is 0.1 mL unless otherwise stated in the text.

Any solvent required in an assay or test in which an indicator is specified is previously neutralised to the indicator, unless a blank test is prescribed.

Caution Statements A number of materials described in the monographs and some of the reagents specified for use in the assays and tests of the Pharmacopoeia may be injurious to health unless adequate precautions are taken. The principles of good laboratory practice and the provisions of any appropriate regulations such as those issued in the United Kingdom in accordance with the Health and Safety at Work *etc.* Act 1974 should be observed at all times in carrying out the assays and tests of the Pharmacopoeia.

Attention is drawn to particular hazards in certain monographs by means of an italicised statement; the absence of such a statement should not however be taken to mean that no hazard exists.

Titles Subsidiary titles, where included, have the same significance as the main titles. An abbreviated title constructed in accordance with the directions given in Appendix XXI A has the same significance as the main title.

Titles that are derived by the suitable inversion of words of a main or subsidiary title, with the addition of a preposition if appropriate, are also official titles. Thus, the following are all official titles: Aspirin Tablets, Tablets of Aspirin; Atropine Injection, Injection of Atropine.

A title of a formulated preparation that includes the full nonproprietary name of the active ingredient or ingredients, where this is not included in the title of the monograph, is also an official title. For example, the title Promethazine Hydrochloride Oral Solution has the same significance as Promethazine Oral Solution and the title Brompheniramine Maleate Tablets has the same significance as Brompheniramine Tablets.

Where the English title at the head of a monograph in the European Pharmacopoeia is different from that at the head of the text incorporated into the British Pharmacopoeia, an Approved Synonym has been created on the recommendation of the British Pharmacopoeia Commission. Approved Synonyms have the same significance as the main title and are thus official titles. A cumulative list of such Approved Synonyms is provided in Appendix XXI B.

Where the names of pharmacopoeial substances, preparations and other materials occur in the text they are printed with capital initial letters and this indicates that materials of Pharmacopoeial quality must be used. Words in the text that name a reagent or other material, a physical characteristic or a process that is described or defined in an appendix are printed in italic type, for example, *methanol*, *absorbance*, *gas chromatography*, and these imply compliance with the requirements specified in the appropriate appendix.

Chemical Formulae When the chemical composition of an official substance is known or generally accepted, the graphic and molecular formulae, the molecular weight and the Chemical Abstracts Service Registry Number are normally given at the beginning of the monograph for information. This information refers to the chemically pure substance and is not to be regarded as an indication of the purity of the official material. Elsewhere, in statements of standards of purity and strength and in descriptions of processes of assay, it is evident from the context that the formulae denote the chemically pure substances.

Where the absolute stereochemical configuration is specified, the International Union of Pure and Applied Chemistry (IUPAC) *R/S* and *E/Z* systems of designation have been used. If the substance is an enantiomer of unknown absolute stereochemistry the sign of the optical rotation, as determined in the solvent and under the conditions specified in the monograph, has been attached to the systematic name. An indication of sign of rotation has also been given where this is incorporated in a trivial name that appears on an IUPAC preferred list.

All amino acids, except glycine, have the L-configuration unless otherwise indicated. The three-letter and one-letter symbols used for amino acids in peptide and protein sequences are those recommended by the Joint Commission on Biochemical Nomenclature of the International Union of Pure and Applied Chemistry and the International Union of Biochemistry and Molecular Biology.

In the graphic formulae the following abbreviations are used:

Me	$-CH_3$	Bu^s	$-CH(CH_3)CH_2CH_3$
Et	$-CH_2CH_3$	Bu^n	$-CH_2CH_2CH_2CH_3$
Pr^i	$-CH(CH_3)_2$	Bu^t	$-C(CH_3)_3$
Pr^n	$-CH_2CH_2CH_3$	Ph	$-C_6H_5$
Bu^i	$-CH_2CH(CH_3)_2$	Ac	$-COCH_3$

Definition Statements given under the heading Definition constitute an official definition of the substance, preparation or other article that is the subject of the monograph. They constitute instructions or requirements and are mandatory in nature.

Certain medicinal or pharmaceutical substances and other articles are defined by reference to a particular method of manufacture. A statement that a substance or article *is* prepared or obtained by a certain method constitutes part of the official definition and implies that other methods are not permitted. A statement that a substance *may be* prepared or obtained by a certain method, however, indicates that this is one possible method and does not imply that other methods are proscribed.

Additional statements concerning the definition of formulated preparations are given in the general notice on Manufacture of Formulated Preparations.

Production Statements given under the heading Production draw attention to particular aspects of the manufacturing process but are not necessarily comprehensive. They constitute mandatory instructions to manufacturers. They may relate, for example, to source materials, to the manufacturing process itself and its validation and control, to in-process testing or to testing that is to be carried out by the manufacturer on the final product (bulk material or dosage form) either on selected batches or on each batch prior to release. These statements cannot necessarily be verified on a sample of the final product by an independent analyst. The competent authority may establish that the instructions have been followed, for example, by examination of data received from the manufacturer, by inspection or by testing appropriate samples.

The absence of a section on Production does not imply that attention to features such as those referred to above is not required. A substance, preparation or article described in a monograph of the Pharmacopoeia is to be manufactured in accordance with the principles of good manufacturing practice and in accordance with relevant international agreements and supranational and national regulations governing medicinal products.

Where in the section under the heading Production a monograph on a vaccine defines the characteristics of the vaccine strain to be used, any test methods given for confirming these characteristics are provided as examples of suitable methods. The use of these methods is not mandatory.

Additional statements concerning the production of formulated preparations are given in the general notice on Manufacture of Formulated Preparations.

Manufacture of Formulated Preparations Attention is drawn to the need to observe adequate hygienic precautions in the preparation and dispensing of pharmaceutical formulations. The principles of good pharmaceutical manufacturing practice should be observed.

The Definition in certain monographs for pharmaceutical preparations is given in terms of the principal ingredients only. Any ingredient, other than those included in the Definition, must comply with the general notice on Excipients and the product must conform with the Pharmacopoeial requirements.

The Definition in other monographs for pharmaceutical preparations is presented as a full formula. No deviation from the stated formula is permitted except those allowed by the general notices on Colouring Agents and Antimicrobial Preservatives. Where additionally directions are given under the heading Extemporaneous Preparation these are intended for the extemporaneous preparation of relatively small quantities for short-term supply and use. When so prepared, no deviation from the stated directions is permitted. If, however, such a pharmaceutical preparation is manufactured on a larger scale with the intention that it may be stored, deviations from the stated directions are permitted provided that the final product meets the following criteria:

(1) compliance with all of the requirements stated in the monograph;

(2) retention of the essential characteristics of the preparation made strictly in accordance with the directions of the Pharmacopoeia.

Monographs for yet other pharmaceutical preparations include both a Definition in terms of the principal ingredients and, under the side-heading Extemporaneous Preparation, a full formula together with, in some cases, directions for their preparation. Such full formulae and directions are intended for the extemporaneous preparation of relatively small quantities for short-term supply and use. When so prepared, no deviation from the stated formula and directions is permitted. If, however, such a pharmaceutical preparation is manufactured on a larger scale with the intention that it may be stored, deviations from the formula and directions stated under the heading Extemporaneous Preparation are permitted provided that any ingredient, other than those included in the Definition, complies with the general notice on Excipients and that the final product meets the following criteria:

(1) accordance with the Definition stated in the monograph;

(2) compliance with all of the requirements stated in the monograph;

(3) retention of the essential characteristics of the preparation made strictly in accordance with the formula and directions of the Pharmacopoeia.

In the manufacture of any official preparation on a large scale with the intention that it should be stored, in addition to following any instruction under the heading Production, it is necessary to ascertain that the product is satisfactory with respect to its physical and chemical stability and its state of preservation over the claimed shelf-life. This applies irrespective of whether the formula of the Pharmacopoeia and any instructions given under the heading Extemporaneous Preparation are followed precisely or modified. Provided that the preparation has been shown to be stable in other respects, deterioration due to microbial contamination may be inhibited by the incorporation of a suitable antimicrobial preservative. In such circumstances the label states appropriate storage conditions, the date after which the product should not be used and the identity and concentration of the antimicrobial preservative.

Freshly and Recently Prepared The direction, given under the heading Extemporaneous Preparation, that a preparation must be freshly prepared indicates that it must be made not more than 24 hours before it is issued for use. The direction that a preparation should be recently prepared indicates that deterioration is likely if the preparation is stored for longer than about 4 weeks at 15° to 25°.

Methods of Sterilisation The methods of sterilisation used in preparing the sterile materials described in the Pharmacopoeia are given in Appendix XVIII. For aqueous preparations, steam sterilisation (heating in an autoclave) is the method of choice wherever it is known to be suitable. Any method of sterilisation must be validated with respect to both the assurance of sterility and the integrity of the product and to ensure that the final product complies with the requirements of the monograph.

Water The term water used without qualification in formulae for formulated preparations means either potable water freshly drawn direct from the public supply and suitable for drinking or freshly boiled and cooled Purified Water. The latter should be used if the public supply is from a local storage tank or if the potable water is unsuitable for a particular preparation.

Excipients Where an excipient for which there is a pharmacopoeial monograph is used in preparing an official preparation it shall comply with that monograph. Any substance added in preparing an official preparation shall be innocuous, shall have no adverse influence on the therapeutic efficacy of the active ingredients and shall not interfere with the assays and tests of the Pharmacopoeia. Particular care should be taken to ensure that such substances are free from harmful organisms.

Colouring Agents If in a monograph for a formulated preparation defined by means of a full formula a specific colouring agent or agents is prescribed, suitable alternatives approved in the country concerned may be substituted.

Antimicrobial Preservatives When the term 'suitable antimicrobial preservative' is used it is implied that the preparation concerned will be effectively preserved according to the appropriate criteria applied and interpreted as described in the test for *efficacy of antimicrobial preservation* (Appendix XVI C). In certain monographs for formulated preparations defined by means of a full formula, a specific antimicrobial agent or agents may be prescribed; suitable alternatives may be substituted provided that their identity and concentration are stated on the label.

Characteristics Statements given under the heading Characteristics are not to be interpreted in a strict sense and are not to be regarded as official requirements. Statements on taste are provided only in cases where this property is a guide to the acceptability of the material (for example, a material used primarily for flavouring). The status of statements on solubility is given in the general notice on Solubility.

Solubility Statements on solubility given under the heading Characteristics are intended as information on the approximate solubility at a temperature between 15° and 25°, unless otherwise stated, and are not to be considered as official requirements.

Statements given under headings such as Solubility in ethanol express exact requirements and constitute part of the standards for the substances under which they occur.

The following table indicates the meanings of the terms used in statements of approximate solubilities.

Descriptive term	Approximate volume of solvent in millilitres per gram of solute
very soluble	less than 1
freely soluble	from 1 to 10
soluble	from 10 to 30
sparingly soluble	from 30 to 100
slightly soluble	from 100 to 1000
very slightly soluble	from 1000 to 10 000
practically insoluble	more than 10 000

The term 'partly soluble' is used to describe a mixture of which only some of the components dissolve.

Identification The tests described or referred to under the heading Identification are not necessarily sufficient to establish absolute proof of identity. They provide a means of verifying that the identity of the material being examined is in accordance with the label on the container.

Unless otherwise prescribed, identification tests are carried out at a temperature between 15° and 25°.

Reference spectra Where a monograph refers to an infrared reference spectrum, this spectrum is provided in a separate section of the Pharmacopoeia. A sample spectrum is considered to be concordant with a reference spectrum if the transmission minima (absorption maxima) of the principal bands in the sample correspond in position, relative intensities and shape to those of the reference. Instrumentation software may be used to calculate concordance with a previously recorded reference spectrum.

When tests for infrared absorption are applied to material extracted from formulated preparations, strict concordance with the specified reference spectrum may not always be possible, but nevertheless a close resemblance between the spectrum of the extracted material and the specified reference spectrum should be achieved.

Assays and Tests The assays and tests described are the official methods upon which the standards of the Pharmacopoeia depend. The analyst is not precluded from employing alternative methods, including methods of micro-analysis, in any assay or test if it is known that the method used will give a result of equivalent accuracy. Local reference materials may be used for routine analysis, provided that these are calibrated against the official reference materials. In the event of doubt or dispute, the methods of analysis, the reference materials and the reference spectra of the Pharmacopoeia are alone authoritative.

Where the solvent used for a solution is not named, the solvent is Purified Water.

Unless otherwise prescribed, the assays and tests are carried out at a temperature between 15° and 25°.

A temperature in a test for Loss on drying, where no temperature range is given, implies a range of ±2° about the stated value.

Visual comparative tests, unless otherwise prescribed, are carried out using identical tubes of colourless, transparent, neutral glass with a flat base. The volumes of liquid prescribed are for use with tubes 16 mm in internal diameter; tubes with a larger internal diameter may be used but the volume of liquid examined must be increased so that the depth of liquid in the tubes is not less than that obtained when the prescribed volume of liquid and tubes 16 mm in internal diameter are used. Equal volumes of the liquids to be compared are examined down the vertical axis of the tubes against a white background or, if necessary, against a black background. The examination is carried out in diffuse light.

Where a direction is given that an analytical operation is to be carried out 'in subdued light', precautions should be taken to avoid exposure to direct sunlight or other strong light. Where a direction is given that an analytical operation is to be carried out 'protected from light', precautions should be taken to exclude actinic light by the use of low-actinic glassware, working in a dark room or similar procedures.

For preparations other than those of fixed strength, the quantity to be taken for an assay or test is usually expressed in terms of the active ingredient. This means that the quantity of the active ingredient expected to

be present and the quantity of the preparation to be taken are calculated from the strength stated on the label.

In assays the approximate quantity to be taken for examination is indicated but the quantity actually used must not deviate by more than 10% from that stated. The quantity taken is accurately weighed or measured and the result of the assay is calculated from this exact quantity. Reagents are measured and the procedures are carried out with an accuracy commensurate with the degree of precision implied by the standard stated for the assay.

In tests the stated quantity to be taken for examination must be used unless any divergence can be taken into account in conducting the test and calculating the result. The quantity taken is accurately weighed or measured with the degree of precision implied by the standard or, where the standard is not stated numerically (for example, in tests for Clarity and colour of solution), with the degree of precision implied by the number of significant figures stated. Reagents are measured and the procedures are carried out with an accuracy commensurate with this degree of precision.

The limits stated in monographs are based on data obtained in normal analytical practice; they take account of normal analytical errors, of acceptable variations in manufacture and of deterioration to an extent considered acceptable. No further tolerances are to be applied to the limits prescribed to determine whether the article being examined complies with the requirements of the monograph.

In determining compliance with a numerical limit, the calculated result of a test or assay is first rounded to the number of significant figures stated, unless otherwise prescribed. The last figure is increased by 1 when the part rejected is equal to or exceeds one half-unit, whereas it is not modified when the part rejected is less than a half-unit.

In certain tests, the concentration of impurity is given in parentheses either as a percentage or in parts per million by weight (ppm). In chromatographic tests such concentrations are stated as a percentage irrespective of the limit. In other tests they are usually stated in ppm unless the limit exceeds 500 ppm. In those chromatographic tests in which a secondary spot or peak in a chromatogram obtained with a solution of the substance being examined is described as corresponding to a named impurity and is compared with a spot or peak in a chromatogram obtained with a reference solution of the same impurity, the percentage given in parentheses indicates the limit for that impurity. In those chromatographic tests in which a spot or peak in a chromatogram obtained with a solution of the substance being examined is described in terms other than as corresponding to a named impurity (commonly, for example, as any (other) *secondary spot* or *peak*) but is compared with a spot or peak in a chromatogram obtained with a reference solution of a named impurity, the percentage given in parentheses indicates an impurity limit expressed in terms of a nominal concentration of the named impurity. In chromatographic tests in which a comparison is made between spots or peaks in chromatograms obtained with solutions of different concentrations of the substance being examined, the percentage given in parentheses indicates an impurity limit expressed in terms of a nominal concentration of the medicinal substance itself. In some monographs, in particular those for certain formulated preparations, the impurity limit is expressed in terms of a nominal concentration of the active moiety rather than of the medicinal

substance itself. Where necessary for clarification the terms in which the limit is expressed are stated within the monograph.

In all cases where an impurity limit is given in parentheses, the figures given are approximations for information only; conformity with the requirements is determined on the basis of compliance or otherwise with the stated test.

The use of a proprietary designation to identify a material used in an assay or test does not imply that another equally suitable material may not be used.

Biological Assays and Tests

Methods of assay described as Suggested methods are not obligatory, but when another method is used its precision must be not less than that required for the Suggested method.

For those antibiotics for which the monograph specifies a microbiological assay the potency requirement is expressed in the monograph in International Units (IU) per milligram. The material is not of pharmacopoeial quality if the upper fiducial limit of error is less than the stated potency. For such antibiotics the required precision of the assay is stated in the monograph in terms of the fiducial limits of error about the estimated potency.

For other substances and preparations for which the monograph specifies a biological assay, unless otherwise stated, the precision of the assay is such that the fiducial limits of error, expressed as a percentage of the estimated potency, are within a range not wider than that obtained by multiplying by a factor of 10 the square roots of the limits given in the monograph for the fiducial limits of error about the stated potency.

In all cases fiducial limits of error are based on a probability of 95% ($P = 0.95$).

Where the biological assay is being used to ascertain the purity of the material, the stated potency means the potency stated on the label in terms of International Units (IU) or other Units per gram, per milligram or per millilitre. When no such statement appears on the label, the stated potency means the fixed or minimum potency required in the monograph. This interpretation of stated potency applies in all cases except where the monograph specifically directs otherwise.

Where the biological assay is being used to determine the total activity in the container, the stated potency means the total number of International Units (IU) or other Units stated on the label or, if no such statement appears, the total activity calculated in accordance with the instructions in the monograph.

Wherever possible the primary standard used in an assay or test is the respective International Standard or Reference Preparation established by the World Health Organization for international use and the biological activity is expressed in International Units (IU).

In other cases, where Units are referred to in an assay or test, the Unit for a particular substance or preparation is, for the United Kingdom, the specific biological activity contained in such an amount of the respective primary standard as the appropriate international or national organisation indicates. The necessary information is provided with the primary standard.

Unless otherwise directed, animals used in an assay or a test are healthy animals, drawn from a uniform stock, that have not previously been treated with any material that will interfere with the assay or test. Unless otherwise stated, guinea-pigs weigh not less than 250 g or, when used in systemic

toxicity tests, not less than 350 g. When used in skin tests they are white or light coloured. Unless otherwise stated, mice weigh not less than 17 g and not more than 22 g.

Certain of the biological assays and tests of the Pharmacopoeia are such that in the United Kingdom they may be carried out only in accordance with the Animals (Scientific Procedures) Act 1986. Instructions included in such assays and tests in the Pharmacopoeia, with respect to the handling of animals, are therefore confined to those concerned with the accuracy and reproducibility of the assay or test.

Reference Substances and Reference Preparations

Certain monographs require the use of a reference substance, a reference preparation or a reference spectrum. These are chosen with regard to their intended use as prescribed in the monographs of the Pharmacopoeia and are not necessarily suitable in other circumstances.

Any information necessary for proper use of the reference substance or reference preparation is given on the label or in the accompanying leaflet or brochure. Where no drying conditions are stated in the leaflet or on the label, the substance is to be used as received. No certificate of analysis or other data not relevant to the prescribed use of the product are provided. The products are guaranteed to be suitable for use for a period of three months from dispatch when stored under the appropriate conditions. The stability of the contents of opened containers cannot be guaranteed. The current lot is listed in the BP Laboratory website catalogue. Additional information is provided in Supplementary Chapter III E.

Chemical Reference Substances The abbreviation BPCRS indicates a Chemical Reference Substance established by the British Pharmacopoeia Commission. The abbreviation CRS or EPCRS indicates a Chemical Reference Substance established by the European Pharmacopoeia Commission. Some Chemical Reference Substances are used for the microbiological assay of antibiotics and their activity is stated, in International Units, on the label or on the accompanying leaflet and defined in the same manner as for Biological Reference Preparations.

Biological Reference Preparations The majority of the primary biological reference preparations referred to are the appropriate International Standards and Reference Preparations established by the World Health Organisation. Because these reference materials are usually available only in limited quantities, the European Pharmacopoeia has established Biological Reference Preparations (indicated by the abbreviation BRP or EPBRP) where appropriate. Where applicable, the potency of the Biological Reference Preparations is expressed in International Units. For some Biological Reference Preparations, where an international standard or reference preparation does not exist, the potency is expressed in European Pharmacopoeia Units.

Storage

Statements under the side-heading Storage constitute non-mandatory advice. The substances and preparations described in the Pharmacopoeia are to be stored under conditions that prevent contamination and, as far as possible, deterioration. Unless otherwise stated in the monograph, the substances and preparations described in the Pharmacopoeia are kept in well-closed containers and stored at a temperature not exceeding 25°. Precautions that should be taken in relation to the effects of the atmosphere, moisture, heat and light are indicated, where appropriate, in

the monographs. Further precautions may be necessary when some materials are stored in tropical climates or under other severe conditions.

The expression 'protected from moisture' means that the product is to be stored in an airtight container. Care is to be taken when the container is opened in a damp atmosphere. A low moisture content may be maintained, if necessary, by the use of a desiccant in the container provided that direct contact with the product is avoided.

The expression 'protected from light' means that the product is to be stored either in a container made of a material that absorbs actinic light sufficiently to protect the contents from change induced by such light or in a container enclosed in an outer cover that provides such protection or stored in a place from which all such light is excluded.

The expression 'tamper-evident container' means a closed container fitted with a device that reveals irreversibly whether the container has been opened.

Labelling The labelling requirements of the Pharmacopoeia are not comprehensive, and the provisions of regulations issued in accordance with the requirements of the territory in which the medicinal product is to be used should be met.

Licensed medicines intended for use within the United Kingdom must comply with the requirements of the Human Medicines Regulations 2012, as amended, in respect of their labelling and packaging leaflets, together with those regulations for the labelling of hazardous materials.

Best practice guidance on the labelling and packaging of medicines for use in the United Kingdom advises that certain items of information are deemed critical for the safe use of the medicine (see "Best Practice Guidance on the Labelling and Packaging of Medicines" issued by the MHRA, 2012). Further information and guidance on the labelling of medicinal products can be found in Supplementary Chapter I G.

Such matters as the exact form of wording to be used and whether a particular item of information should appear on the primary label and additionally, or alternatively, on the package or exceptionally in a leaflet are, in general, outside the scope of the Pharmacopoeia. When the term 'label' is used in Labelling statements of the Pharmacopoeia, decisions as to where the particular statement should appear should therefore be made in accordance with relevant legislation.

The label of every official formulated preparation other than those of fixed strength also states the content of the active ingredient or ingredients expressed in the terms required by the monograph. Where the content of active ingredient is required to be expressed in terms other than the weight of the official medicinal substance used in making the formulation, this is specifically stated under the heading Labelling. Unless otherwise stated in the monograph, the content of the active ingredient is expressed in terms of the official medicinal substance used in making the formulation.

These requirements do not necessarily apply to unlicensed preparations supplied in accordance with a prescription. For requirements for unlicensed medicines see the general monograph on Unlicensed Medicines.

Action and Use The statements given under this heading in monographs are intended only as information on the principal pharmacological actions or the uses of the materials in medicine or pharmacy. It should not be assumed that the

substance has no other action or use. The statements are not intended to be binding on prescribers or to limit their discretion.

Crude Drugs; Traditional Herbal and Complementary Medicines

Herbal and complementary medicines are classed as medicines under the Human Medicines Regulations 2012, as amended. It is emphasised that, although requirements for the quality of the material are provided in the monograph to assist the registration scheme by the UK Licensing Authority, the British Pharmacopoeia Commission has not assessed the safety or efficacy of the material in traditional use.

Monograph Title For traditional herbal medicines, the monograph title is a combination of the binomial name together with a description of use. Monographs for the material that has not been processed (the herbal drug) and the processed material (the herbal drug preparation) are published where possible. To distinguish between the two, the word 'Processed' is included in the relevant monograph title.

Definition Under the heading Definition, the botanical name together with any synonym is given. Where appropriate, for material that has not been processed, information on the collection/harvesting and/or treatment/drying of the whole herbal drug may be given. For processed materials, the method of processing, where appropriate, will normally be given in a separate section.

Characteristics References to odour are included only where this is highly characteristic. References to taste are not included.

Control methods Where applicable, the control methods to be used in monographs are:

(a) macroscopical and microscopical descriptions and chemical/chromatographic tests for identification
(b) tests for absence of any related species
(c) microbial test to assure microbial quality
(d) tests for inorganic impurities and non-specific purity tests, including extractive tests, Sulfated ash and Heavy metals, where appropriate
(e) test for Loss on drying or Water
(f) wherever possible, a method for assaying the active constituent(s) or suitable marker constituent(s).

The macroscopical characteristics include those features that can be seen by the unaided eye or by the use of a hand lens. When two species/subspecies of the same plant are included in the Definition, individual differences between the two are indicated where possible.

The description of the microscopical characteristics of the powdered drug includes information on the dominant or the most specific characters. Where it is considered to be an aid to identification, illustrations of the powdered drug may be provided.

The following aspects are controlled by the general monograph for Herbal Drugs: they are required to be free from moulds, insects, decay, animal matter and animal excreta. Unless otherwise prescribed the amount of foreign matter is not more than 2% w/w. Microbial contamination should be minimal.

In determining the content of the active constituents or the suitable marker substances measurements are made with reference to the dried or anhydrous herbal drug. In the tests for Acid-insoluble ash, Ash, Extractive soluble in ethanol, Loss on drying, Sulfated ash, Water, Water-soluble ash and Water-soluble extractive of herbal drugs, the calculations are made with

reference to the herbal drug that has not been specifically dried unless otherwise prescribed in the monograph.

Homoeopathic Medicines

Homoeopathic medicines are classed as medicines under the Human Medicines Regulations 2012, as amended. It is emphasised that, although requirements for the quality of the material are provided in the relevant monograph in order to assist the simplified registration scheme by the UK Licensing Authority, the British Pharmacopoeia Commission has not assessed the safety or efficacy of the material in use.

All materials used for the production of homoeopathic medicines, including excipients, must comply with European Pharmacopoeia or British Pharmacopoeia monographs for those materials. Where such European Pharmacopoeia or British Pharmacopoeia monographs do not exist, each material used for the production of homoeopathic medicines must comply with an official national pharmacopoeia of a Member State.

British Pharmacopoeia monographs for homoeopathic medicines apply to homoeopathic stocks and mother tinctures only, but may be prefaced by a section which details the quality requirements applicable to the principle component where there is no European Pharmacopoeia or British Pharmacopoeia monograph for the material. These monographs also include either general statements on the methods of preparation or refer to specific methods of preparation given in the European Pharmacopoeia. Homoeopathic stocks and mother tinctures undergo the further process referred to as potentisation. Potentisation is a term specific to homoeopathic medicine and is a process of dilution of stocks and mother tinctures to produce the final product.

Identification tests are established for the components in homoeopathic stocks and usually relate to those applied to the materials used in the production of the homoeopathic stocks. An assay is included for the principal component(s) where possible. For mother tinctures, an identification test, usually chromatographic, is established and, where applicable, an assay for the principle component(s); where appropriate, other tests, related to the solvent, dry matter or known adulterants, are included.

Specifications have not been set for final homoeopathic products due to the high dilution used in their preparation and the subsequent difficulty in applying analytical methodology.

Statements under Crude Drugs; Traditional Herbal and Complementary Medicines also apply to homoeopathic stocks and mother tinctures, when appropriate.

Unlicensed Medicines

The General Monograph for Unlicensed Medicines applies to those formulations used in human medicine that are prepared under a Manufacturer's 'Specials' Licence or prepared extemporaneously under the supervision of a pharmacist, whether or not there is a published monograph for the specific dosage form.

An article intended for medicinal use that is described by means of an official title must comply with the requirements of the relevant monograph. A formulated preparation must comply throughout its assigned shelf-life (period of validity). The subject of any other monograph must comply throughout its period of use.

Unlicensed medicines that are prepared under a Manufacturer's 'Specials' Licence comply with the requirements of the General Monograph

for Pharmaceutical Preparations, the requirements of the General Monograph for Unlicensed Medicines and, where applicable, the requirements of the individual monograph for the specific dosage form.

Unlicensed medicines prepared extemporaneously under the supervision of a pharmacist comply with the requirements of the General Monograph for Pharmaceutical Preparations, the requirements of the General Monograph for Unlicensed Medicines and, where applicable, the requirements of the individual monograph for the specific dosage form. While it is expected that extemporaneous preparations will demonstrate pharmacopoeial compliance when tested, it is recognised that it might not be practicable to carry out the pharmacopoeial tests routinely on such formulations. In the event of doubt or dispute, the methods of analysis, the reference materials and the reference spectra of the Pharmacopoeia are alone authoritative.

Part III

Monographs and other texts of the European Pharmacopoeia that are incorporated in this edition of the British Pharmacopoeia are governed by the general notices of the European Pharmacopoeia; these are reproduced below.

GENERAL NOTICES OF THE EUROPEAN PHARMACOPOEIA

1.1. GENERAL STATEMENTS

The General Notices apply to all monographs and other texts of the European Pharmacopoeia.

The official texts of the European Pharmacopoeia are published in English and French. Translations in other languages may be prepared by the signatory States of the European Pharmacopoeia Convention. In case of doubt or dispute, the English and French versions are alone authoritative.

In the texts of the European Pharmacopoeia, the word 'Pharmacopoeia' without qualification means the European Pharmacopoeia. The official abbreviation Ph. Eur. may be used to indicate the European Pharmacopoeia.

The use of the title or the subtitle of a monograph implies that the article complies with the requirements of the relevant monograph. Such references to monographs in the texts of the Pharmacopoeia are shown using the monograph title and reference number in *italics*.

A preparation must comply throughout its period of validity; a distinct period of validity and/or specifications for opened or broached containers may be decided by the competent authority. The subject of any other monograph must comply throughout its period of use. The period of validity that is assigned to any given article and the time from which that period is to be calculated are decided by the competent authority in light of experimental results of stability studies.

Unless otherwise indicated in the General Notices or in the monographs, statements in monographs constitute mandatory requirements. General chapters become mandatory when referred to in a monograph, unless such reference is made in a way that indicates that it is not the intention to make the text referred to mandatory but rather to cite it for information.

The active substances, excipients, pharmaceutical preparations and other articles described in the monographs are intended for human and veterinary use (unless explicitly restricted to one of these uses).

Quality systems The quality standards represented by monographs are valid only where the articles in question are produced within the framework of a suitable quality system. The quality system must assure that the articles consistently meet the requirements of the Pharmacopoeia.

Alternative methods The tests and assays described are the official methods upon which the standards of the Pharmacopoeia are based. With the agreement of the competent authority, alternative methods of analysis may be used for control purposes, provided that the methods used enable an unequivocal decision to be made as to whether compliance with the standards of the

monographs would be achieved if the official methods were used. In the event of doubt or dispute, the methods of analysis of the Pharmacopoeia are alone authoritative.

Demonstration of compliance with the Pharmacopoeia

(1) An article is not of Pharmacopoeia quality unless it complies with all the requirements stated in the monograph. This does not imply that performance of all the tests in a monograph is necessarily a prerequisite for a manufacturer in assessing compliance with the Pharmacopoeia before release of a product. The manufacturer may obtain assurance that a product is of Pharmacopoeia quality on the basis of its design, together with its control strategy and data derived, for example, from validation studies of the manufacturing process.

(2) An enhanced approach to quality control could utilise process analytical technology (PAT) and/or real-time release testing (including parametric release) strategies as alternatives to end-product testing alone. Real-time release testing in circumstances deemed appropriate by the competent authority is thus not precluded by the need to comply with the Pharmacopoeia.

(3) Reduction of animal testing: the European Pharmacopoeia is dedicated to phasing out the use of animals for test purposes, in accordance with the 3Rs (Replacement, Reduction, Refinement) set out in the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes. In demonstrating compliance with the Pharmacopoeia as indicated above (1), manufacturers may consider establishing additional systems to monitor consistency of production. With the agreement of the competent authority, the choice of tests performed to assess compliance with the Pharmacopoeia when animal tests are prescribed is established in such a way that animal usage is minimised as much as possible.

Grade of materials

Certain materials that are the subject of a pharmacopoeial monograph may exist in different grades suitable for different purposes. Unless otherwise indicated in the monograph, the requirements apply to all grades of the material. In some monographs, particularly those on excipients, a list of functionality-related characteristics that are relevant to the use of the substance may be appended to the monograph for information. Test methods for determination of one or more of these characteristics may be given, also for information.

General monographs

Substances and preparations that are the subject of an individual monograph are also required to comply with relevant, applicable general monographs. Cross-references to applicable general monographs are not normally given in individual monographs.

General monographs apply to all substances and preparations within the scope of the Definition section of the general monograph, except where a preamble limits the application, for example to substances and preparations that are the subject of a monograph of the Pharmacopoeia.

General monographs on dosage forms apply to all preparations of the type defined. The requirements are not necessarily comprehensive for a given specific preparation and requirements additional to those prescribed in the general monograph may be imposed by the competent authority.

General monographs and individual monographs are complementary. If the provisions of a general monograph do not apply to a particular product, this is expressly stated in the individual monograph.

Validation of pharmacopoeial methods The test methods given in monographs and general chapters have been validated in accordance with accepted scientific practice and current recommendations on analytical validation. Unless otherwise stated in the monograph or general chapter, validation of the test methods by the analyst is not required.

Implementation of pharmacopoeial methods When implementing a pharmacopoeial method, the user must assess whether and to what extent the suitability of the method under the actual conditions of use needs to be demonstrated according to relevant monographs, general chapters and quality systems.

Conventional terms The term 'competent authority' means the national, supranational or international body or organisation vested with the authority for making decisions concerning the issue in question. It may, for example, be a national pharmacopoeia authority, a licensing authority or an official control laboratory.

The expression 'unless otherwise justified and authorised' means that the requirements have to be met, unless the competent authority authorises a modification or an exemption where justified in a particular case.

Statements containing the word 'should' are informative or advisory.

In certain monographs or other texts, the terms 'suitable' and 'appropriate' are used to describe a reagent, micro-organism, test method etc.; if criteria for suitability are not described in the monograph, suitability is demonstrated to the satisfaction of the competent authority.

Medicinal product (a) Any substance or combination of substances presented as having properties for treating or preventing disease in human beings and/or animals; or (b) any substance or combination of substances that may be used in or administered to human beings and/or animals with a view either to restoring, correcting or modifying physiological functions by exerting a pharmacological, immunological or metabolic action, or to making a medical diagnosis.

Herbal medicinal product Any medicinal product, exclusively containing as active ingredients one or more herbal drugs or one or more herbal drug preparations, or one or more such herbal drugs in combination with one or more such herbal drug preparations.

Active substance Any substance intended to be used in the manufacture of a medicinal product and that, when so used, becomes an active ingredient of the medicinal product. Such substances are intended to furnish a pharmacological activity or other direct effect in the diagnosis, cure, mitigation, treatment or prevention of disease, or to affect the structure and function of the body.

Excipient (auxiliary substance). Any constituent of a medicinal product that is not an active substance. Adjuvants, stabilisers, antimicrobial preservatives, diluents, antioxidants, for example, are excipients.

Interchangeable methods Certain general chapters contain a statement that the text in question is harmonised with the corresponding text of the Japanese Pharmacopoeia and/or the United States Pharmacopeia and that these texts are interchangeable. This implies that if a substance or preparation is found to

comply with a requirement using an interchangeable method from one of these pharmacopoeias it complies with the requirements of the European Pharmacopoeia. In the event of doubt or dispute, the text of the European Pharmacopoeia is alone authoritative.

References to regulatory documents Monographs and general chapters may contain references to documents issued by regulatory authorities for medicines, for example directives and notes for guidance of the European Union. These references are provided for information for users for the Pharmacopoeia. Inclusion of such a reference does not modify the status of the documents referred to, which may be mandatory or for guidance.

1.2. OTHER PROVISIONS APPLYING TO GENERAL CHAPTERS AND MONOGRAPHS

Quantities In tests with numerical limits and assays, the quantity stated to be taken for examination is approximate. The amount actually used, which may deviate by not more than 10 per cent from that stated, is accurately weighed or measured and the result is calculated from this exact quantity. In tests where the limit is not numerical, but usually depends upon comparison with the behaviour of a reference substance in the same conditions, the stated quantity is taken for examination. Reagents are used in the prescribed amounts.

Quantities are weighed or measured with an accuracy commensurate with the indicated degree of precision. For weighings, the precision corresponds to plus or minus 5 units after the last figure stated (for example, 0.25 g is to be interpreted as 0.245 g to 0.255 g). For the measurement of volumes, if the figure after the decimal point is a zero or ends in a zero (for example, 10.0 mL or 0.50 mL), the volume is measured using a pipette, a volumetric flask or a burette, as appropriate; otherwise, a graduated measuring cylinder or a graduated pipette may be used. Volumes stated in microlitres are measured using a micropipette or microsyringe.

It is recognised, however, that in certain cases the precision with which quantities are stated does not correspond to the number of significant figures stated in a specified numerical limit. The weighings and measurements are then carried out with a sufficiently improved accuracy.

Apparatus and procedures Volumetric glassware complies with Class A requirements of the appropriate International Standard issued by the International Organisation for Standardisation.

Unless otherwise prescribed, analytical procedures are carried out at a temperature between 15 °C and 25 °C.

Unless otherwise prescribed, comparative tests are carried out using identical tubes of colourless, transparent, neutral glass with a flat base; the volumes of liquid prescribed are for use with tubes having an internal diameter of 16 mm, but tubes with a larger internal diameter may be used provided the volume of liquid used is adjusted (*2.1.5*). Equal volumes of the liquids to be compared are examined down the vertical axis of the tubes against a white background, or if necessary against a black background. The examination is carried out in diffuse light.

Any solvent required in a test or assay in which an indicator is to be used is previously neutralised to the indicator, unless a blank test is prescribed.

Water-bath The term 'water-bath' means a bath of boiling water unless water at another temperature is indicated. Other methods of heating may be substituted provided the temperature is near to but not higher than 100 °C or the indicated temperature.

Drying and ignition to constant mass The terms 'dried to constant mass' and 'ignited to constant mass' mean that 2 consecutive weighings do not differ by more than 0.5 mg, the 2nd weighing following an additional period of drying or of ignition respectively appropriate to the nature and quantity of the residue.

Where drying is prescribed using one of the expressions 'in a desiccator' or '*in vacuo*', it is carried out using the conditions described in chapter *2.2.32. Loss on drying*.

Reagents The proper conduct of the analytical procedures described in the Pharmacopoeia and the reliability of the results depend, in part, upon the quality of the reagents used. The reagents are described in general chapter 4. It is assumed that reagents of analytical grade are used; for some reagents, tests to determine suitability are included in the specifications.

Solvents Where the name of the solvent is not stated, the term 'solution' implies a solution in water.

Where the use of water is specified or implied in the analytical procedures described in the Pharmacopoeia or for the preparation of reagents, water complying with the requirements of the monograph *Purified water (0008)* is used, except that for many purposes the requirements for bacterial endotoxins (*Purified water in bulk*) and microbial contamination (*Purified water in containers*) are not relevant. The term 'distilled water' indicates purified water prepared by distillation.

The term 'ethanol' without qualification means anhydrous ethanol. The term 'alcohol' without qualification means ethanol (96 per cent). Other dilutions of ethanol are indicated by the term 'ethanol' or 'alcohol' followed by a statement of the percentage by volume of ethanol (C_2H_6O) required.

Expression of content In defining content, the expression 'per cent' is used according to circumstances with one of 2 meanings:

— per cent *m/m* (percentage, mass in mass) expresses the number of grams of substance in 100 g of final product;

— per cent *V/V* (percentage, volume in volume) expresses the number of millilitres of substance in 100 mL of final product.

The expression 'parts per million' (or ppm) refers to mass in mass, unless otherwise specified.

Temperature Where an analytical procedure describes temperature without a figure, the general terms used have the following meaning:

— in a deep-freeze: below −15 °C;

— in a refrigerator: 2 °C to 8 °C;

— cold or cool: 8 °C to 15 °C;

— room temperature: 15 °C to 25 °C.

1.3. GENERAL CHAPTERS

Containers Materials used for containers are described in general chapter *3.1*. General names used for materials, particularly plastic materials, each cover a range

of products varying not only in the properties of the principal constituent but also in the additives used. The test methods and limits for materials depend on the formulation and are therefore applicable only for materials whose formulation is covered by the preamble to the specification. The use of materials with different formulations, and the test methods and limits applied to them, are subject to agreement by the competent authority.

The specifications for containers in general chapter *3.2* have been developed for general application to containers of the stated category, but in view of the wide variety of containers available and possible new developments, the publication of a specification does not exclude the use, in justified circumstances, of containers that comply with other specifications, subject to agreement by the competent authority.

Reference may be made within the monographs of the Pharmacopoeia to the definitions and specifications for containers provided in chapter *3.2. Containers*. The general monographs for pharmaceutical dosage forms may, under the heading Definition/Production, require the use of certain types of container; certain other monographs may, under the heading Storage, indicate the type of container that is recommended for use.

1.4. MONOGRAPHS

Titles Monograph titles are in English and French in the respective versions and there is a Latin subtitle.

Relative Atomic And Molecular Masses The relative atomic mass (A_r) or the relative molecular mass (M_r) is shown, as and where appropriate, at the beginning of each monograph. The relative atomic and molecular masses and the molecular and graphic formulae do not constitute analytical standards for the substances described.

Chemical Abstracts Service (CAS) Registry Number CAS registry numbers are included for information in monographs, where applicable, to provide convenient access to useful information for users. CAS Registry Number® is a registered trademark of the American Chemical Society.

Definition Statements under the heading Definition constitute an official definition of the substance, preparation or other article that is the subject of the monograph.

Limits of content Where limits of content are prescribed, they are those determined by the method described under Assay.

Herbal drugs In monographs on herbal drugs, the definition indicates whether the subject of the monograph is, for example, the whole drug or the drug in powdered form. Where a monograph applies to the drug in several states, for example both to the whole drug and the drug in powdered form, the definition states this.

Production Statements under the heading Production draw attention to particular aspects of the manufacturing process but are not necessarily comprehensive. They constitute mandatory requirements for manufacturers, unless otherwise stated. They may relate, for example, to source materials; to the manufacturing process itself and its validation and control; to in-process testing; or to testing that is to be carried out by the manufacturer on the final article, either on selected batches or on each batch prior to release. These statements cannot necessarily be verified on a sample of the final article by an independent analyst. The competent authority may establish

that the instructions have been followed, for example, by examination of data received from the manufacturer, by inspection of manufacture or by testing appropriate samples.

The absence of a Production section does not imply that attention to features such as those referred to above is not required.

Choice of vaccine strain, Choice of vaccine composition The Production section of a monograph may define the characteristics of a vaccine strain or vaccine composition. Unless otherwise stated, test methods given for verification of these characteristics are provided for information as examples of suitable methods. Subject to approval by the competent authority, other test methods may be used without validation against the method shown in the monograph.

Potential Adulteration

Due to the increasing number of fraudulent activities and cases of adulteration, information may be made available to Ph. Eur. users to help detect adulterated materials (i.e. active substances, excipients, intermediate products, bulk products and finished products).

To this purpose, a method for the detection of potential adulterants and relevant limits, together with a reminder that all stages of production and sourcing are subjected to a suitable quality system, may be included in this section of monographs on substances for which an incident has occurred or that present a risk of deliberate contamination. The frequency of testing by manufacturers or by users (e.g. manufacturers of intermediate products, bulk products and finished products, where relevant) depends on a risk assessment, taking into account the level of knowledge of the whole supply chain and national requirements.

This section constitutes requirements for the whole supply chain, from manufacturers to users (e.g. manufacturers of intermediate products, bulk products and finished products, where relevant). The absence of this section does not imply that attention to features such as those referred to above is not required.

Characters

The statements under the heading Characters are not to be interpreted in a strict sense and are not requirements.

Solubility In statements of solubility in the Characters section, the terms used have the following significance, referred to a temperature between 15 °C and 25 °C.

Descriptive term	Approximate volume of solvent in millilitres per gram of solute			
Very soluble	less than	1		
Freely soluble	from	1	to	10
Soluble	from	10	to	30
Sparingly soluble	from	30	to	100
Slightly soluble	from	100	to	1000
Very slightly soluble	from	1000	to	10 000
Practically insoluble	more than			10 000

The term 'partly soluble' is used to describe a mixture where only some of the components dissolve. The term 'miscible' is used to describe a liquid that is miscible in all proportions with the stated solvent.

Identification

Scope The tests given in the Identification section are not designed to give a full confirmation of the chemical structure or composition of the product; they are intended to give confirmation, with an acceptable degree of assurance, that the article conforms to the description on the label.

First and second identifications Certain monographs have subdivisions entitled 'First identification' and 'Second identification'. The test or tests that constitute the 'First identification' may be used in all circumstances. The test or tests that constitute the 'Second identification' may be used in pharmacies provided it can be demonstrated that the substance or preparation is fully traceable to a batch certified to comply with all the other requirements of the monograph.

Certain monographs give two or more sets of tests for the purpose of the first identification, which are equivalent and may be used independently. One or more of these sets usually contain a cross-reference to a test prescribed in the Tests section of the monograph. It may be used to simplify the work of the analyst carrying out the identification and the prescribed tests. For example, one identification set cross-refers to a test for enantiomeric purity while the other set gives a test for specific optical rotation: the intended purpose of the two is the same, that is, verification that the correct enantiomer is present.

Powdered herbal drugs Monographs on herbal drugs may contain schematic drawings of the powdered drug. These drawings complement the description given in the relevant identification test.

Tests And Assays

Scope The requirements are not framed to take account of all possible impurities. It is not to be presumed, for example, that an impurity that is not detectable by means of the prescribed tests is tolerated if common sense and good pharmaceutical practice require that it be absent. See also below under Impurities.

Calculation Where the result of a test or assay is required to be calculated with reference to the dried or anhydrous substance or on some other specified basis, the determination of loss on drying, water content or other property is carried out by the method prescribed in the relevant test in the monograph. The words 'dried substance' or 'anhydrous substance' etc. appear in parentheses after the result.Where a quantitative determination of a residual solvent is carried out and a test for loss on drying is not carried out, the content of residual solvent is taken into account for the calculation of the assay content of the substance, the specific optical rotation and the specific absorbance. No further indication is given in the specific monograph.

Limits The limits prescribed are based on data obtained in normal analytical practice; they take account of normal analytical errors, of acceptable variations in manufacture and compounding and of deterioration to an extent considered acceptable. No further tolerances are to be applied to the limits prescribed to determine whether the article being examined complies with the requirements of the monograph.

In determining compliance with a numerical limit, the calculated result of a test or assay is first rounded to the number of significant figures stated, unless otherwise prescribed. The limits, regardless of whether the values are expressed as percentages or as absolute values, are considered significant to the last digit shown (for example 140 indicates 3 significant figures). The last figure of the result is increased by one when the part rejected is equal to

or exceeds one half-unit, whereas it is not modified when the part rejected is less than a half-unit.

Indication of permitted limit of impurities The acceptance criteria for related substances are expressed in monographs either in terms of comparison of peak areas (comparative tests) or as numerical values. For comparative tests, the approximate content of impurity tolerated, or the sum of impurities, may be indicated in brackets for information only. Acceptance or rejection is determined on the basis of compliance or non-compliance with the stated test. If the use of a reference substance for the named impurity is not prescribed, this content may be expressed as a nominal concentration of the substance used to prepare the reference solution specified in the monograph, unless otherwise described.

Herbal Drugs For herbal drugs, the sulfated ash, total ash, water-soluble matter, alcohol-soluble matter, water content, content of essential oil and content of active principle are calculated with reference to the drug that has not been specially dried, unless otherwise prescribed in the monograph.

Equivalents Where an equivalent is given, for the purposes of the Pharmacopoeia only the figures shown are to be used in applying the requirements of the monograph.

Culture media The culture media described in monographs and general chapters have been found to be satisfactory for the intended purpose. However, the components of media, particularly those of biological origin, are of variable quality, and it may be necessary for optimal performance to modulate the concentration of some ingredients, notably:

— peptones and meat or yeast extracts, with respect to their nutritive properties;
— buffering substances;
— bile salts, bile extract, deoxycholate, and colouring matter, depending on their selective properties;
— antibiotics, with respect to their activity.

Storage The information and recommendations given under the heading Storage do not constitute a pharmacopoeial requirement but the competent authority may specify particular storage conditions that must be met.

The articles described in the Pharmacopoeia are stored in such a way as to prevent contamination and, as far as possible, deterioration. Where special conditions of storage are recommended, including the type of container (see section 1.3. General chapters) and limits of temperature, they are stated in the monograph.

The following expressions are used in monographs under Storage with the meaning shown.

In an airtight container Means that the product is stored in an airtight container (*3.2*). Care is to be taken when the container is opened in a damp atmosphere. A low moisture content may be maintained, if necessary, by the use of a desiccant in the container provided that direct contact with the product is avoided.

Protected from light Means that the product is stored either in a container made of a material that absorbs actinic light sufficiently to protect the contents from change induced by such light, or in a container enclosed in an outer cover that provides such protection, or is stored in a place from which all such light is excluded.

Labelling In general, labelling of medicines is subject to supranational and national regulation and to international agreements. The statements under the heading Labelling are not therefore comprehensive and, moreover, for the purposes of the Pharmacopoeia only those statements that are necessary to demonstrate compliance or non-compliance with the monograph are mandatory. Any other labelling statements are included as recommendations. When the term 'label' is used in the Pharmacopoeia, the labelling statements may appear on the container, the package, a leaflet accompanying the package, or a certificate of analysis accompanying the article, as decided by the competent authority.

Warnings Materials described in monographs and reagents specified for use in the Pharmacopoeia may be injurious to health unless adequate precautions are taken. The principles of good quality control laboratory practice and the provisions of any appropriate regulations are to be observed at all times. Attention is drawn to particular hazards in certain monographs by means of a warning statement; absence of such a statement is not to be taken to mean that no hazard exists.

Impurities A list of all known and potential impurities that have been shown to be detected by the tests in a monograph may be given. See also chapter *5.10. Control of impurities in substances for pharmaceutical use*. The impurities are designated by a letter or letters of the alphabet. Where a letter appears to be missing, the impurity designated by this letter has been deleted from the list during monograph development prior to publication or during monograph revision.

Functionality-related Characteristics of Excipients Monographs on excipients may have a section on functionality-related characteristics. The characteristics, any test methods for determination and any tolerances are not mandatory requirements; they may nevertheless be relevant for use of the excipient and are given for information (see also section 1.1. General statements).

Reference Standards Certain monographs require the use of reference standards (chemical reference substances, herbal reference standards, biological reference preparations, reference spectra). See also chapter *5.12. Reference standards*. The European Pharmacopoeia Commission establishes the official reference standards, which are alone authoritative in case of arbitration. These reference standards are available from the European Directorate for the Quality of Medicines & HealthCare (EDQM). Information on the available reference standards and a batch validity statement can be obtained via the EDQM website.

1.5. ABBREVIATIONS AND SYMBOLS

A	Absorbance	mp	Melting point
$A_{1\ cm}^{1\ per\ cent}$	Specific absorbance	n_D^{20}	Refractive index
A_r	Relative atomic mass	Ph. Eur. U.	European Pharmacopoeia Unit
$[\alpha]_D^{20}$	Specific optical rotation	ppb	Parts per billion (micrograms per kilogram)
bp	Boiling point	ppm	Parts per million (milligrams per kilogram)
BRP	Biological reference preparation	R	Substance or solution defined under 4. *Reagents*
CRS	Chemical reference substance	R_F	Retardation factor (see chapter *2.2.46*)
d_{20}^{20}	Relative density	R_{st}	Used in chromatography to indicate the ratio of the distance travelled by a substance to the distance travelled by a reference substance
λ	Wavelength		
HRS	Herbal reference standard	RV	Substance used as a primary standard in volumetric analysis (chapter *4.2.1*)
IU	International Unit		
M	Molarity		
M_r	Relative molecular mass		

Abbreviations used in the monographs on immunoglobulins, immunosera and vaccines

CFU	Colony-forming units	Lo/10 dose	The largest quantity of a toxin that, in the conditions of the test, when mixed with 0.1 IU of antitoxin and administered by the specified route, does not cause symptoms of toxicity in the test animals within a given period
LD_{50}	The statistically determined quantity of a substance that, when administered by the specified route, may be expected to cause the death of 50 per cent of the test animals within a given period	Lf dose	The quantity of toxin or toxoid that flocculates in the shortest time with 1 IU of antitoxin
MLD	Minimum lethal dose	$CCID_{50}$	The statistically determined quantity of virus that may be expected to infect 50 per cent of the cell cultures to which it is added
L+/10 dose	The smallest quantity of a toxin that, in the conditions of the test, when mixed with 0.1 IU of antitoxin and administered by the specified route, causes the death of the test animals within a given period	EID_{50}	The statistically determined quantity of virus that may be expected to infect 50 per cent of the fertilised eggs into which it is inoculated
L+ dose	The smallest quantity of a toxin that, in the conditions of the test, when mixed with 1 IU of antitoxin and administered by the specified route, causes the death of the test animals within a given period	ID_{50}	The statistically determined quantity of a virus that may be expected to infect 50 per cent of the animals into which it is inoculated
In/100 dose	The smallest quantity of a toxin that, in the conditions of the test, when mixed with 0.01 IU of antitoxin and injected intracutaneously causes a characteristic reaction at the site of injection within a given period	PD_{50}	The statistically determined dose of a vaccine that, in the conditions of the test, may be expected to protect 50 per cent of the animals against a challenge dose of the micro-organisms or toxins against which it is active
Lp/10 dose	The smallest quantity of toxin that, in the conditions of the test, when mixed with 0.1 IU of antitoxin and administered by the specified route, causes paralysis in the test animals within a given period	ED_{50}	The statistically determined dose of a vaccine that, in the conditions of the test, may be expected to induce specific antibodies in 50 per cent of the animals for the relevant vaccine antigens
		PFU	Pock-forming units or plaque-forming units
		SPF	Specified-pathogen-free

Collections of micro-organisms

ATCC	American Type Culture Collection 10801 University Boulevard Manassas, Virginia 20110-2209, USA
C.I.P.	Collection de Bactéries de l'Institut Pasteur B.P. 52, 25 rue du Docteur Roux 75724 Paris Cedex 15, France
IMI	International Mycological Institute Bakeham Lane Surrey TW20 9TY, Great Britain
I.P.	Collection Nationale de Culture de Microorganismes (C.N.C.M.) Institut Pasteur 25, rue du Docteur Roux 75724 Paris Cedex 15, France
NCIMB	National Collection of Industrial and Marine Bacteria Ltd 23 St Machar Drive Aberdeen AB2 1RY, Great Britain
NCPF	National Collection of Pathogenic Fungi London School of Hygiene and Tropical Medicine Keppel Street London WC1E 7HT, Great Britain
NCTC	National Collection of Type Cultures Central Public Health Laboratory Colindale Avenue London NW9 5HT, Great Britain
NCYC	National Collection of Yeast Cultures AFRC Food Research Institute Colney Lane Norwich NR4 7UA, Great Britain
NITE	Biological Resource Center Department of Biotechnology National Institute of Technology and Evaluation 2-5-8 Kazusakamatari, Kisarazu-shi, Chiba, 292-0818 Japan
S.S.I.	Statens Serum Institut 80 Amager Boulevard, Copenhagen, Denmark

1.6. UNITS OF THE INTERNATIONAL SYSTEM (SI) USED IN THE PHARMACOPOEIA AND EQUIVALENCE WITH OTHER UNITS

International System Of Units (SI)

The International System of Units comprises 2 main classes of units, namely base units and derived units[1]. The base units are the metre, the kilogram, the second, the ampere, the kelvin, the mole and the candela.

The derived units are formed as products of powers of the base units according to the algebraic relationships linking the corresponding quantities. Some of these derived units have special names and symbols. The derived units used in the Pharmacopoeia are shown in Table 1.6.-1.

Some important and widely used units outside the International System are shown in Table 1.6.-2.

The prefixes shown in Table 1.6.-3 are used to form the names and symbols of the decimal multiples and submultiples of SI units.

Table 1.6.-1. – *Derived units used in the European Pharmacopoeia and equivalence with other units*

Quantity		Unit				Conversion of other units into SI units
Name	Symbol	Name	Symbol	Expression in SI base units	Expression in other SI units	
Wave number	ν	one per metre	1/m	m^{-1}		
Wavelength	λ	micrometre nanometre	µm nm	10^{-6} m 10^{-9} m		
Area	A, S	square metre	m^2	m^2		
Volume	V	cubic metre	m^3	m^3		1 mL = 1 cm^3 = 10^{-6} m^3
Frequency	ν	hertz	Hz	s^{-1}		
Density	ρ	kilogram per cubic metre	kg/m^3	$kg \cdot m^{-3}$		1 g/mL = 1 g/cm^3 = 10^3 $kg \cdot m^{-3}$
Velocity, speed	v	metre per second	m/s	$m \cdot s^{-1}$		
Force	F	newton	N	$m \cdot kg \cdot s^{-2}$		1 dyne = 1 $g \cdot cm \cdot s^{-2}$ = 10^{-5} N 1 kp = 9.806 65 N
Pressure, stress	p	pascal	Pa	$m^{-1} \cdot kg \cdot s^{-2}$	$N \cdot m^{-2}$	1 dyne/cm^2 = 10^{-1} Pa = 10^{-1} $N \cdot m^{-2}$ 1 atm = 101 325 Pa = 101.325 kPa 1 bar = 10^5 Pa = 0.1 MPa 1 mm Hg = 133.322 387 Pa 1 Torr = 133.322 368 Pa 1 psi = 6.894 757 kPa
Dynamic viscosity	η	pascal second	Pa·s	$m^{-1} \cdot kg \cdot s^{-1}$	$N \cdot s \cdot m^{-2}$	1 P = 10^{-1} Pa·s = 10^{-1} $N \cdot s \cdot m^{-2}$ 1 cP = 1 mPa·s
Kinematic viscosity	ν	square metre per second	m^2/s	$m^2 \cdot s^{-1}$	$Pa \cdot s \cdot m^3 \cdot kg^{-1}$ $N \cdot m \cdot s \cdot kg^{-1}$	1 St = 1 $cm^2 \cdot s^{-1}$ = 10^{-4} $m^2 \cdot s^{-1}$

[1] *The definitions of the units used in the International System are given in the booklet 'Le Système International d'Unités (SI)', published by the Bureau International des Poids et Mesures, Pavillon de Breteuil, F-92310 Sèvres.*

Quantity		Unit				Conversion of other units into SI units
Name	Symbol	Name	Symbol	Expression in SI base units	Expression in other SI units	
Energy	W	joule	J	$m^2 \cdot kg \cdot s^{-2}$	N·m	1 erg = 1 $cm^2 \cdot g \cdot s^{-2}$ = 1 dyne·cm = 10^{-7} J 1 cal = 4.1868 J
Power, radiant flux	P	watt	W	$m^2 \cdot kg \cdot s^{-3}$	$N \cdot m \cdot s^{-1}$ $J \cdot s^{-1}$	1 erg/s = 1 dyne·$cm \cdot s^{-1}$ = 10^{-7} W = 10^{-7} $N \cdot m \cdot s^{-1}$ = 10^{-7} $J \cdot s^{-1}$
Absorbed dose (of radiant energy)	D	gray	Gy	$m^2 \cdot s^{-2}$	$J \cdot kg^{-1}$	1 rad = 10^{-2} Gy
Electric potential difference, voltage	U	volt	V	$m^2 \cdot kg \cdot s^{-3} \cdot A^{-1}$	$W \cdot A^{-1}$	
Electric resistance	R	ohm	Ω	$m^2 \cdot kg \cdot s^{-3} \cdot A^{-2}$	$V \cdot A^{-1}$	
Electric charge	Q	coulomb	C	A·s		
Activity referred to a radionuclide	A	becquerel	Bq	s^{-1}		1 Ci = $37 \cdot 10^9$ Bq = $37 \cdot 10^9$ s^{-1}
Concentration (of amount of substance), molar concentration	c	mole per cubic metre	mol/m^3	$mol \cdot m^{-3}$		1 mol/L = 1 M = 1 mol/dm^3 = 10^3 $mol \cdot m^{-3}$
Mass concentration	ρ	kilogram per cubic metre	kg/m^3	$kg \cdot m^{-3}$		1 g/L = 1 g/dm^3 = 1 $kg \cdot m^{-3}$
Catalytic activity	Z	katal	kat	$mol \cdot s^{-1}$		

Table 1.6.-2. – *Non-SI units accepted for use with the SI units*

Quantity	Unit		Value in SI units
	Name	Symbol	
Time	minute	min	1 min = 60 s
	hour	h	1 h = 60 min = 3600 s
	day	d	1 d = 24 h = 86 400 s
Plane angle	degree	°	$1° = (\pi/180)$ rad
Volume	litre	L	1 L = 1 dm^3 = 10^{-3} m^3
Mass	tonne	t	1 t = 10^3 kg
	dalton	Da	1 Da = 1.660539040(20) × 10^{-27} kg
Rotational frequency	revolution per minute	r/min	1 r/min = (1/60) s^{-1}
Energy	electronvolt	eV	1eV=1.602176634 × 10^{-19}J

Table 1.6.-3. – *Decimal multiples and sub-multiples of SI units*

Factor	Prefix	Symbol	Factor	Prefix	Symbol
10^{18}	exa	E	10^{-1}	deci	d
10^{15}	peta	P	10^{-2}	centi	c
10^{12}	tera	T	10^{-3}	milli	m
10^{9}	giga	G	10^{-6}	micro	μ
10^{6}	mega	M	10^{-9}	nano	n
10^{3}	kilo	k	10^{-12}	pico	p
10^{2}	hecto	h	10^{-15}	femto	f
10^{1}	deca	da	10^{-18}	atto	a

Notes

1. In the Pharmacopoeia, the Celsius temperature is used (symbol t). This is defined by the following equation:

$$t = T - T_0$$

where T_0 = 273.15 K by definition. The Celsius or centigrade temperature is expressed in degrees Celsius (symbol °C). The unit 'degree Celsius' is equal to the unit 'kelvin'.

2. The practical expressions of concentrations used in the Pharmacopoeia are defined in the General Notices.

3. The radian is the plane angle between two radii of a circle that cut off on the circumference an arc equal in length to the radius.

4. In the Pharmacopoeia, conditions of centrifugation are defined by reference to the acceleration due to gravity (g):

$$g = 9.806\ 65\ m \cdot s^{-2}$$

5. Certain quantities without dimensions are used in the Pharmacopoeia: relative density (*2.2.5*), absorbance (*2.2.25*), specific absorbance (*2.2.25*) and refractive index (*2.2.6*).

6. The microkatal is defined as the enzymic activity that, under defined conditions, produces the transformation (e.g. hydrolysis) of 1 micromole of the substrate per second.

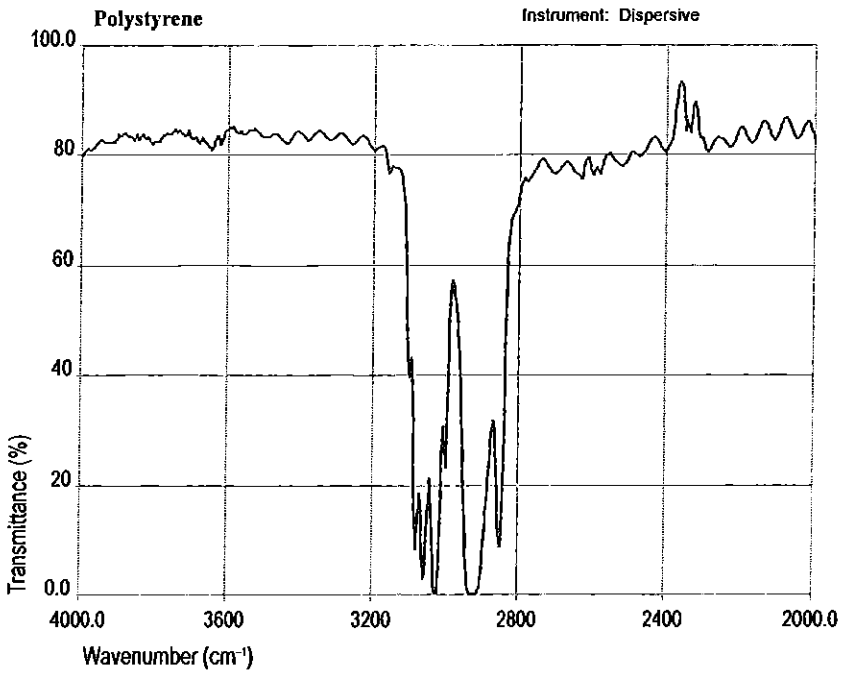
Infrared Reference Spectra

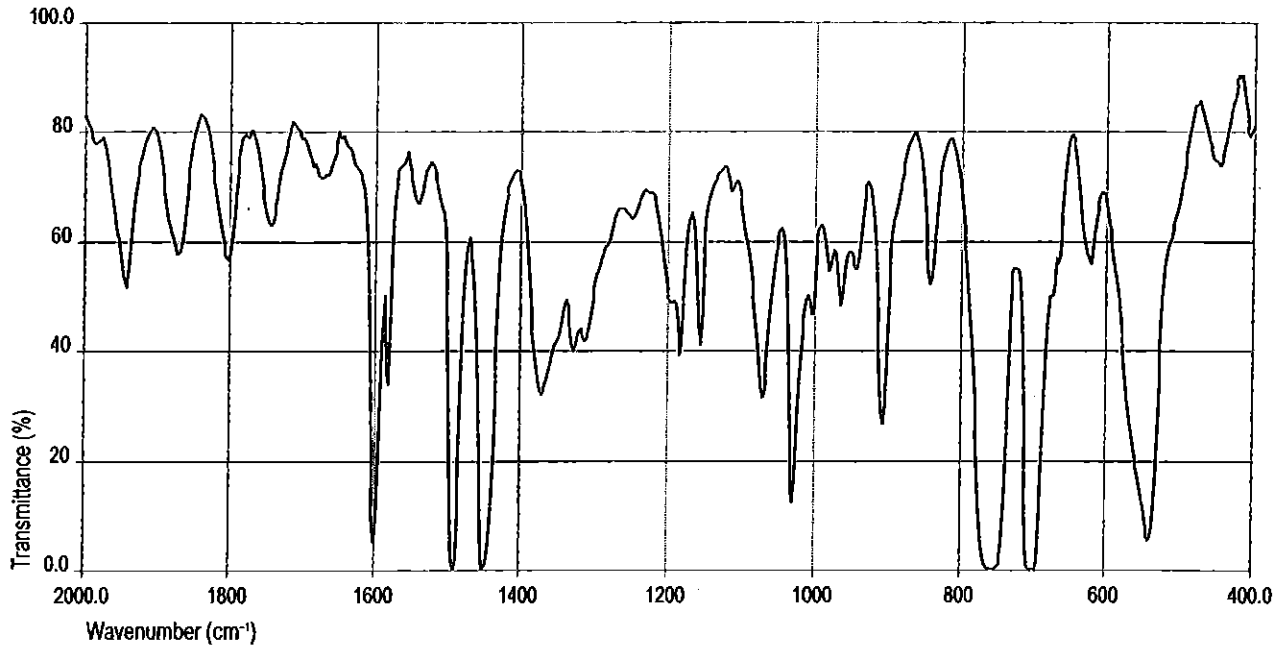
Preparation of Infrared Reference Spectra

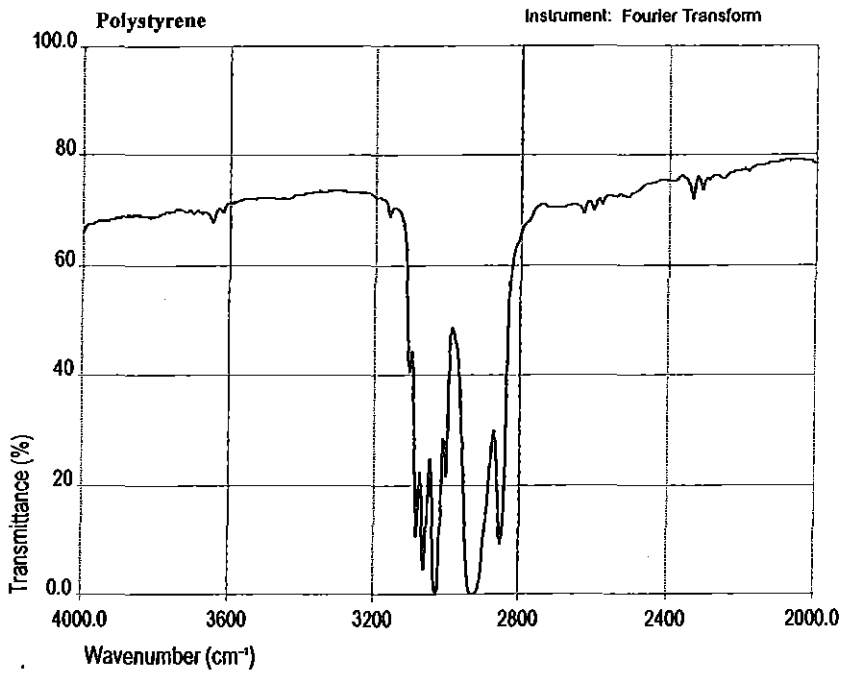
All spectra presented in this section were recorded using either a Perkin-Elmer model 682 dispersive infrared spectrophotometer, a Perkin Elmer model 16PC Fourier transform infrared spectrophotometer, a Perkin Elmer model Spectrum 100 Fourier transform infrared spectrophotometer, or a Perkin Elmer model Spectrum One Fourier transform infrared spectrophotometer.

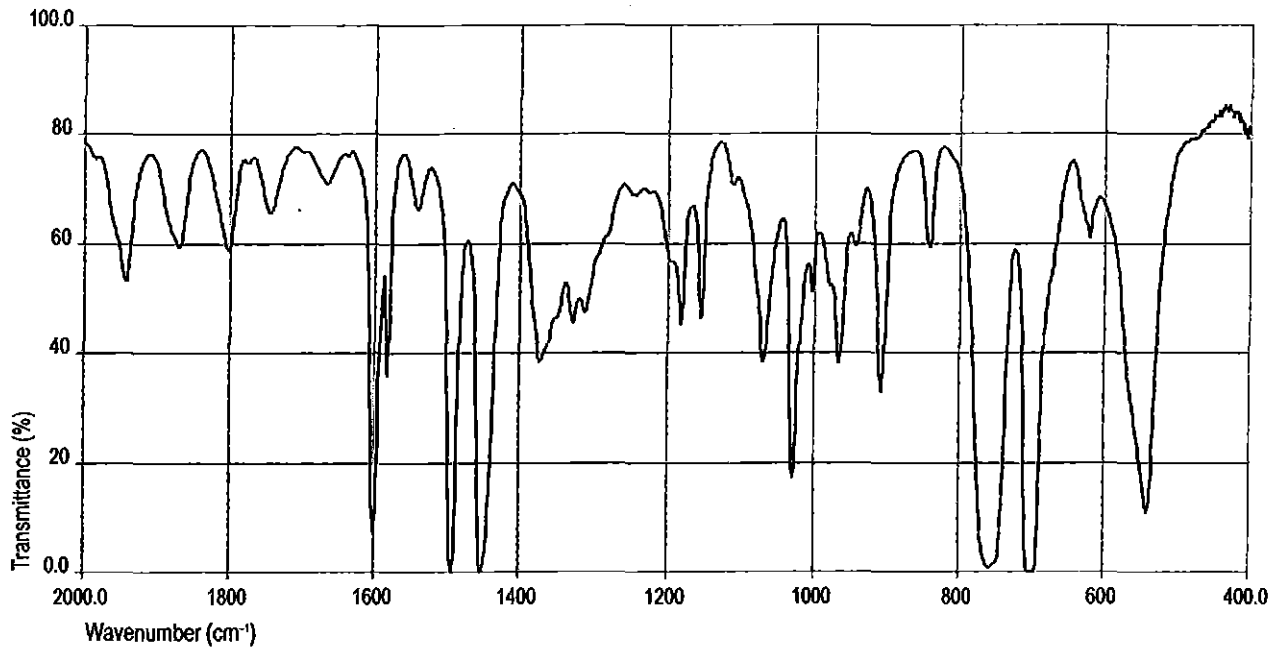
Pressed discs, 13 mm in diameter, were prepared using potassium bromide or potassium chloride. Liquid paraffin mulls and thin films were prepared between potassium bromide plates, and gas and solution spectra were prepared using cells with potassium bromide windows. Solution spectra were prepared against a solvent reference and all other spectra were recorded against air. Attenuated Total Reflectance (ATR) spectra were recorded by pressing the sample against a horizontal diamond ATR crystal.

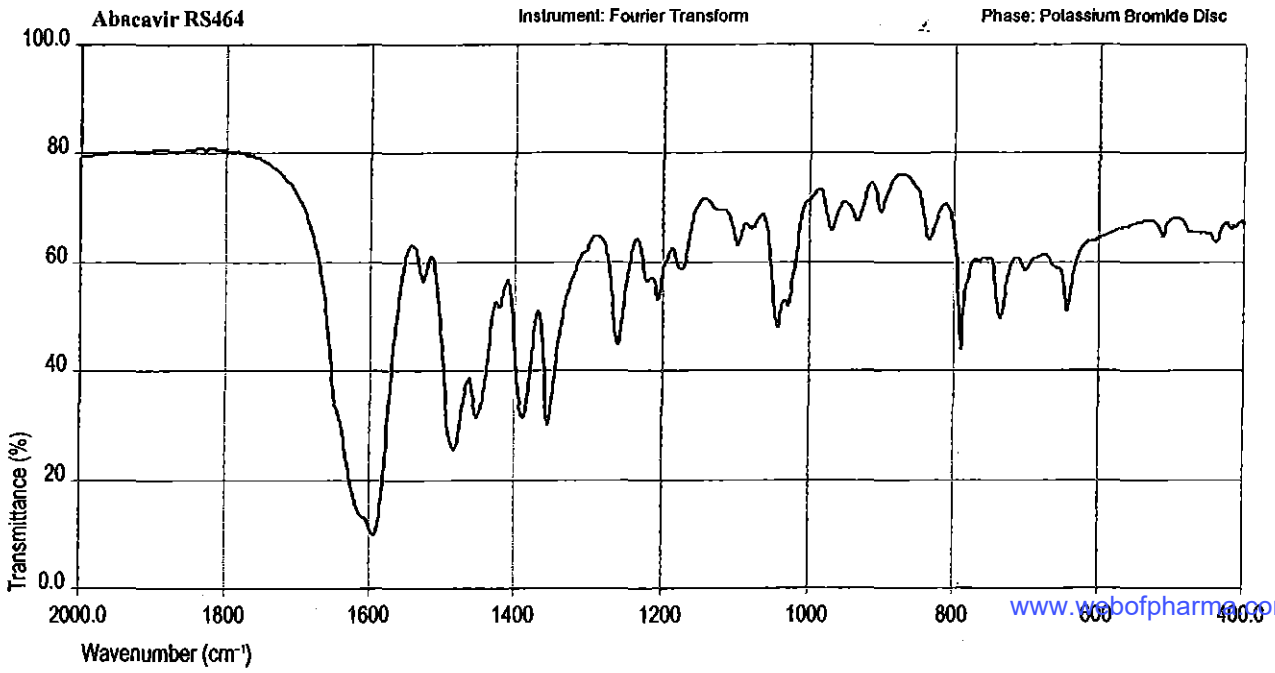
For solution spectra the regions of the spectrum within which the solvent shows strong absorption should be disregarded. Solvent 'cut-offs' in the reference spectra may be recorded as horizontal straight lines or may appear as blank regions on the spectrum.

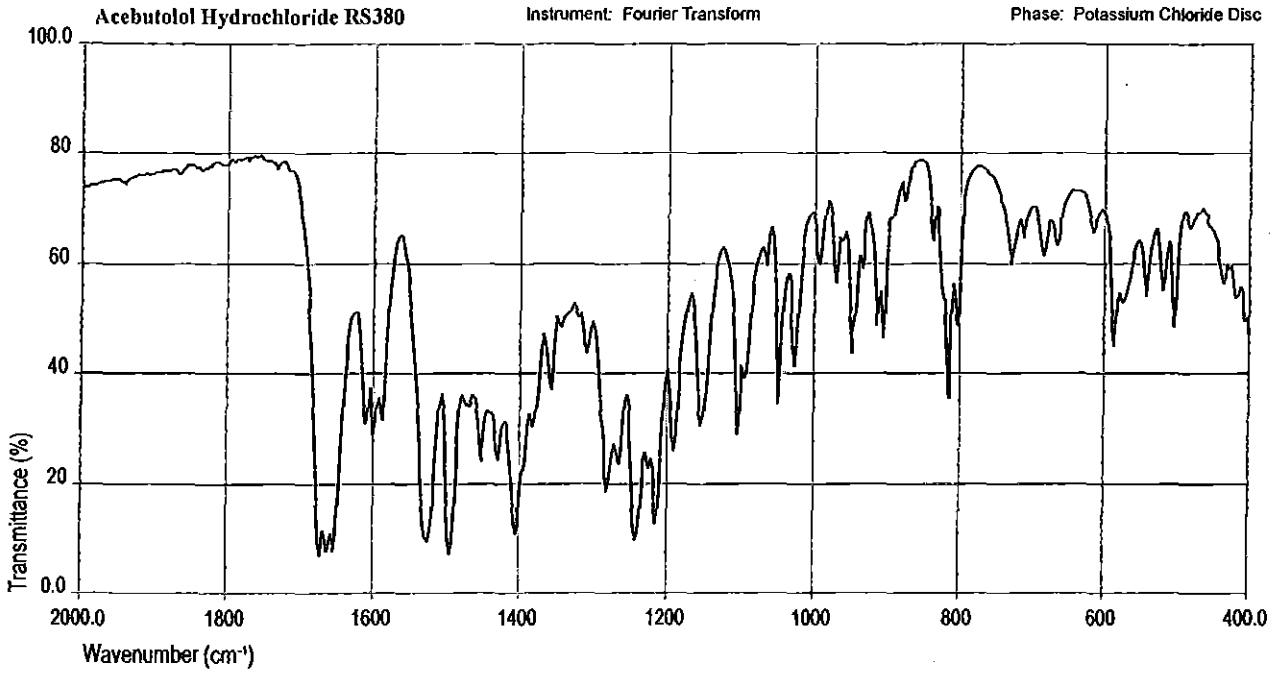


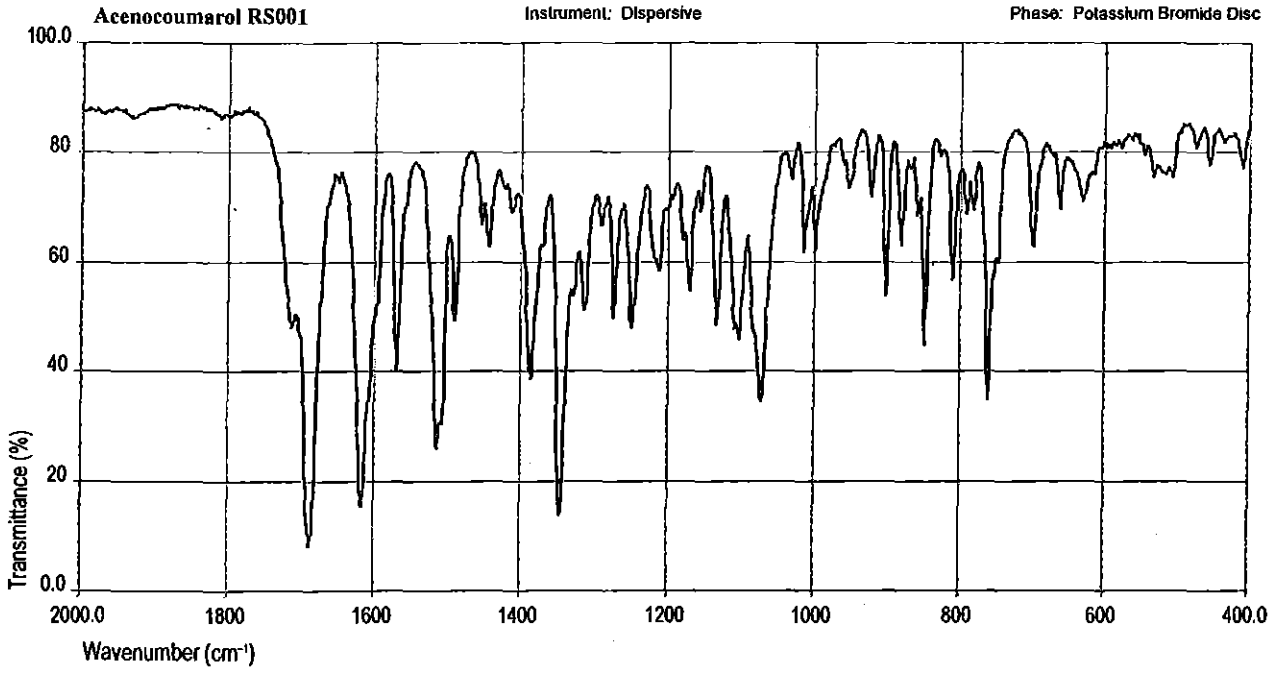


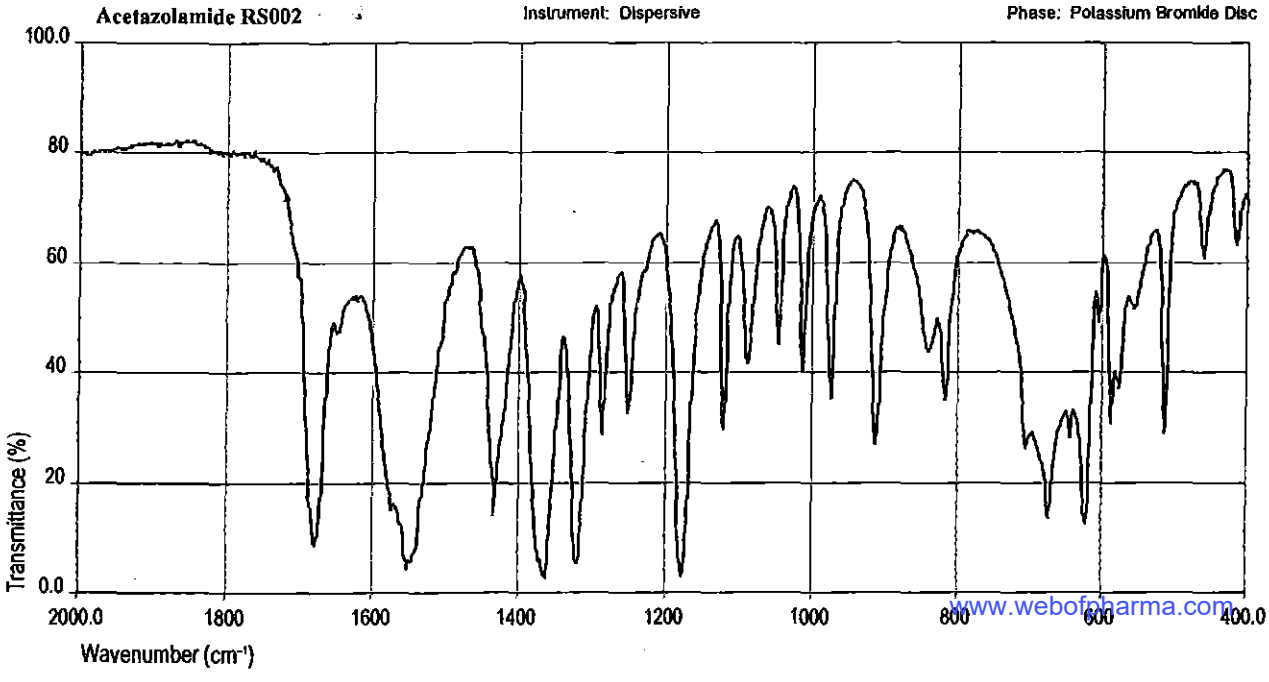


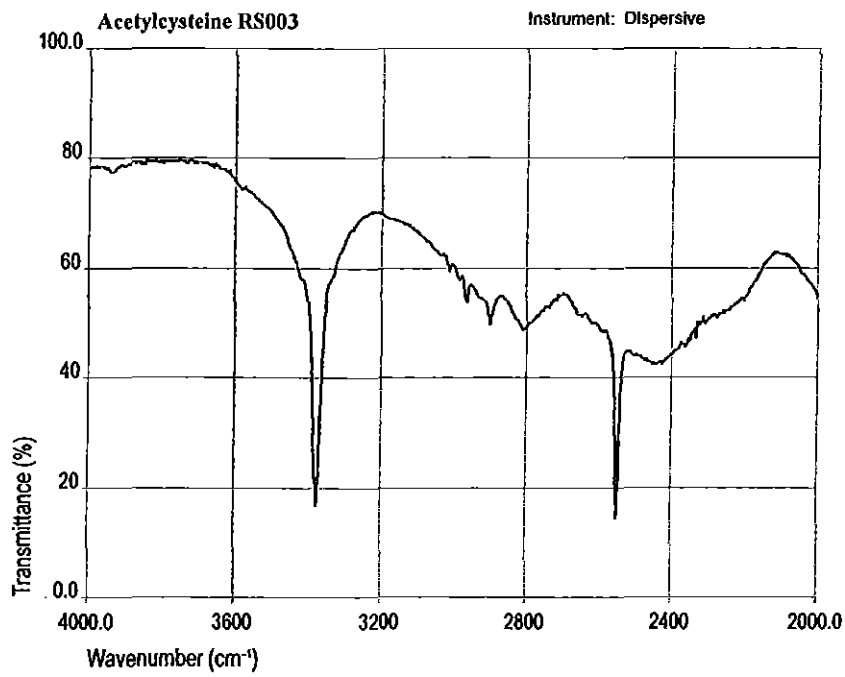


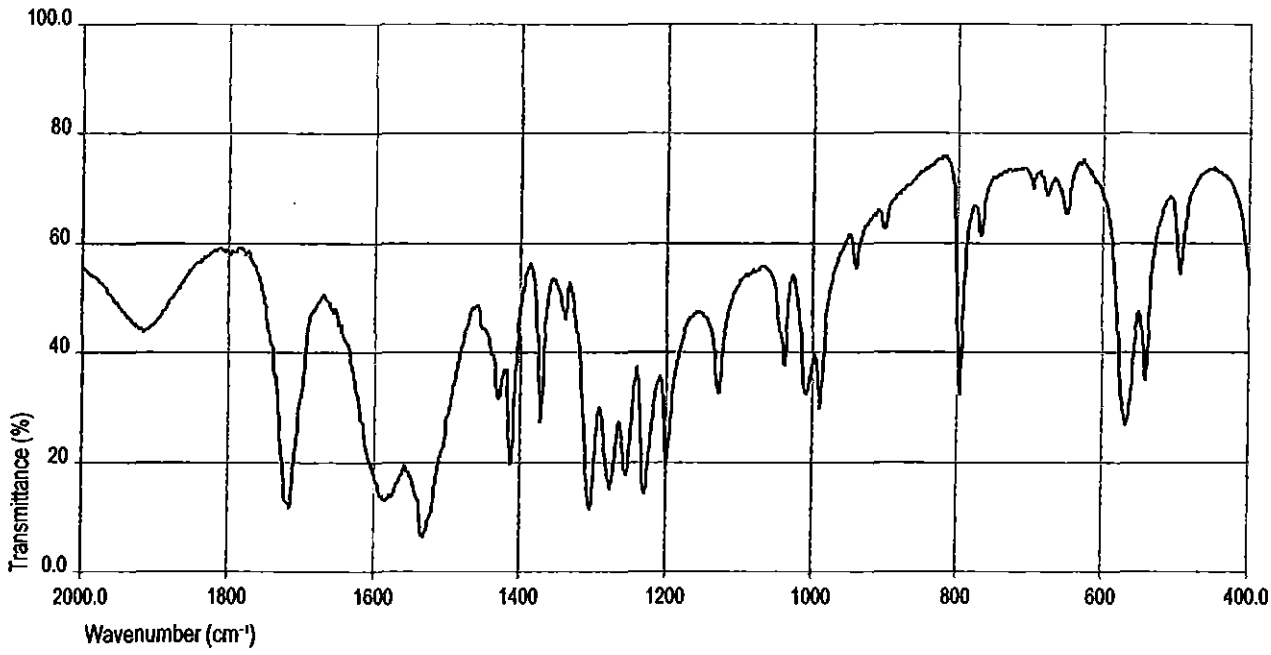


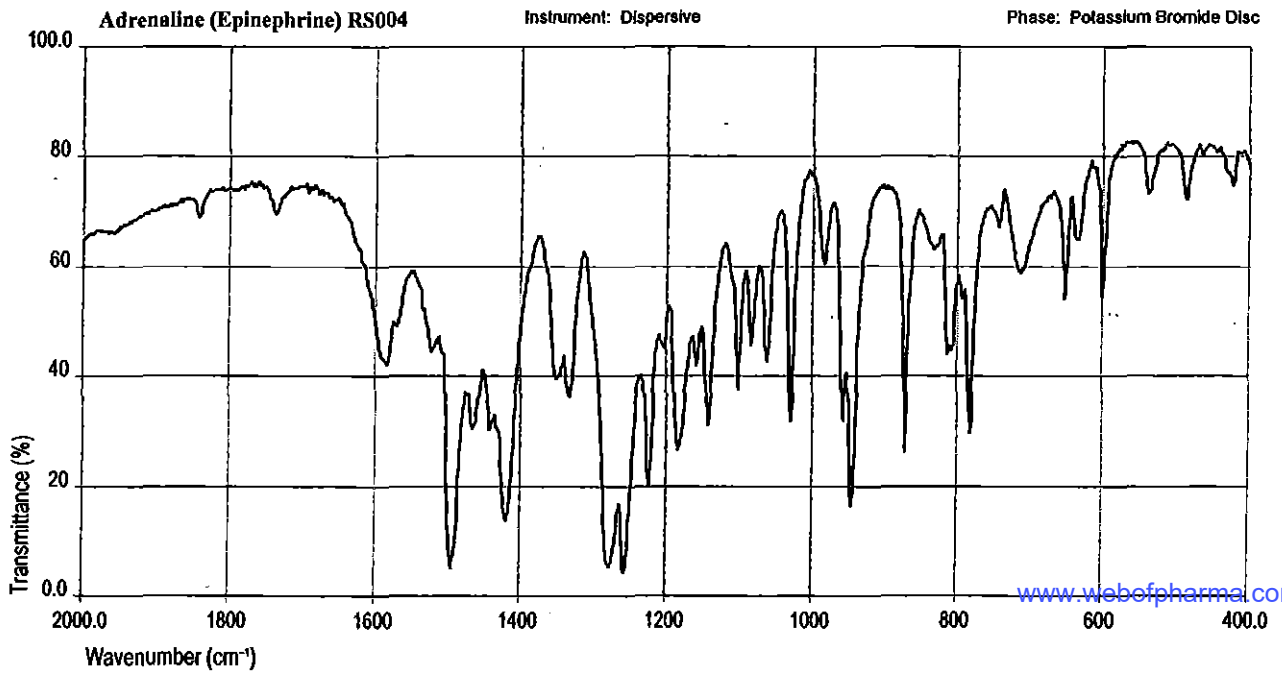








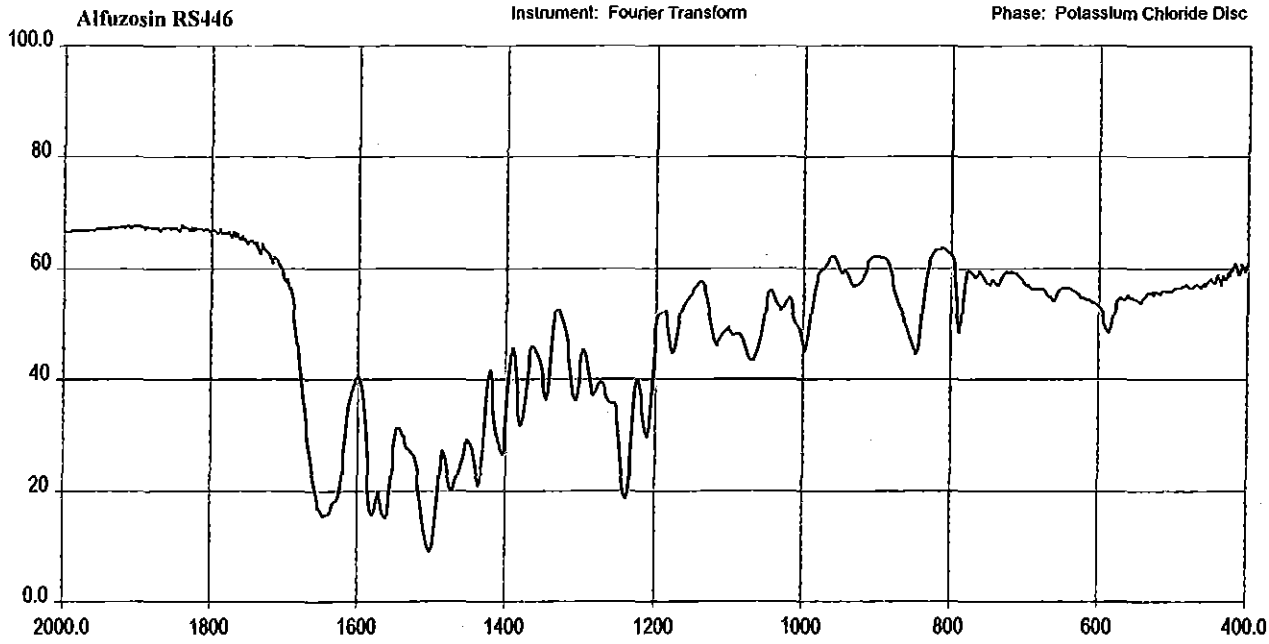




Alfuzosin RS446

Instrument: Fourier Transform

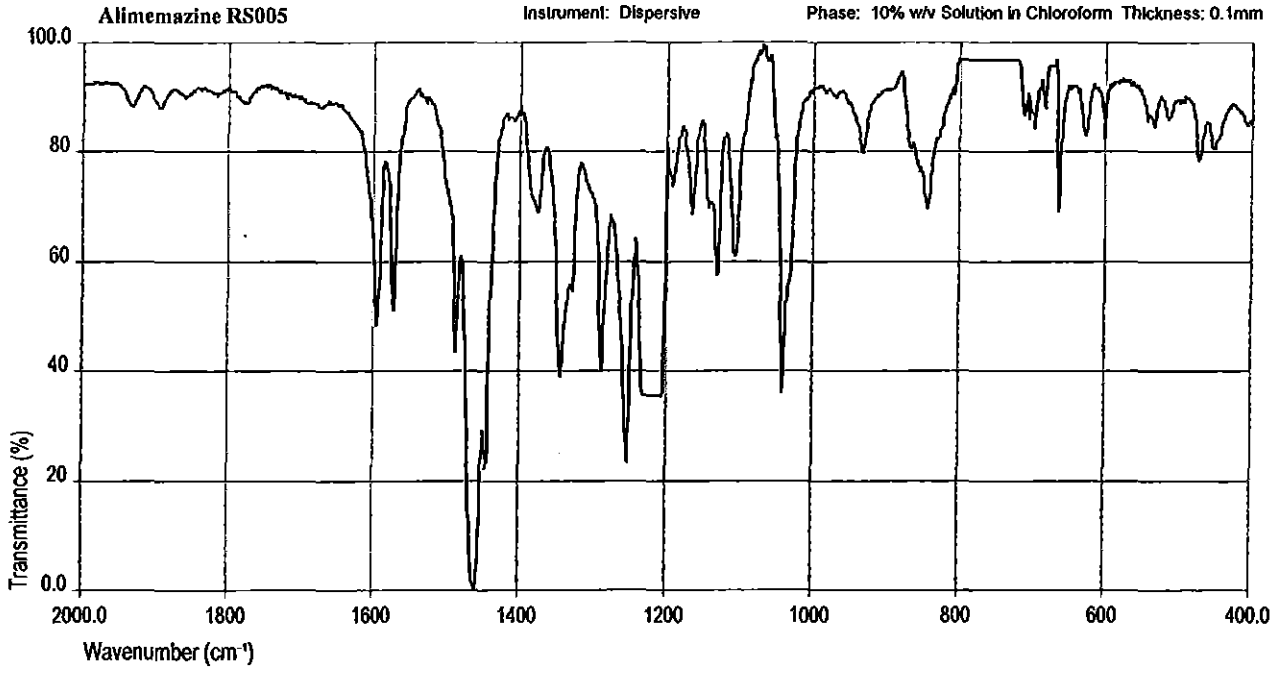
Phase: Potassium Chloride Disc



Alimemazine RS005

Instrument: Dispersive

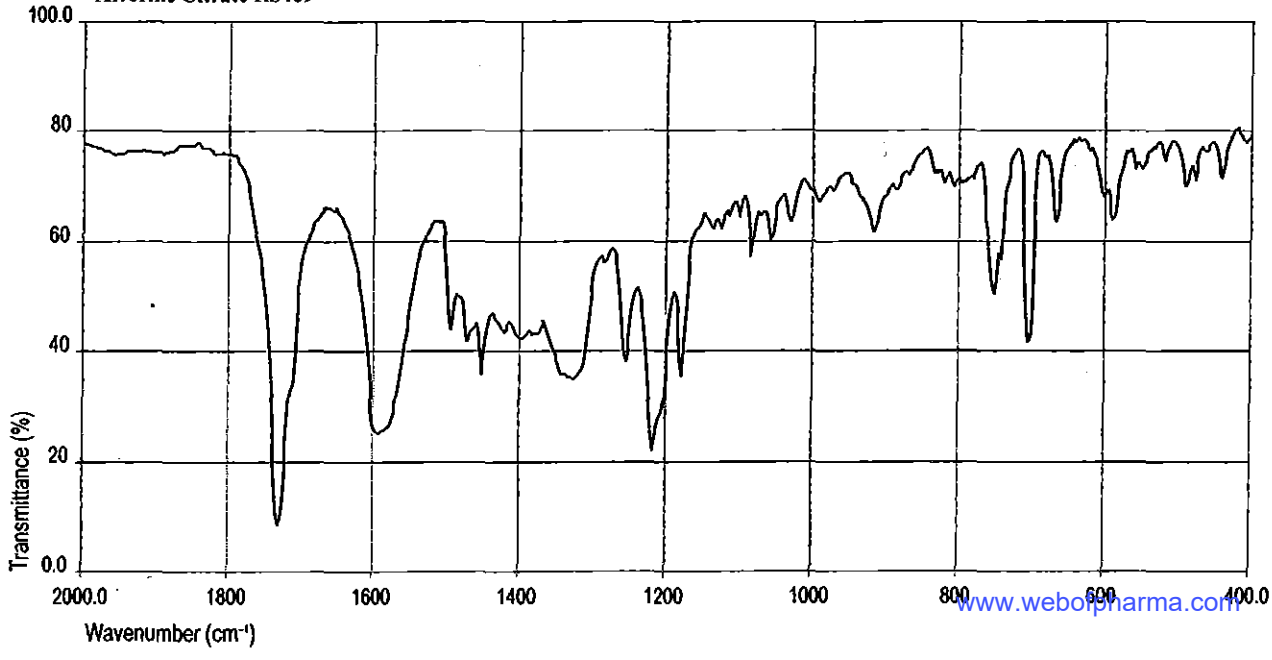
Phase: 10% w/v Solution in Chloroform Thickness: 0.1mm

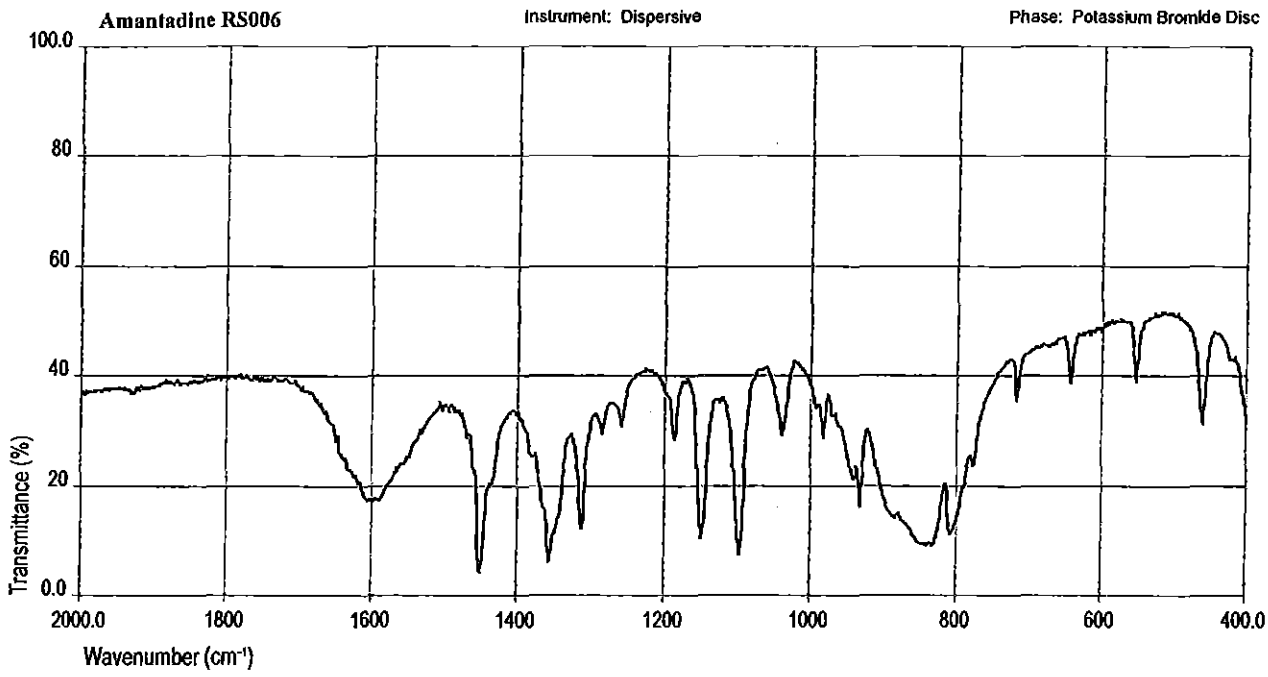


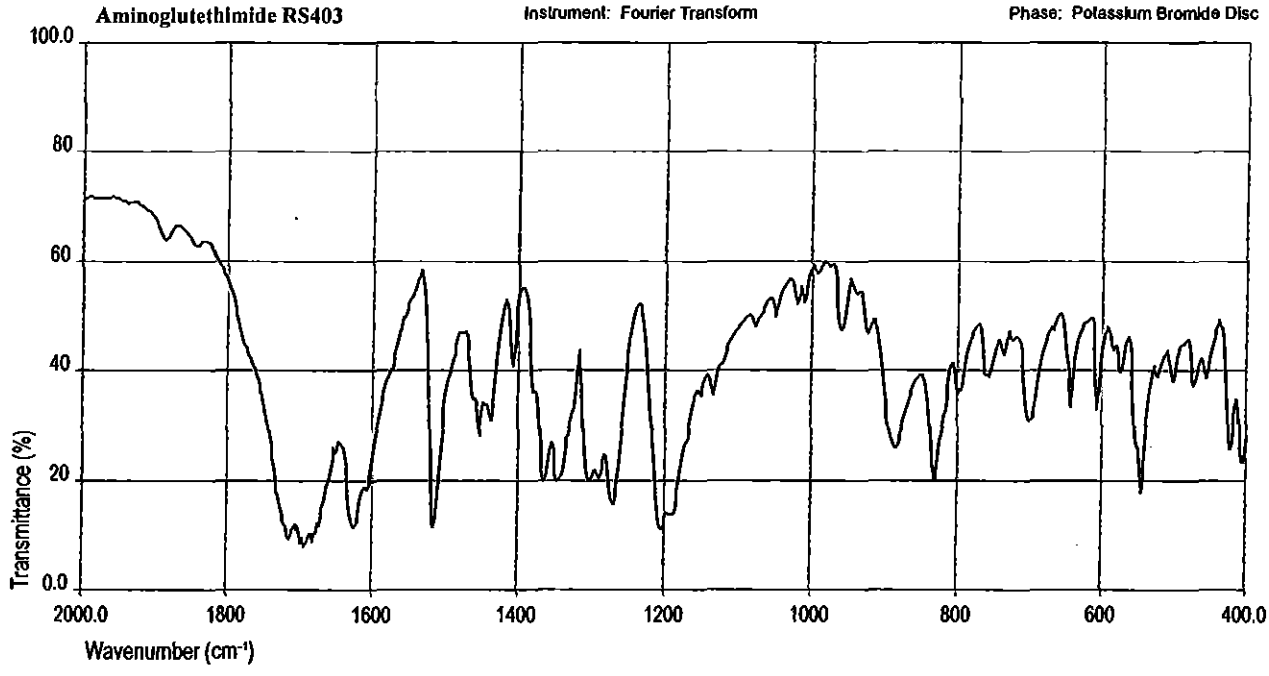
Alverine Citrate RS409

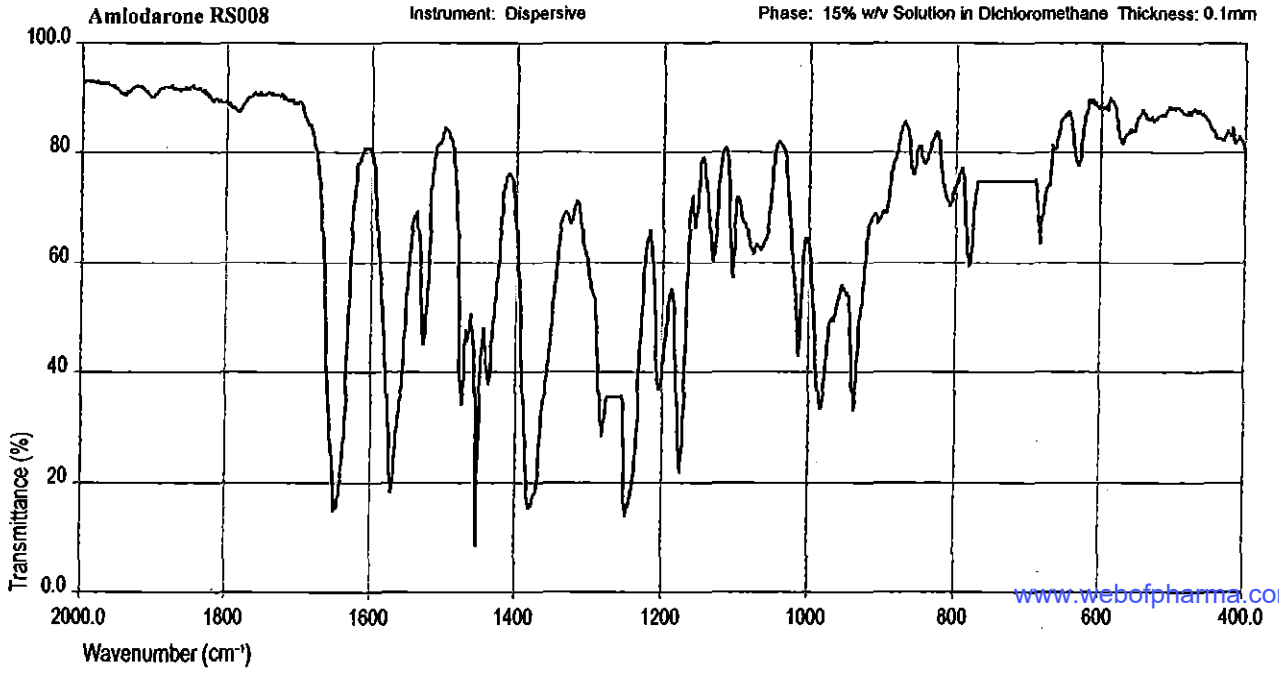
Instrument: Fourier Transform

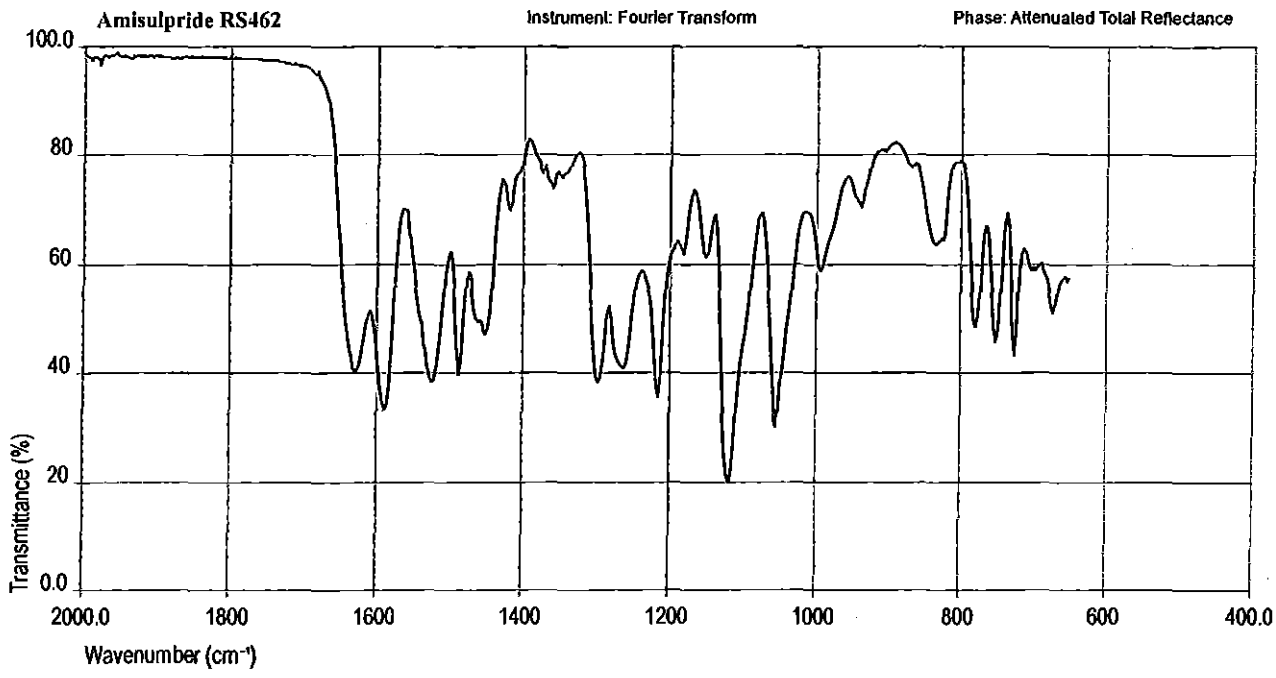
Phase: Potassium Bromide Disc

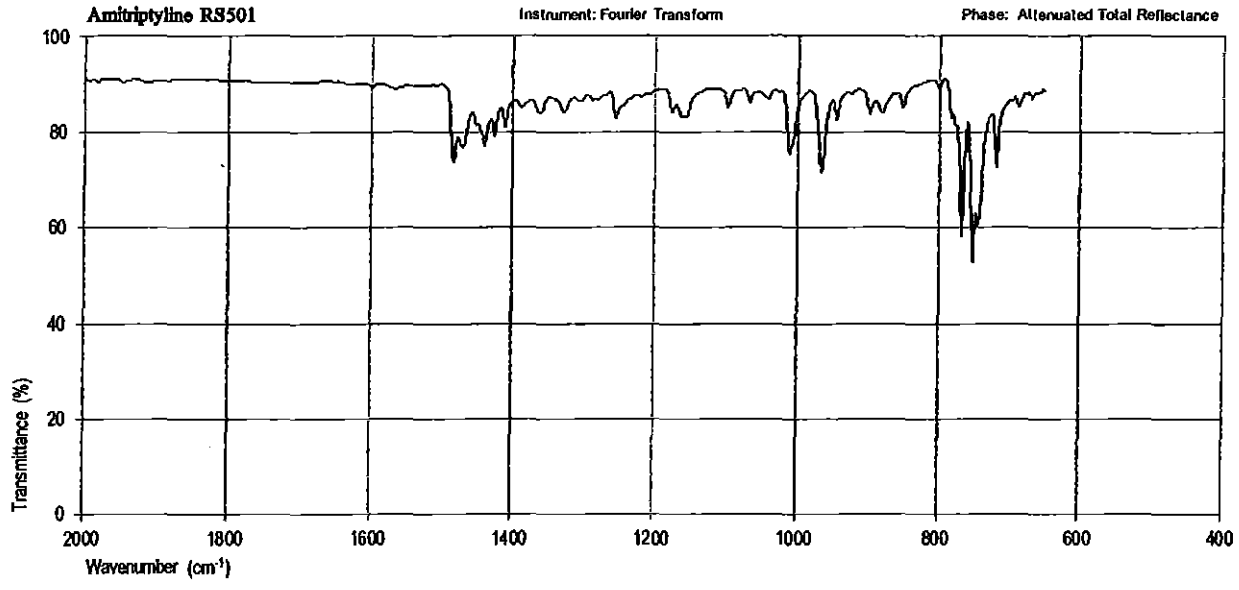


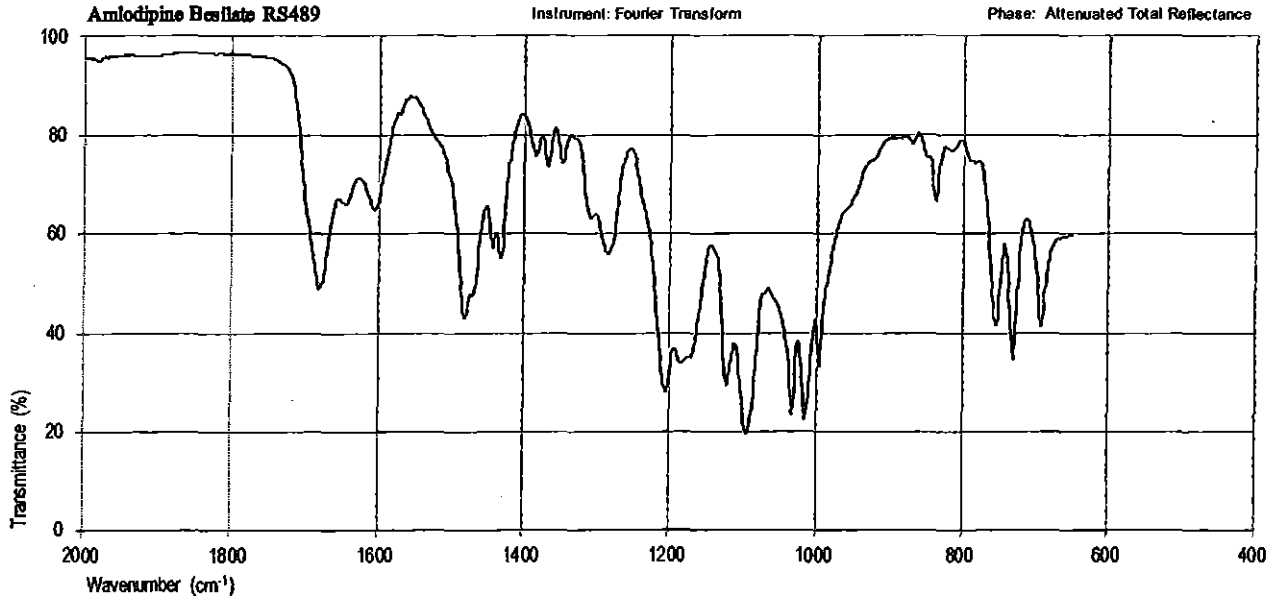


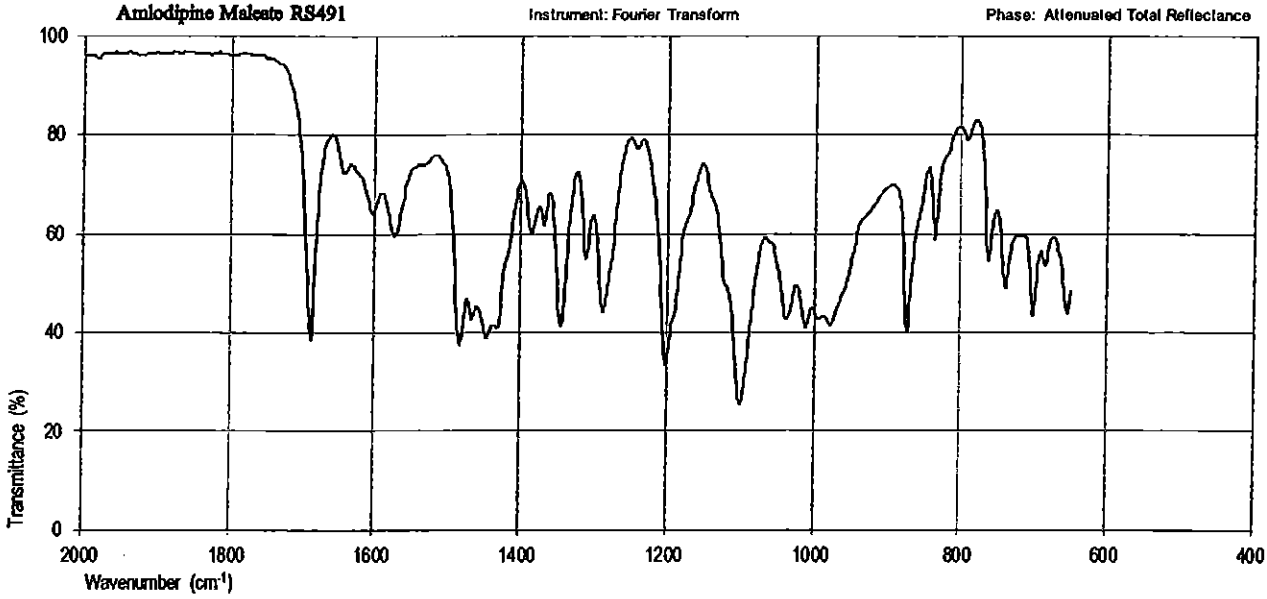


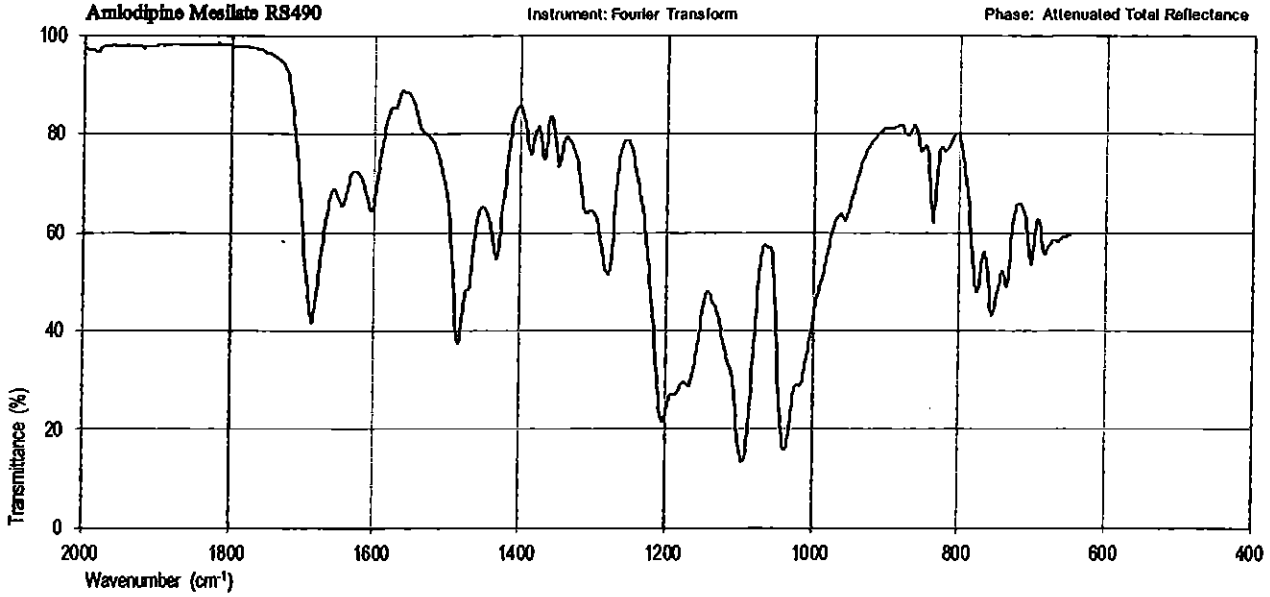


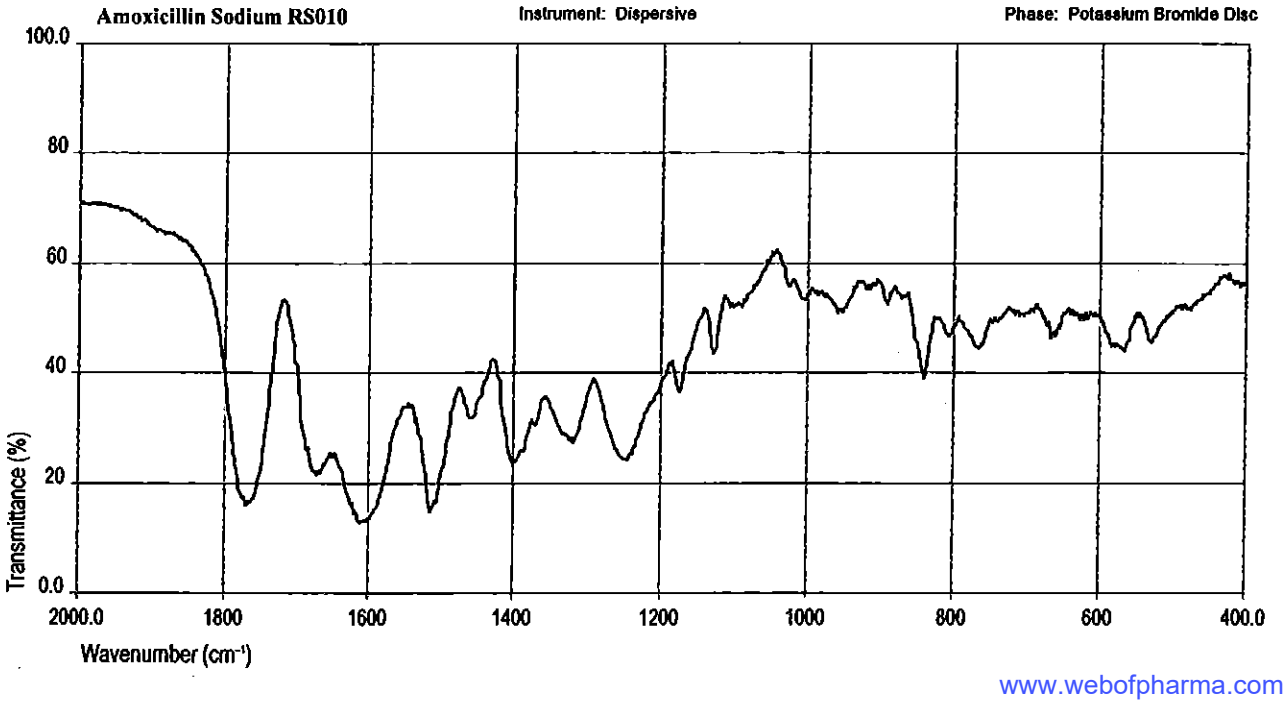


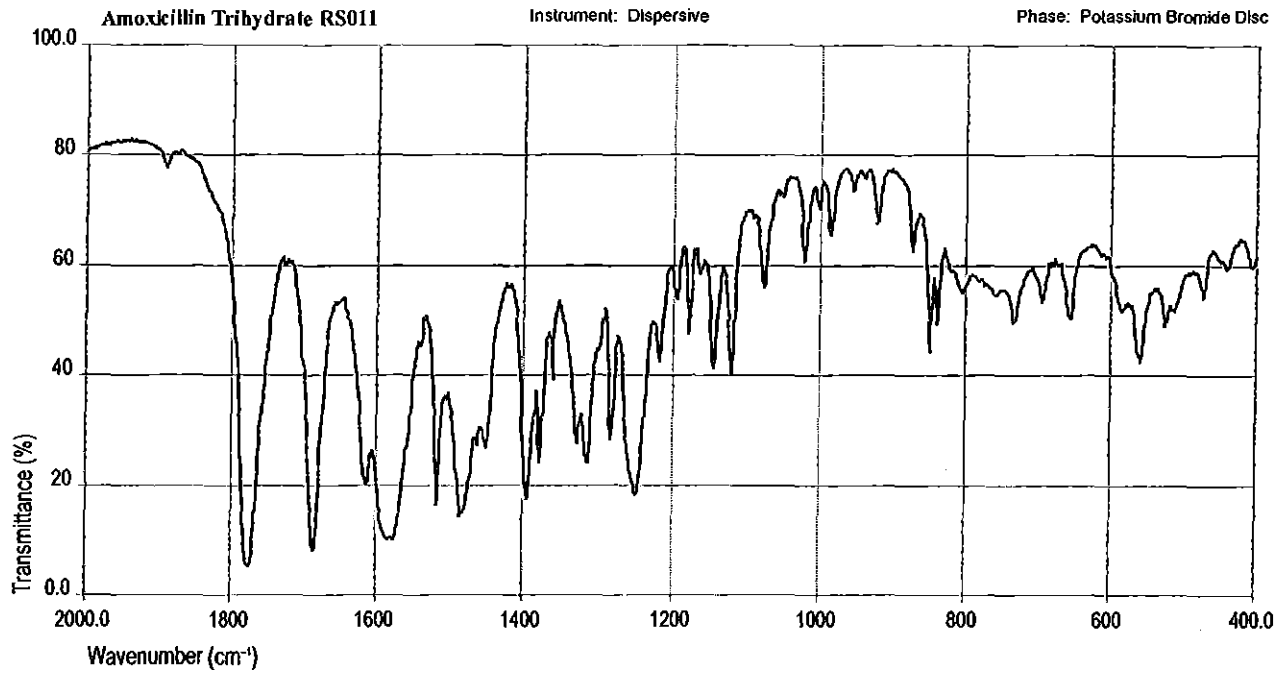


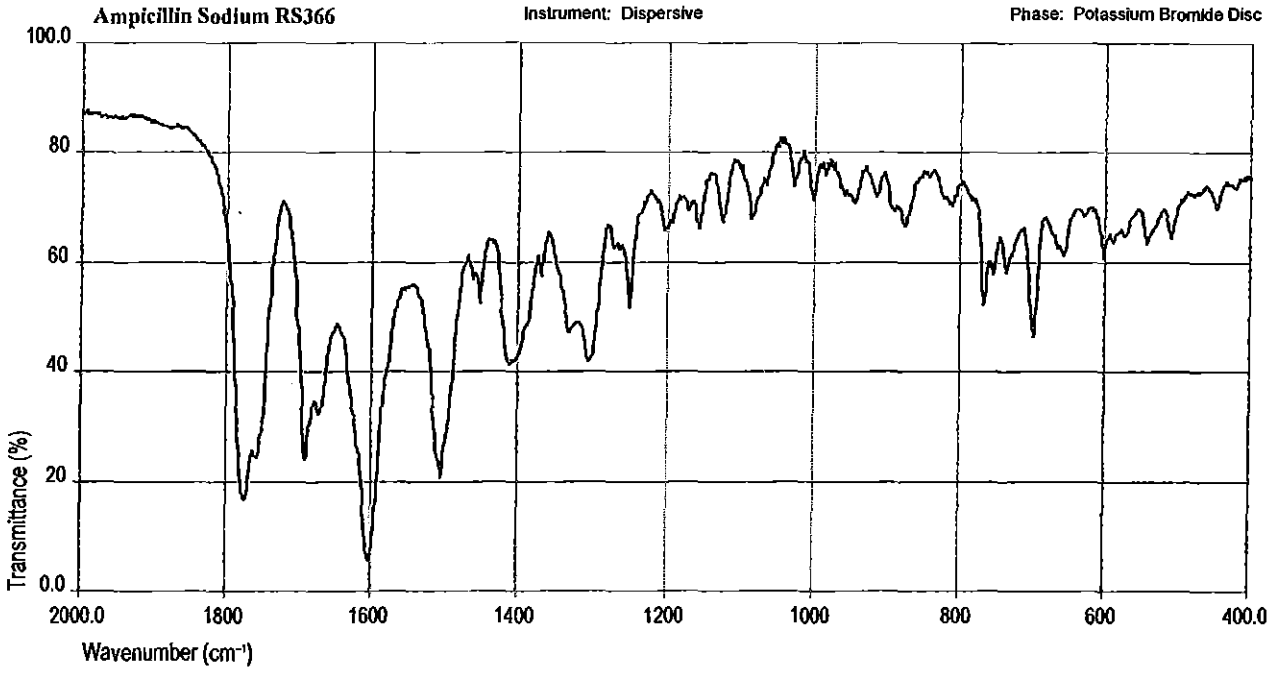


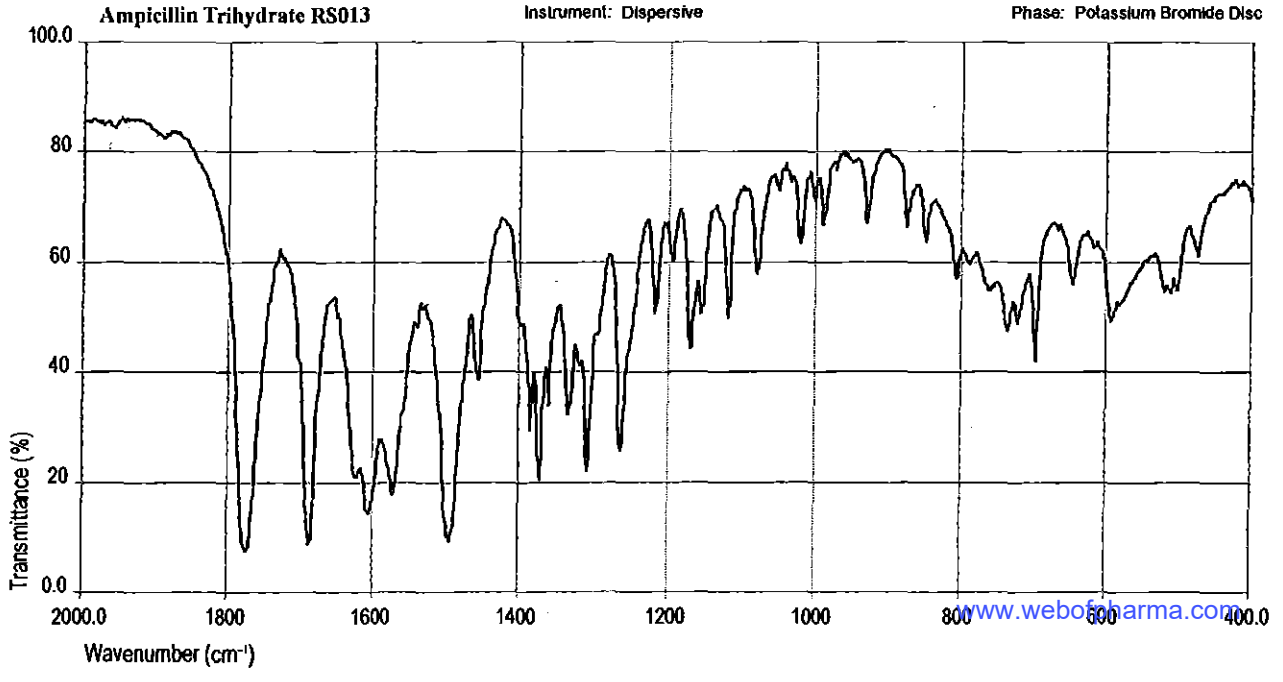


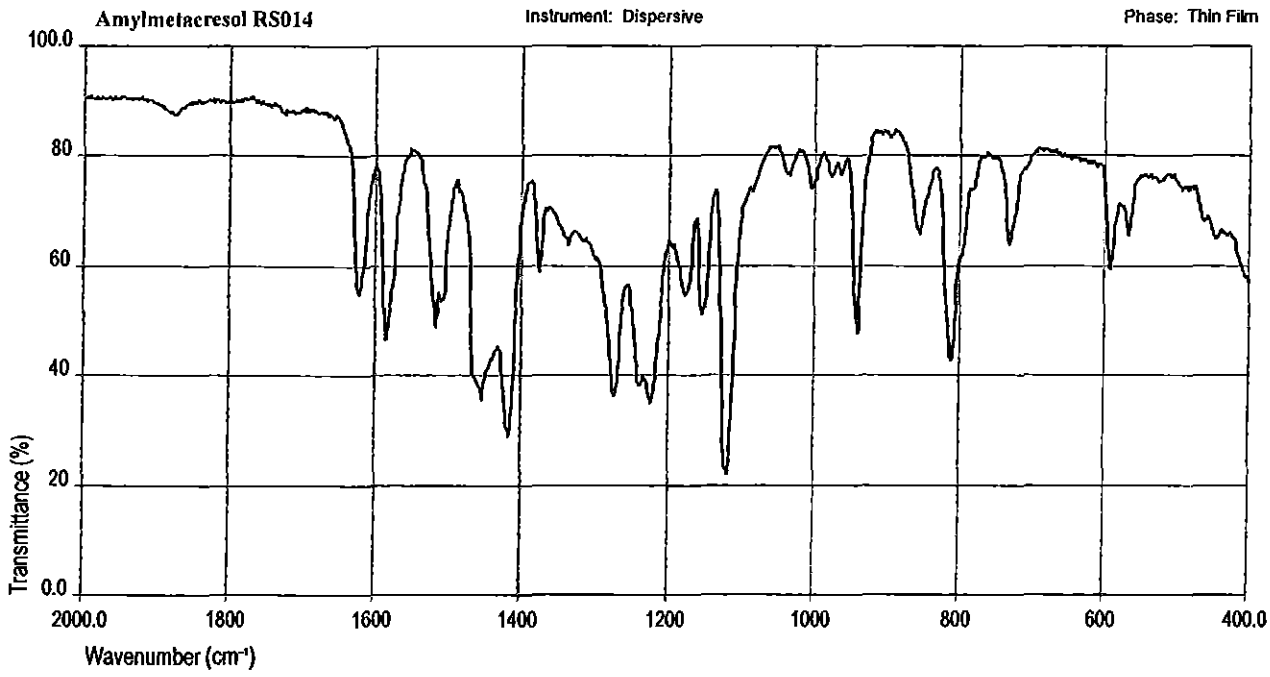


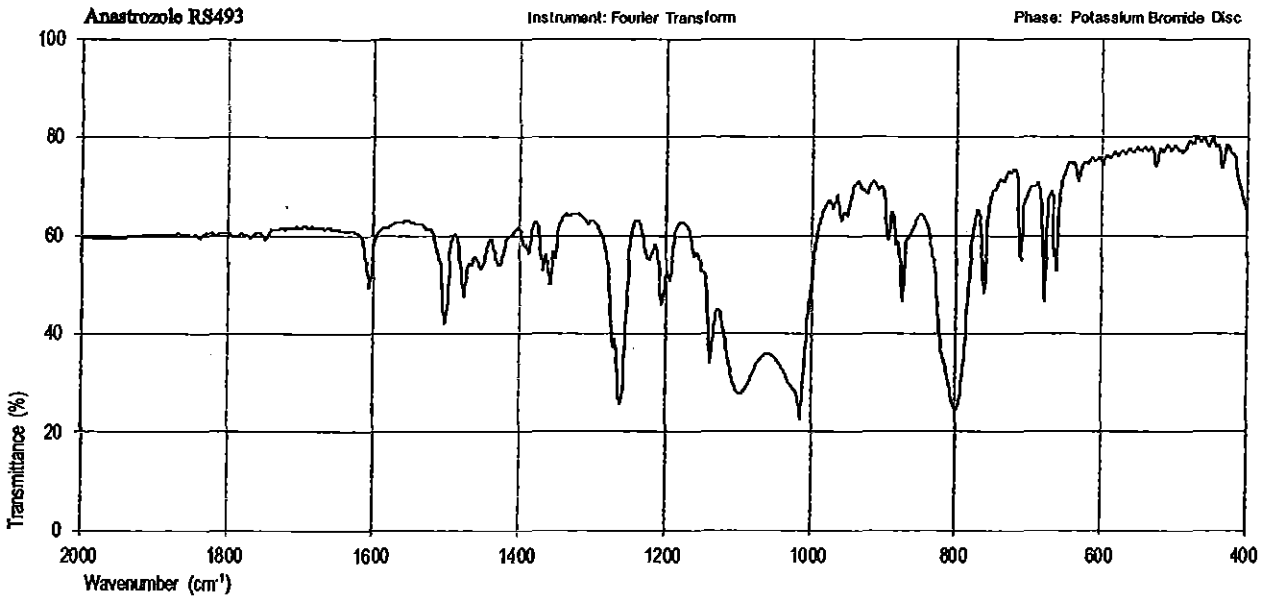


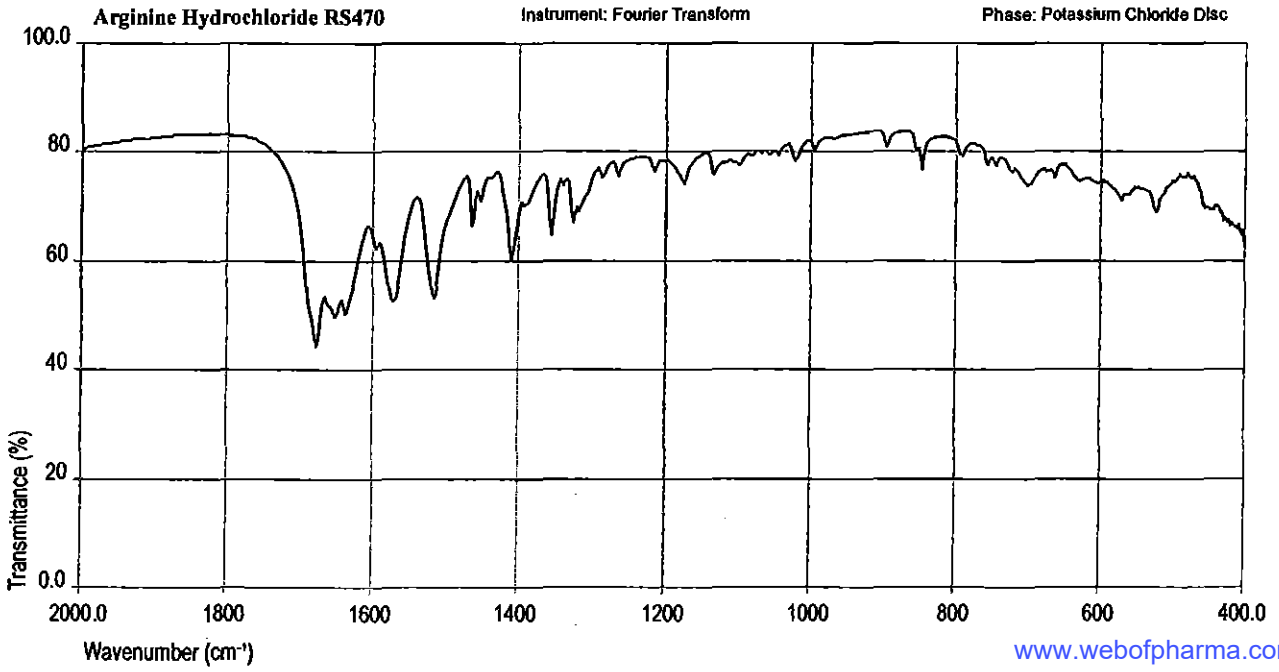


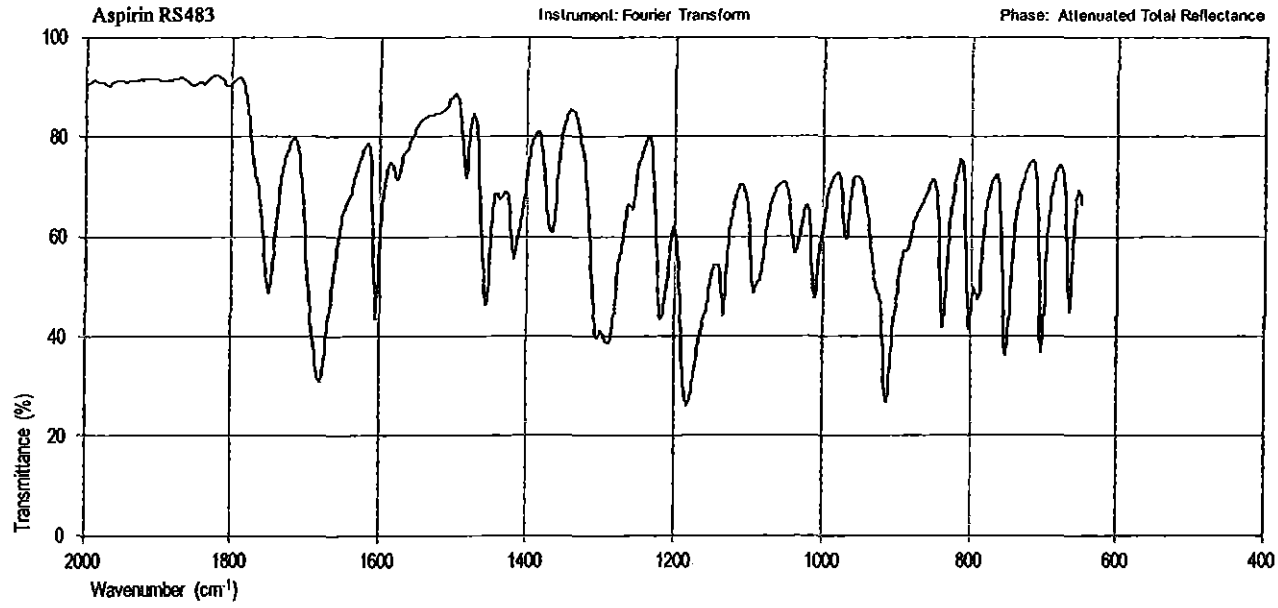


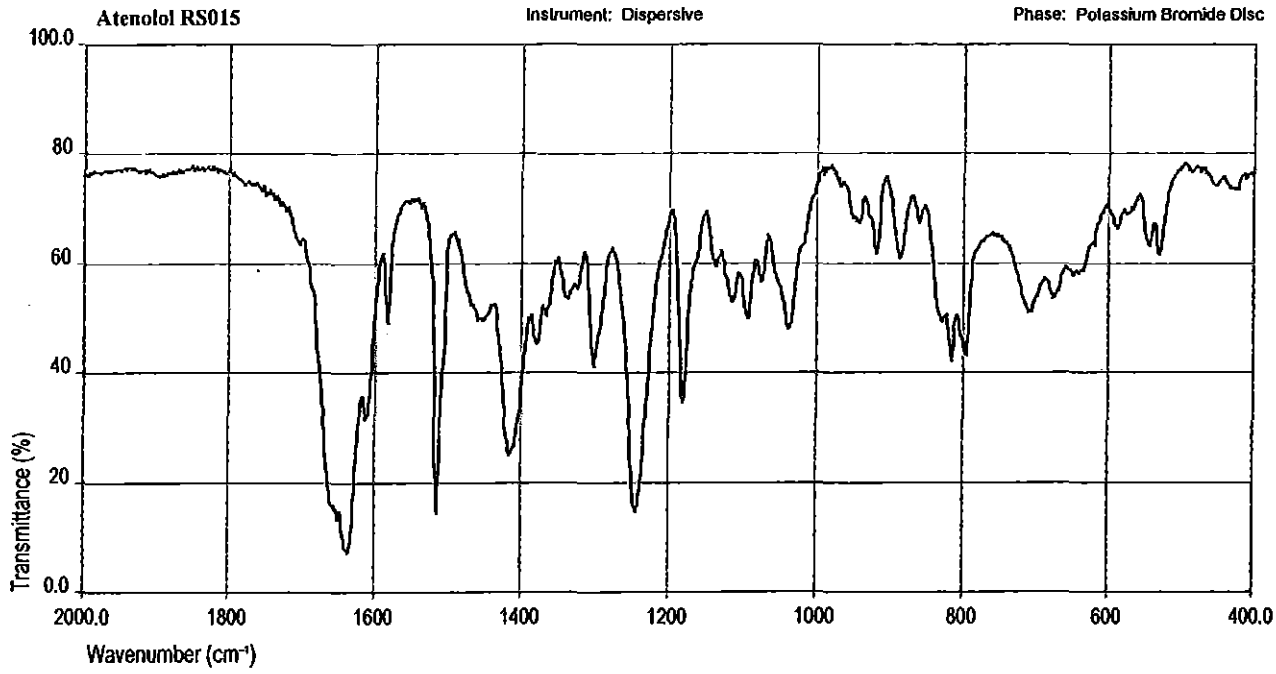


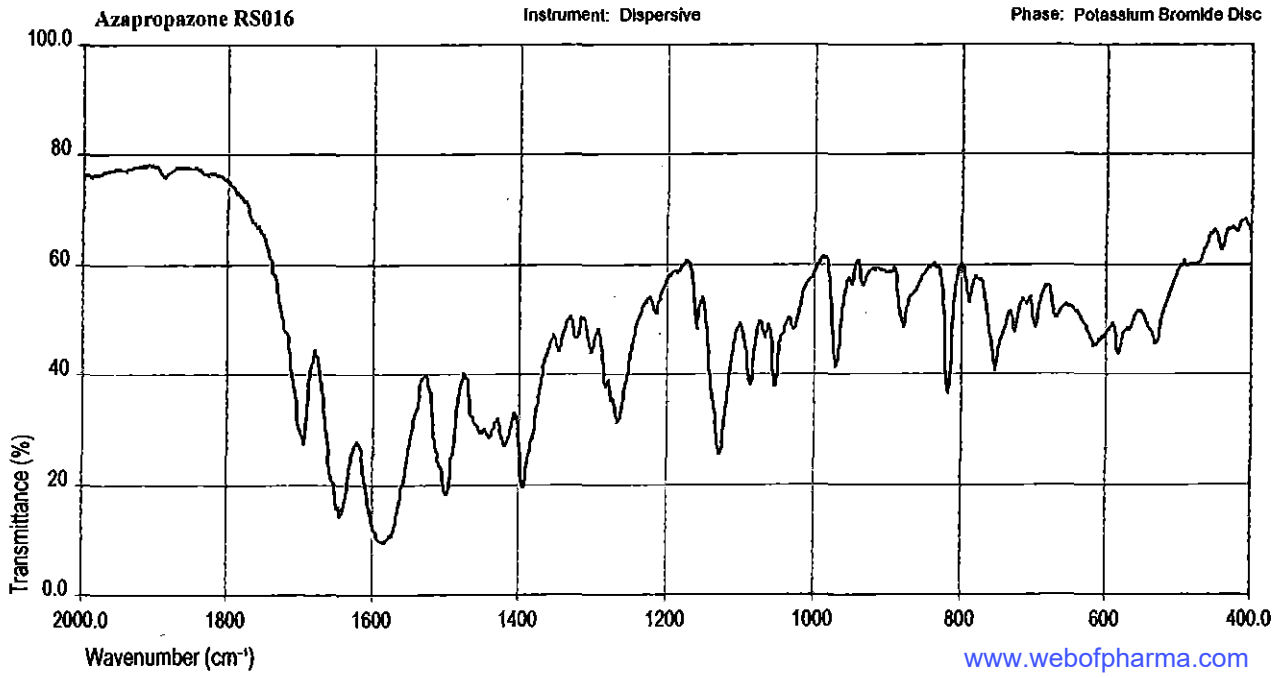


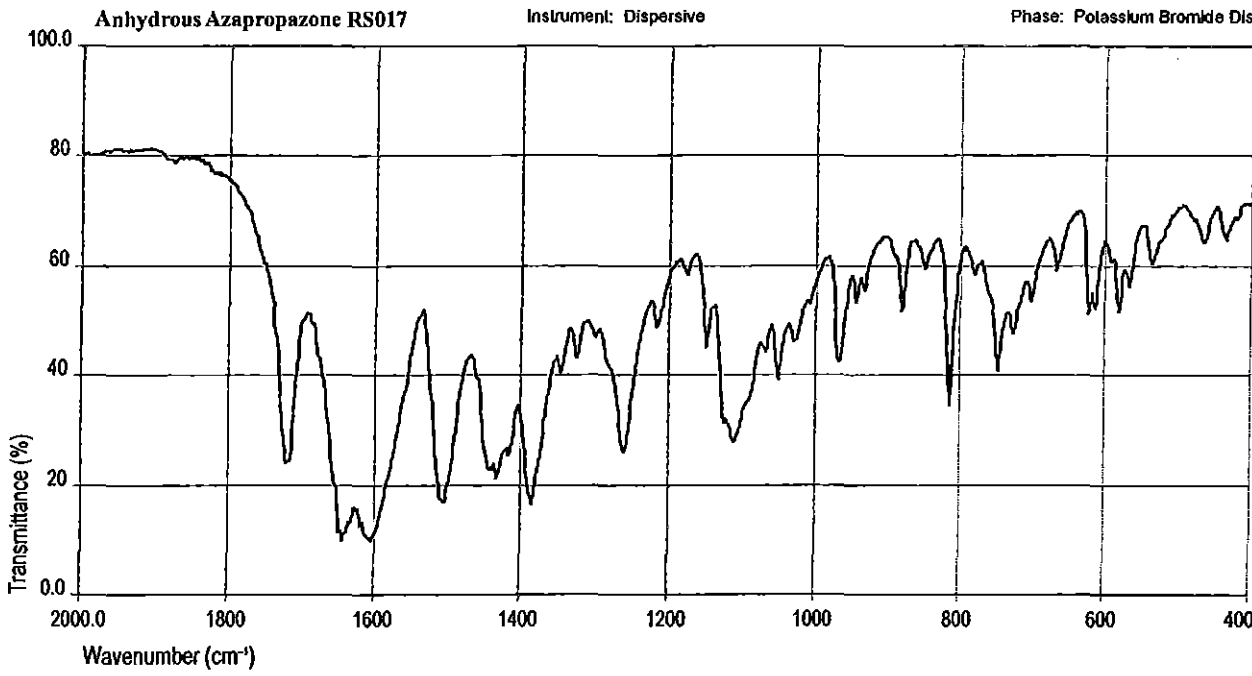


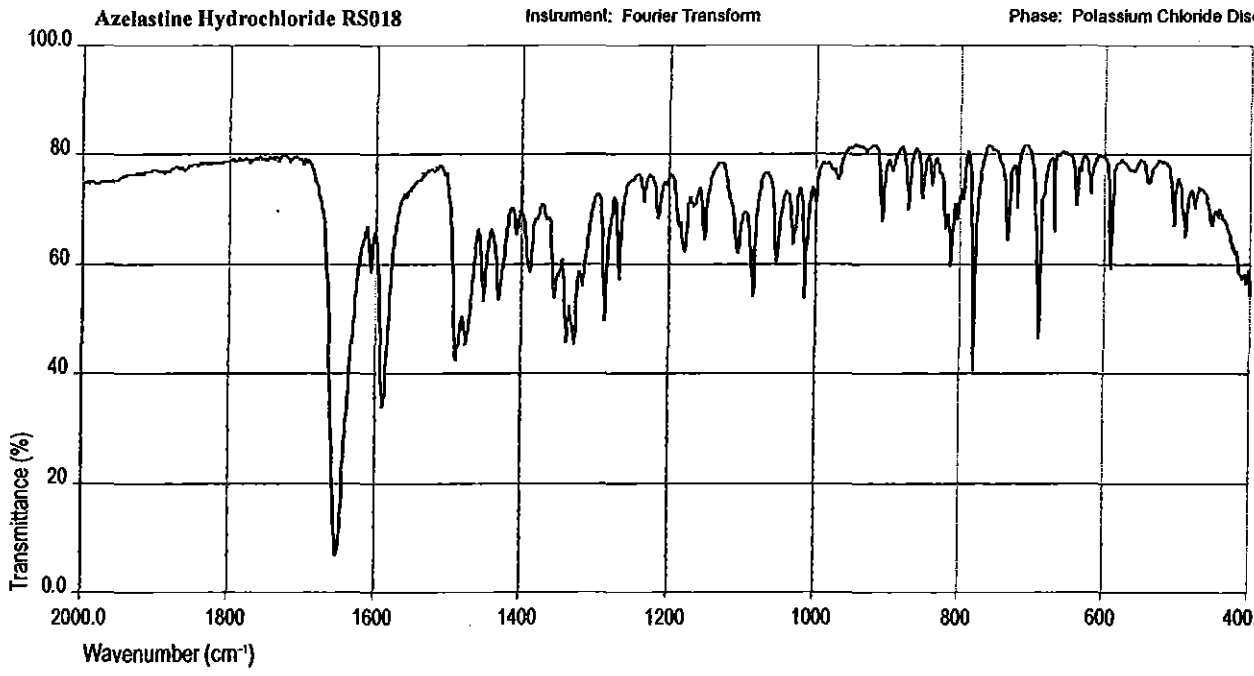


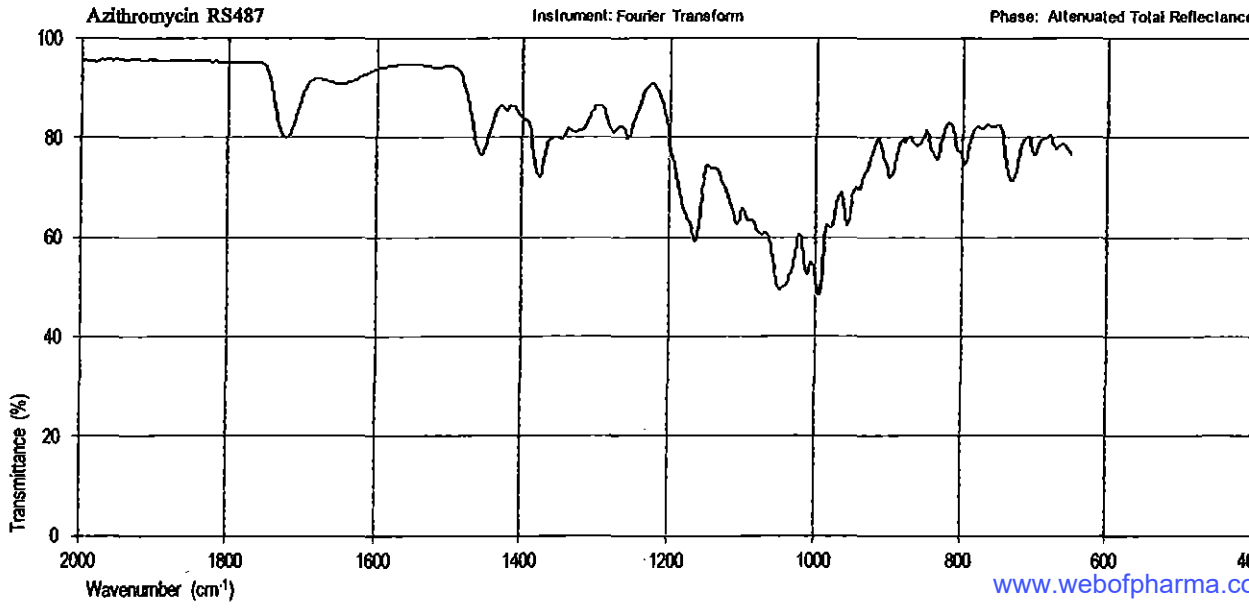


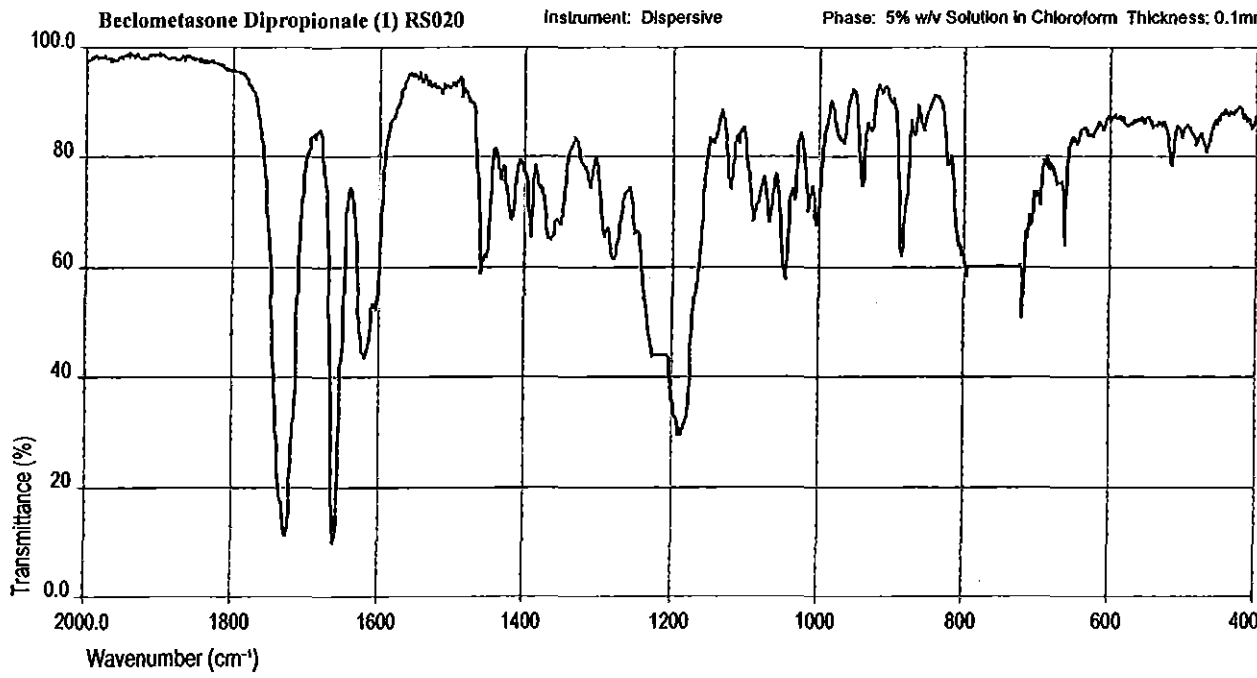


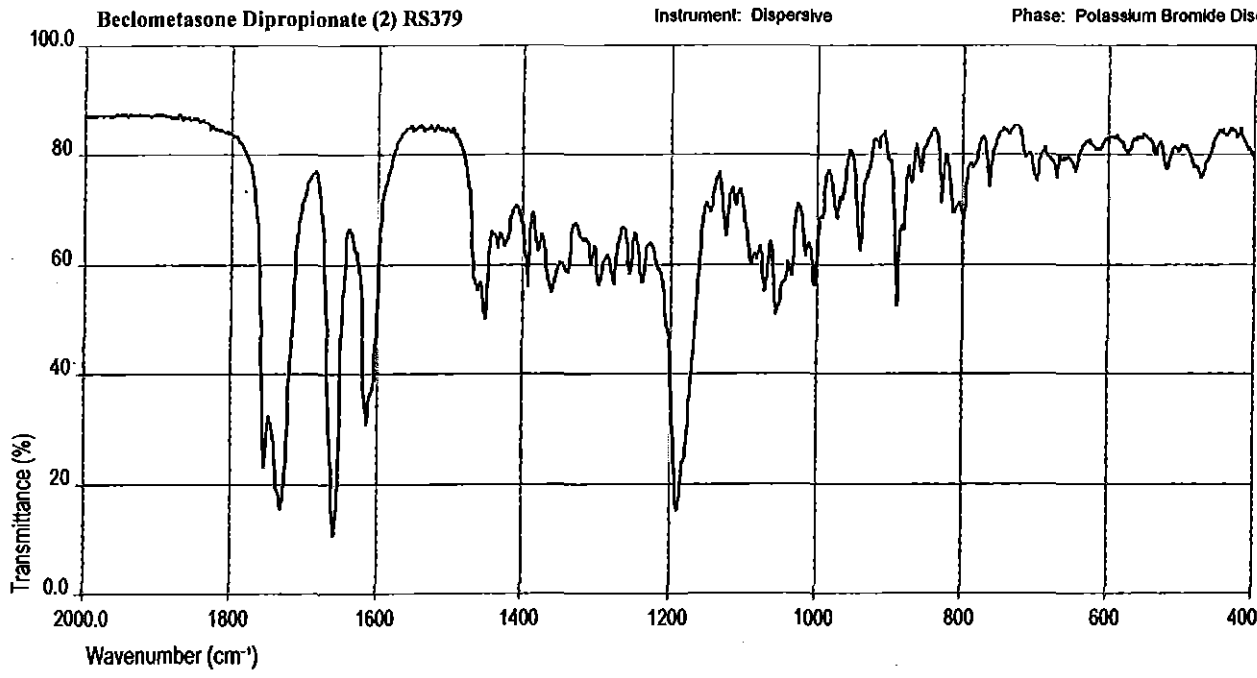


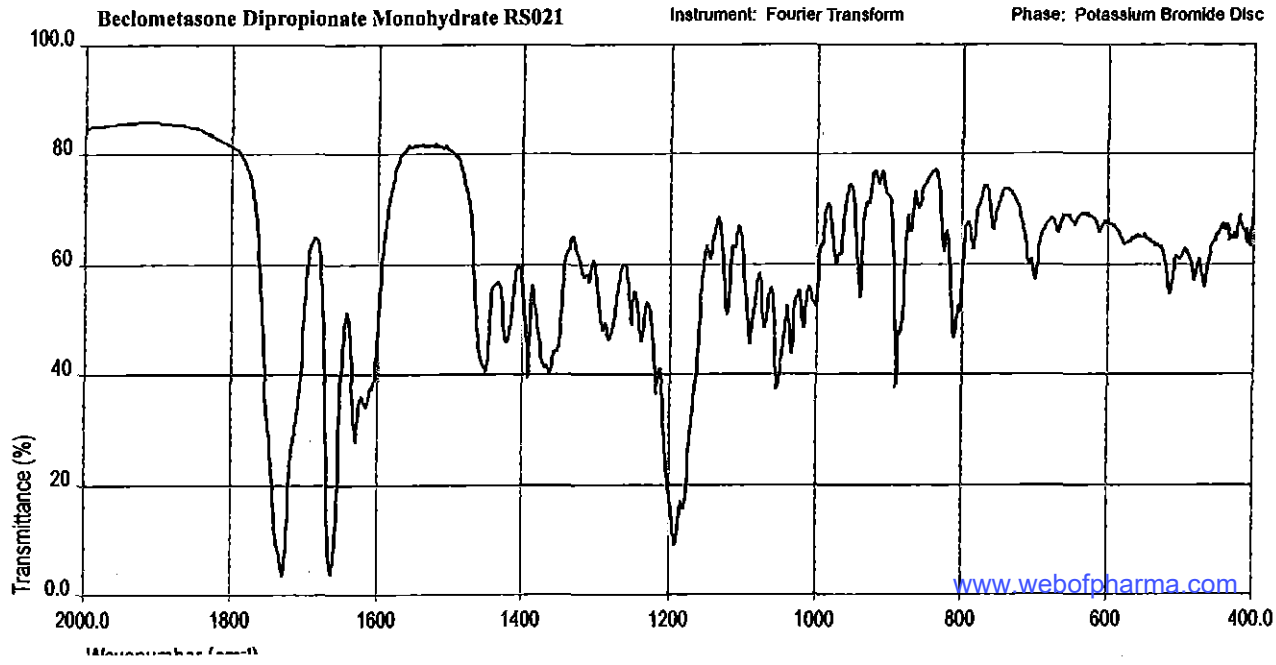


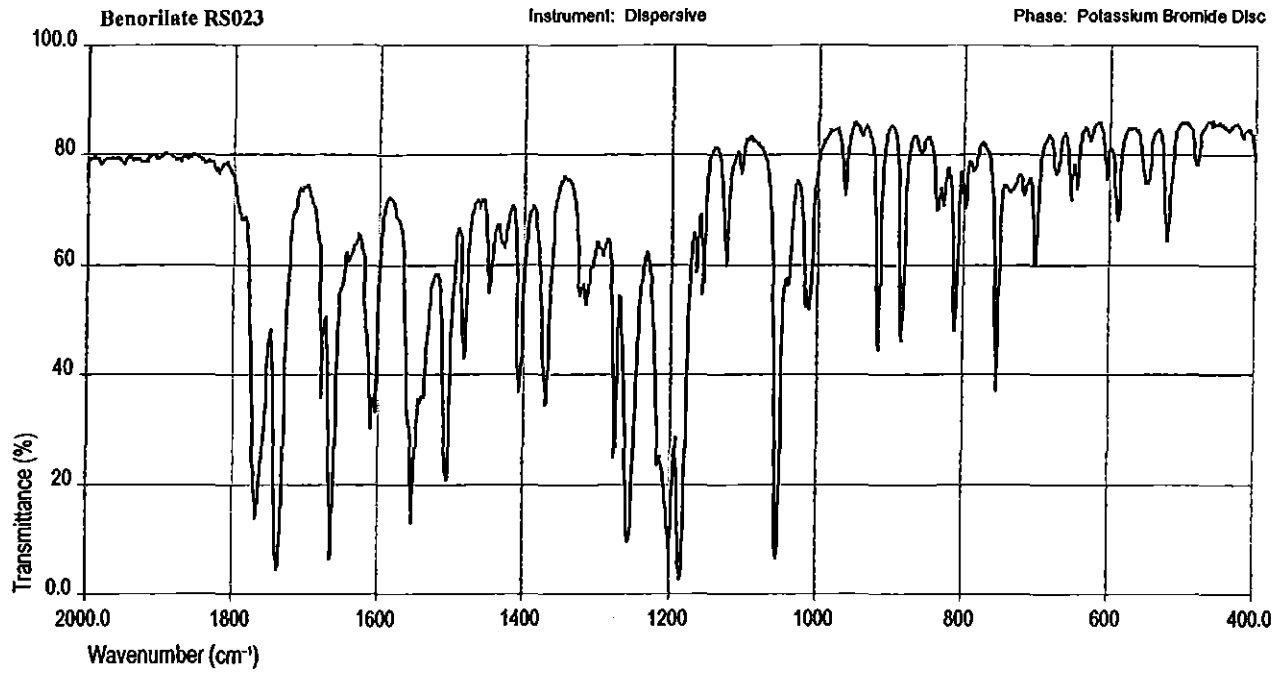


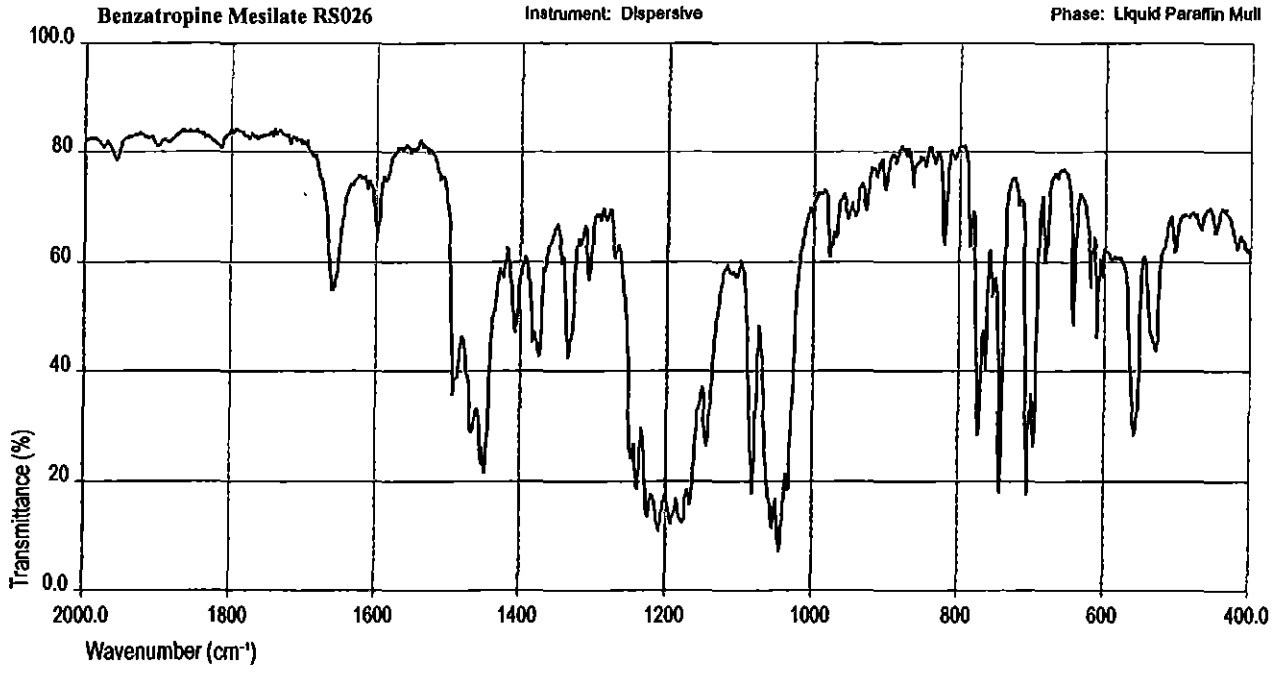


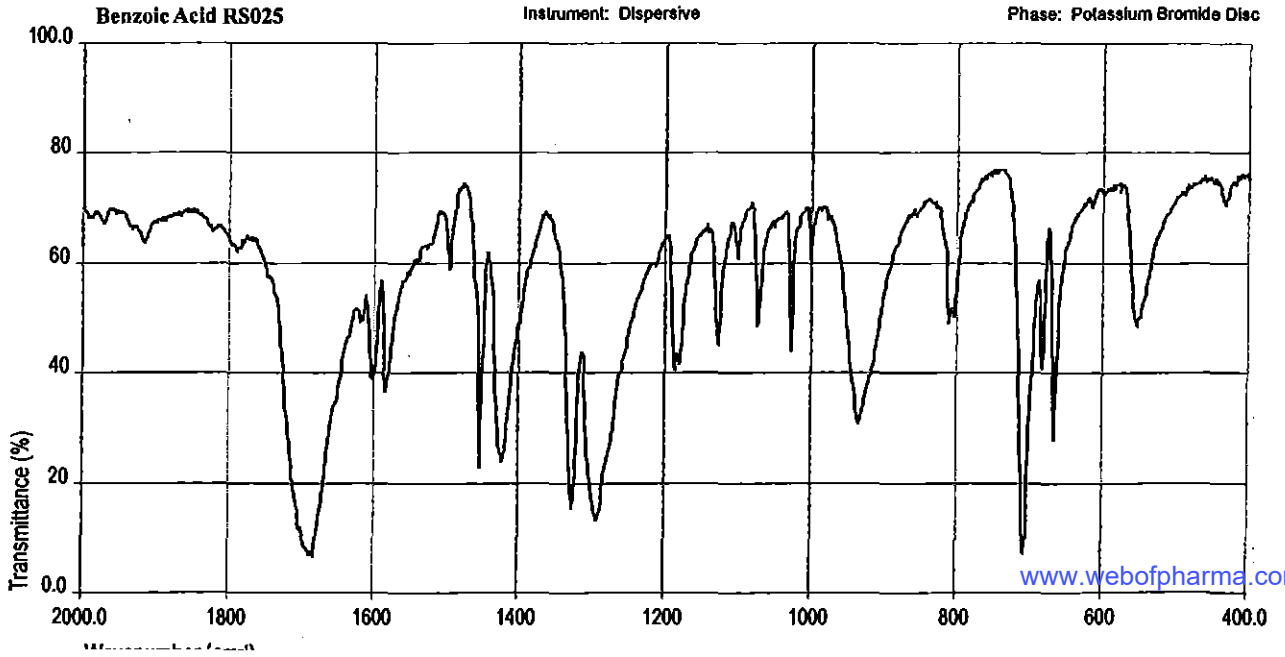




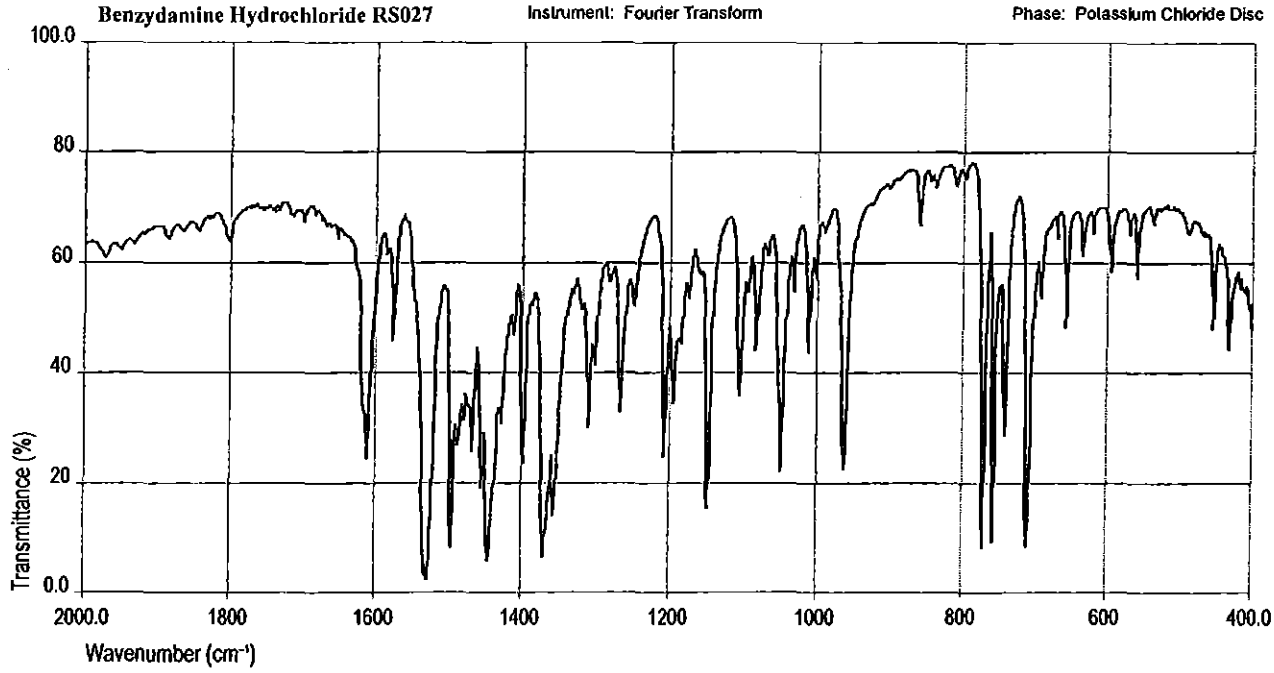




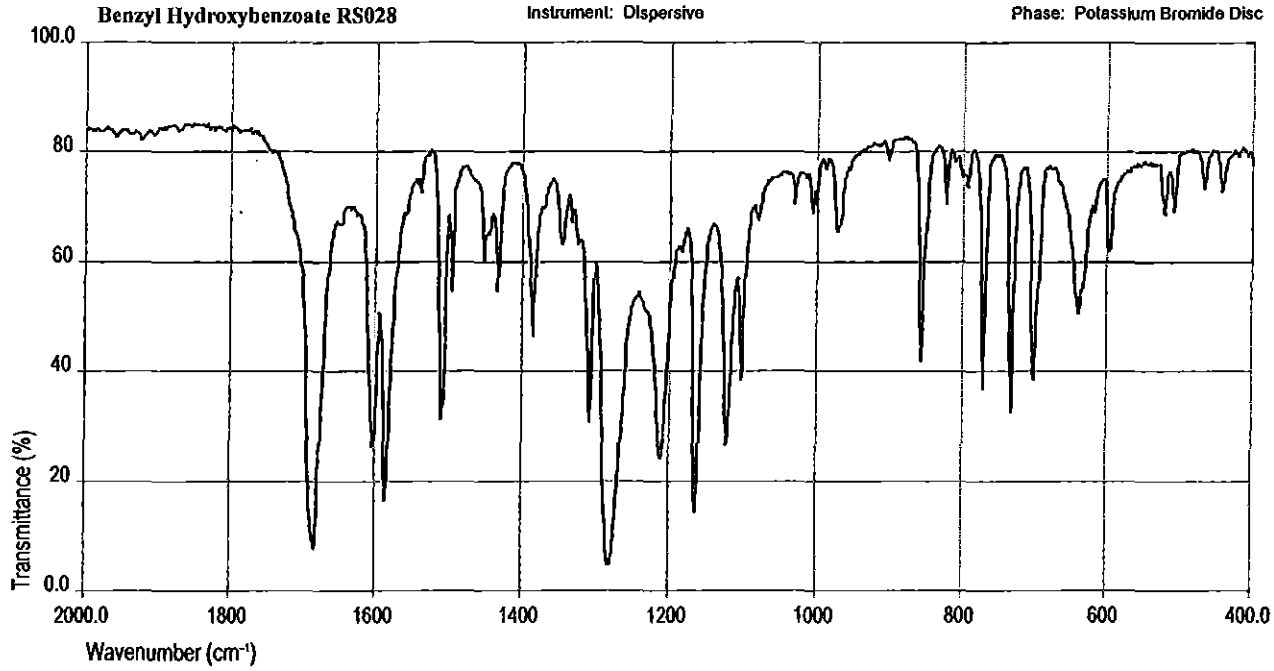




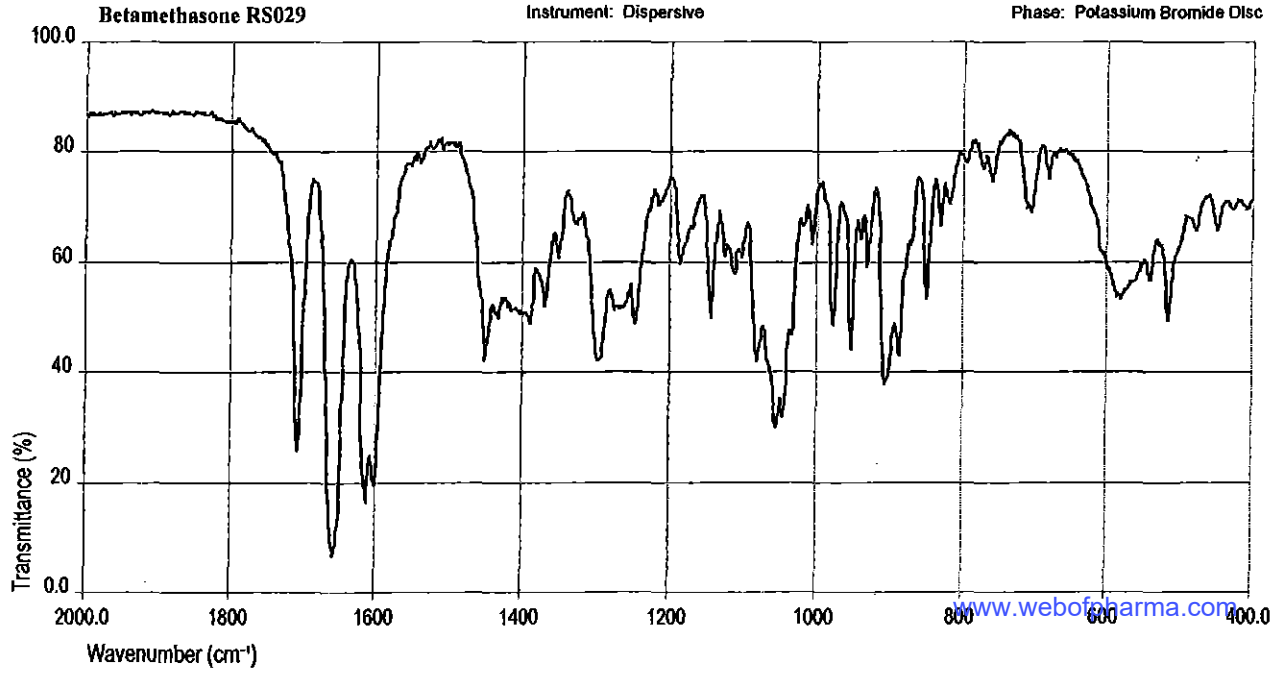
Benzydamine Hydrochloride RS027 Instrument: Fourier Transform Phase: Potassium Chloride Disc

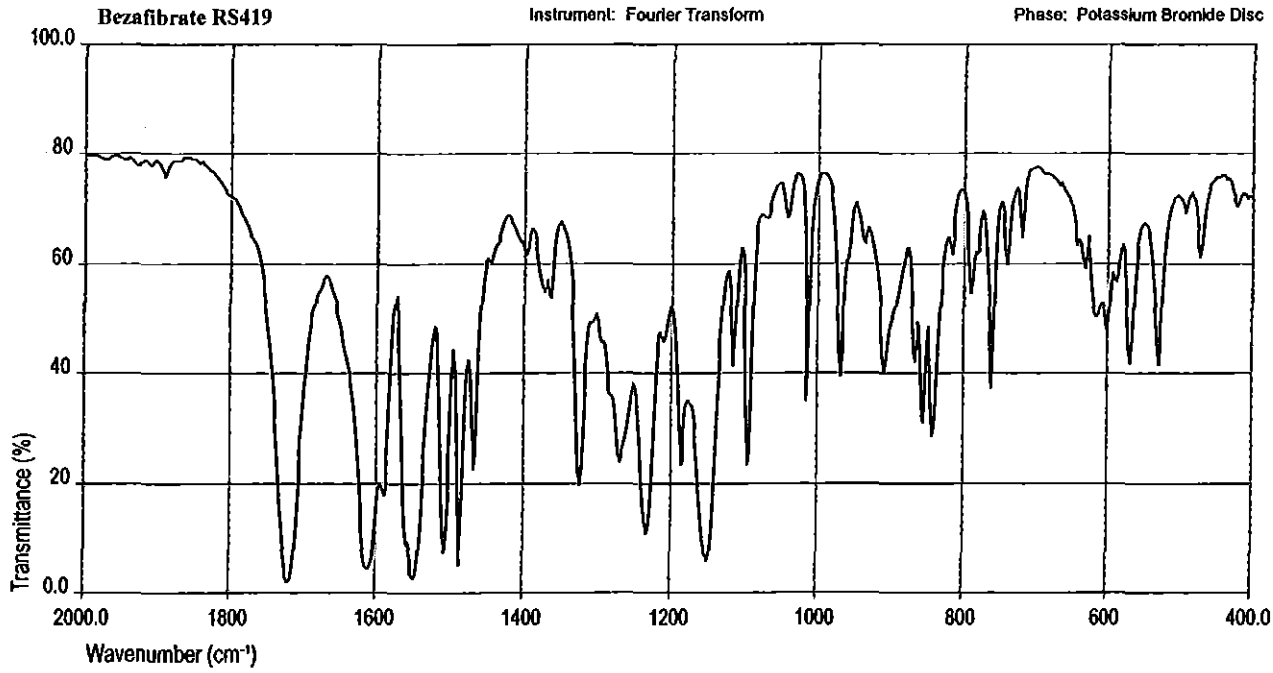


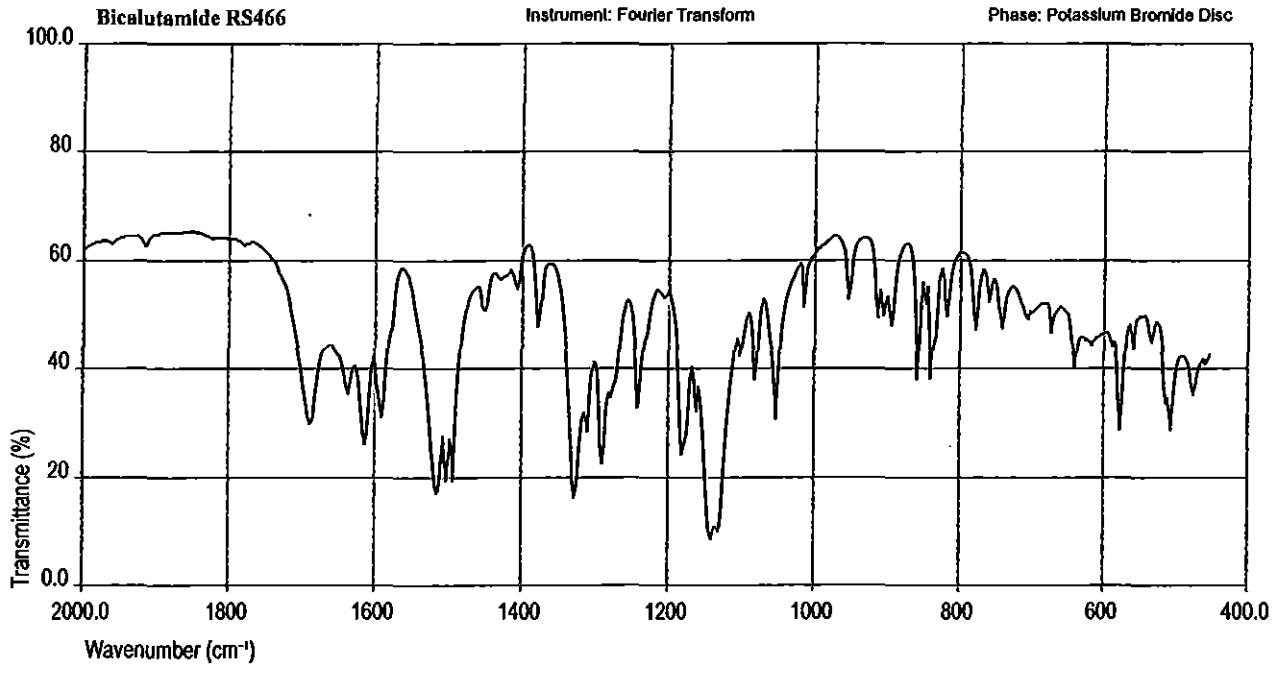
Benzyl Hydroxybenzoate RS028 Instrument: Dispersive Phase: Potassium Bromide Disc

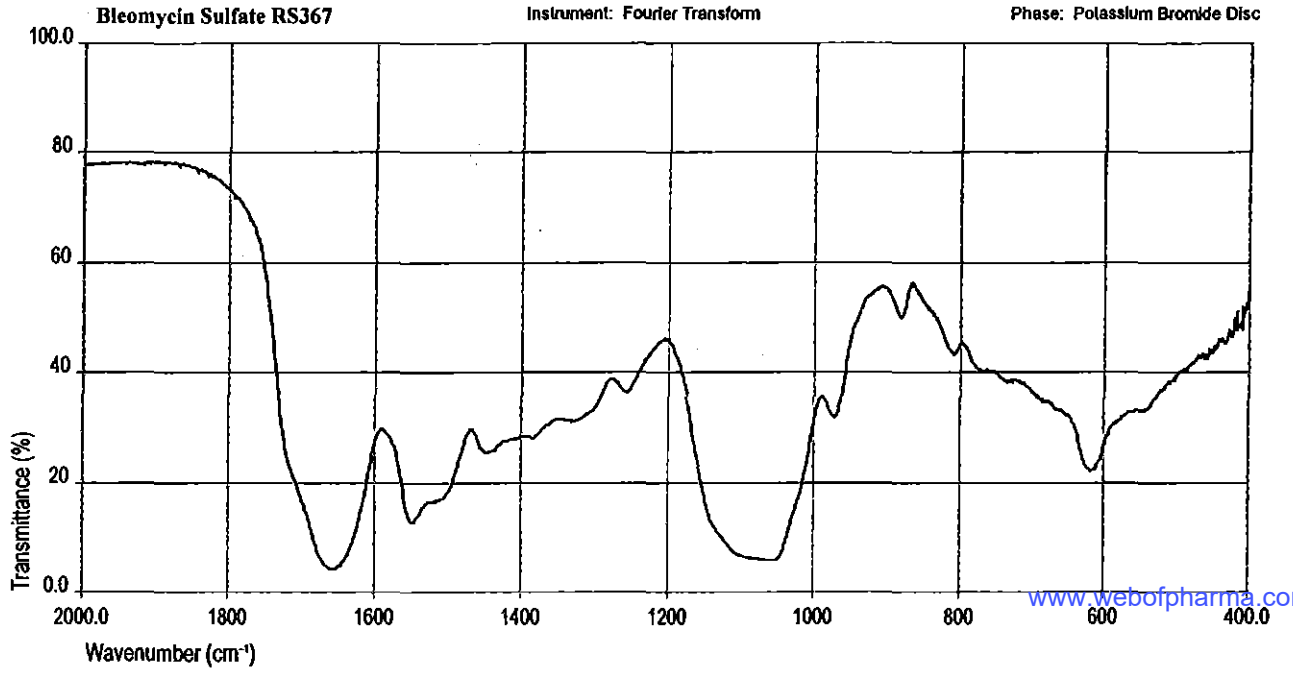


Betamethasone RS029 Instrument: Dispersive Phase: Potassium Bromide Disc





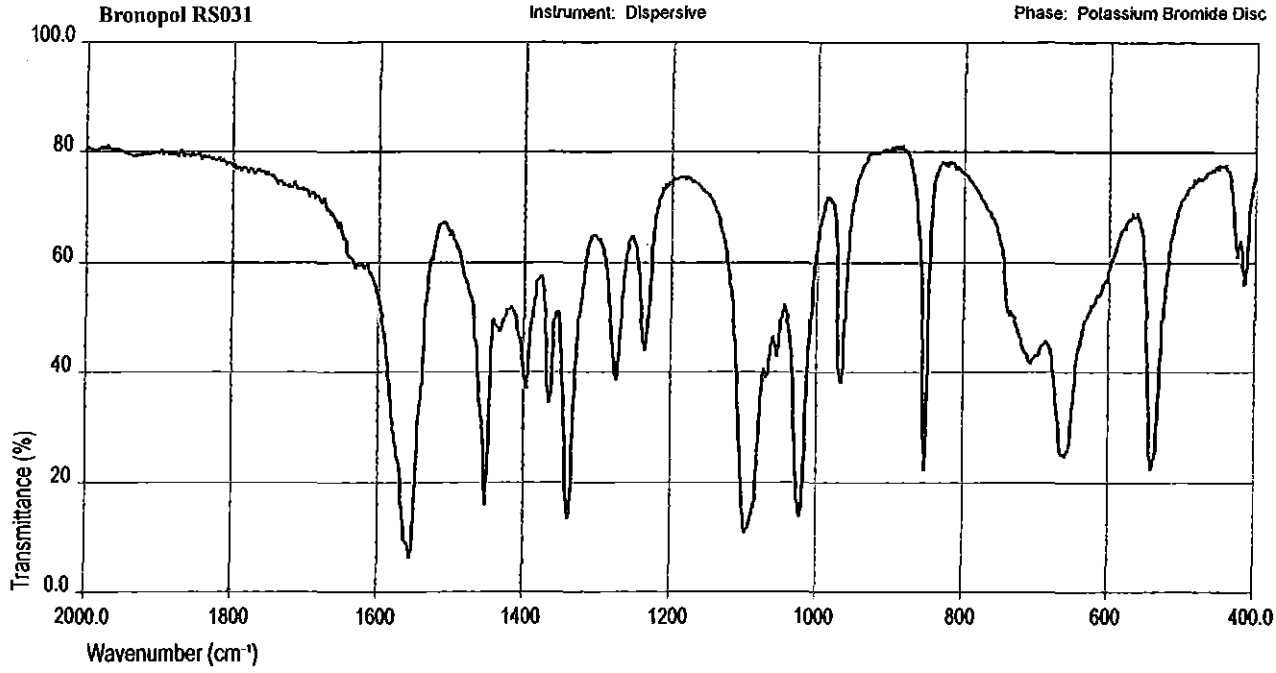




Bronopol RS031

Instrument: Dispersive

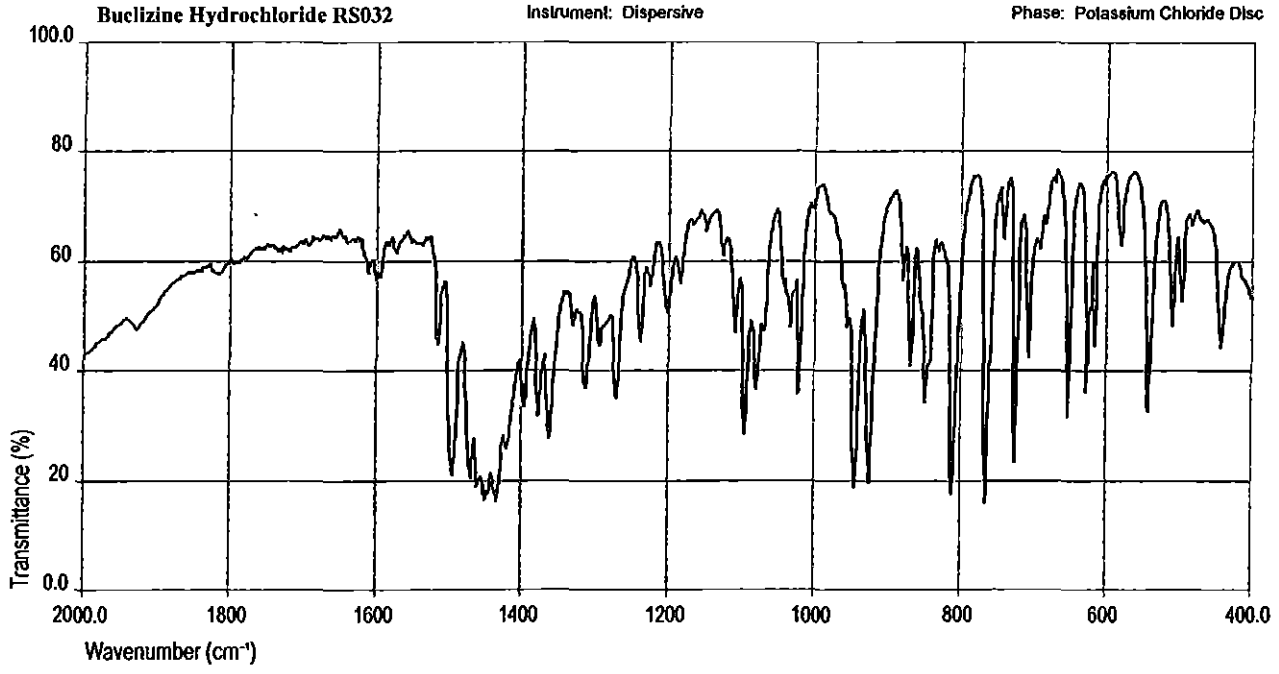
Phase: Potassium Bromide Disc



Buclizine Hydrochloride RS032

Instrument: Dispersive

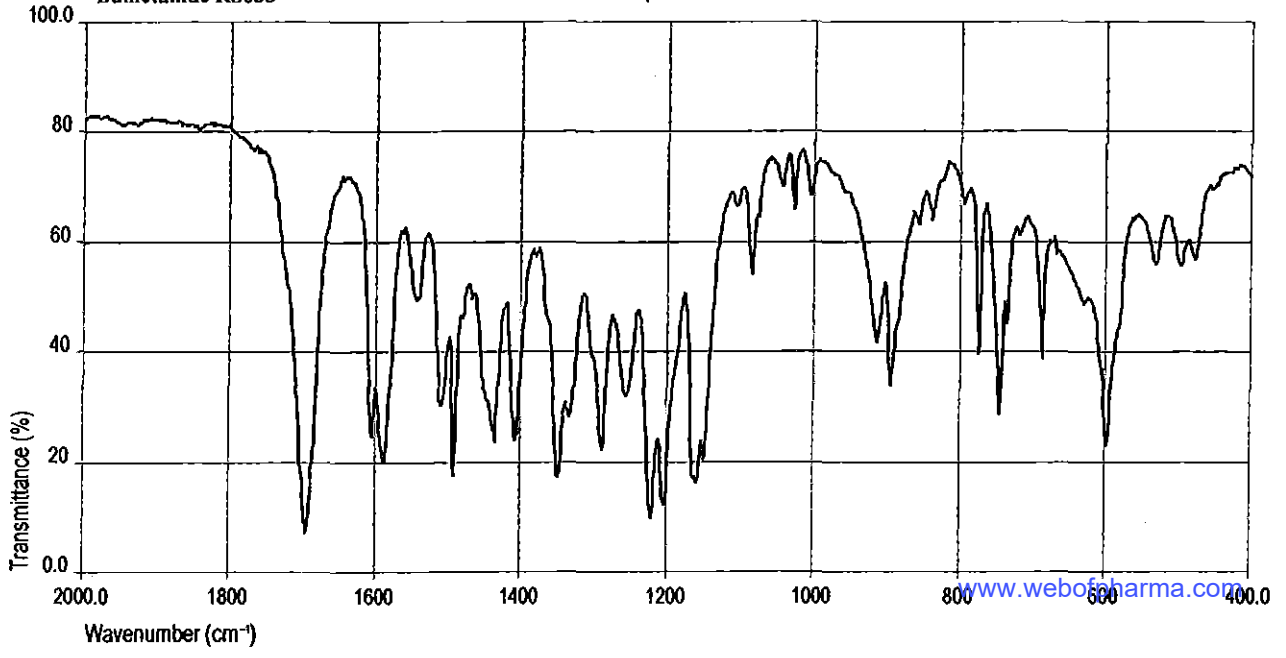
Phase: Potassium Chloride Disc

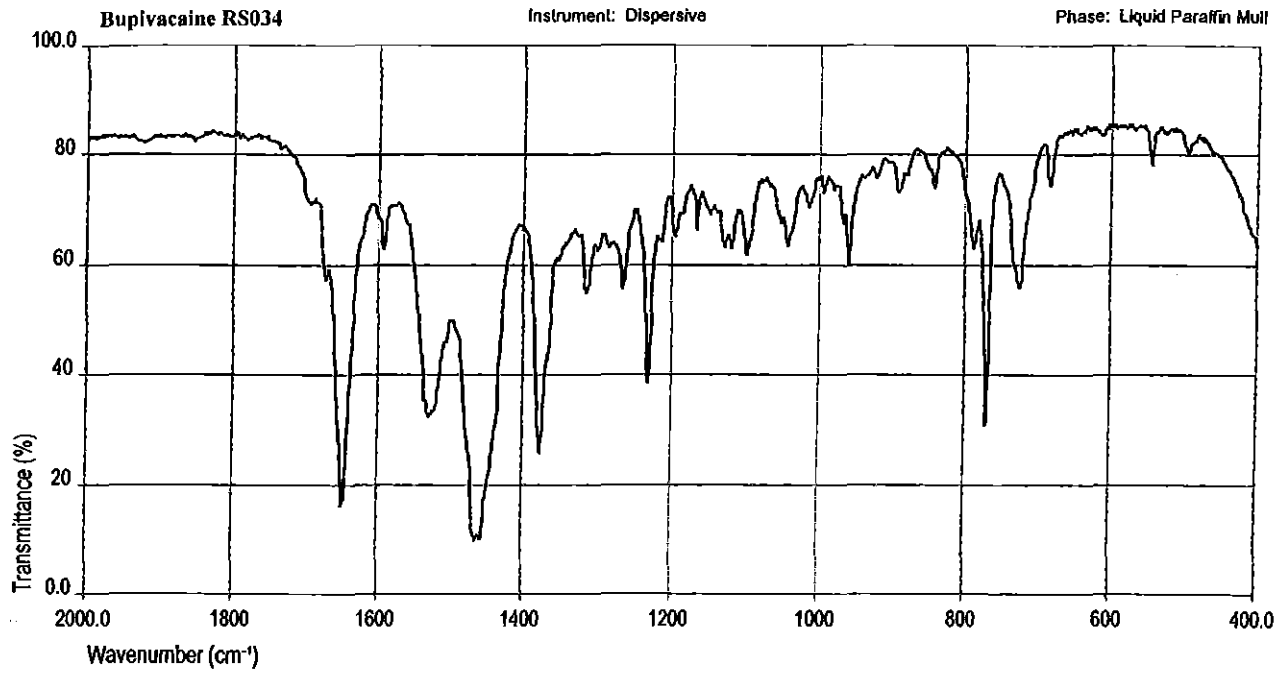


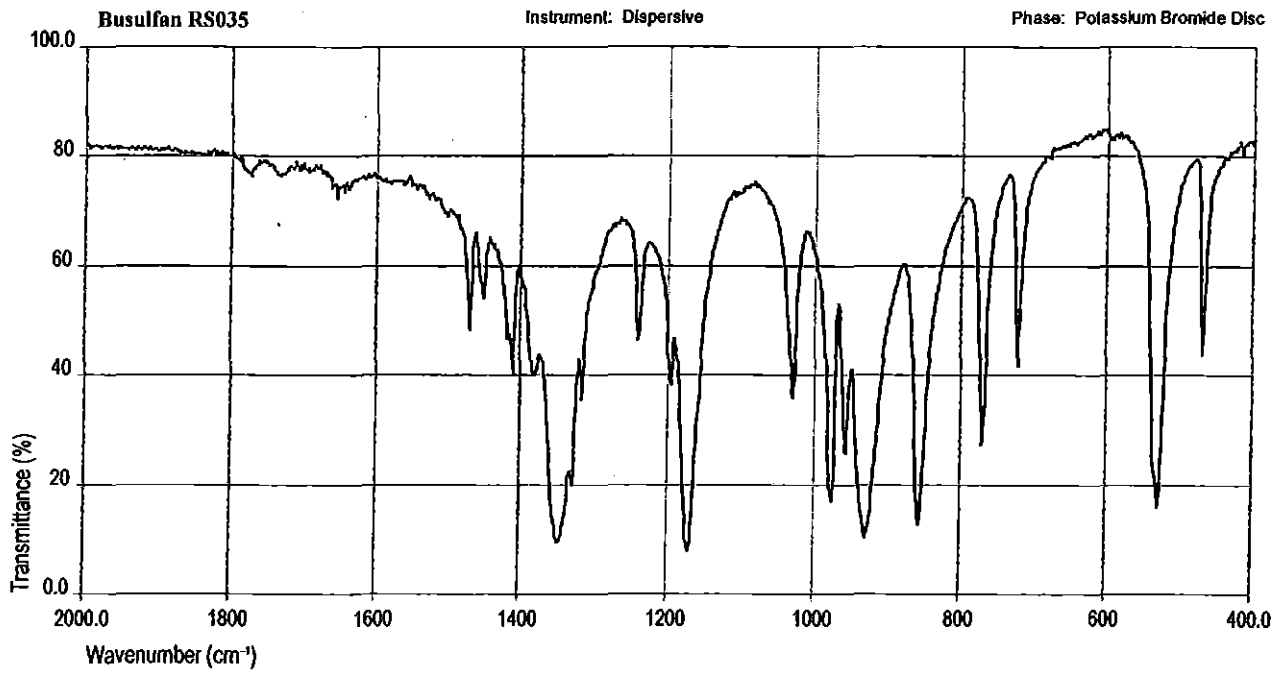
Bumetanide RS033

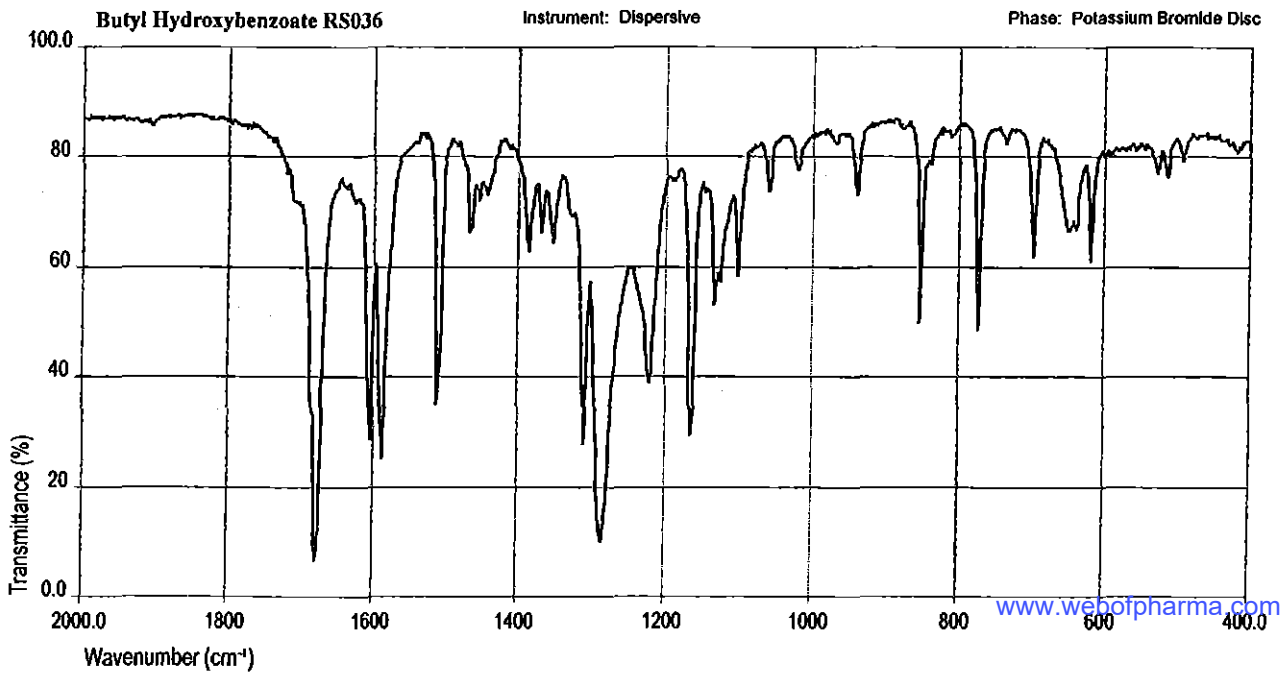
Instrument: Dispersive

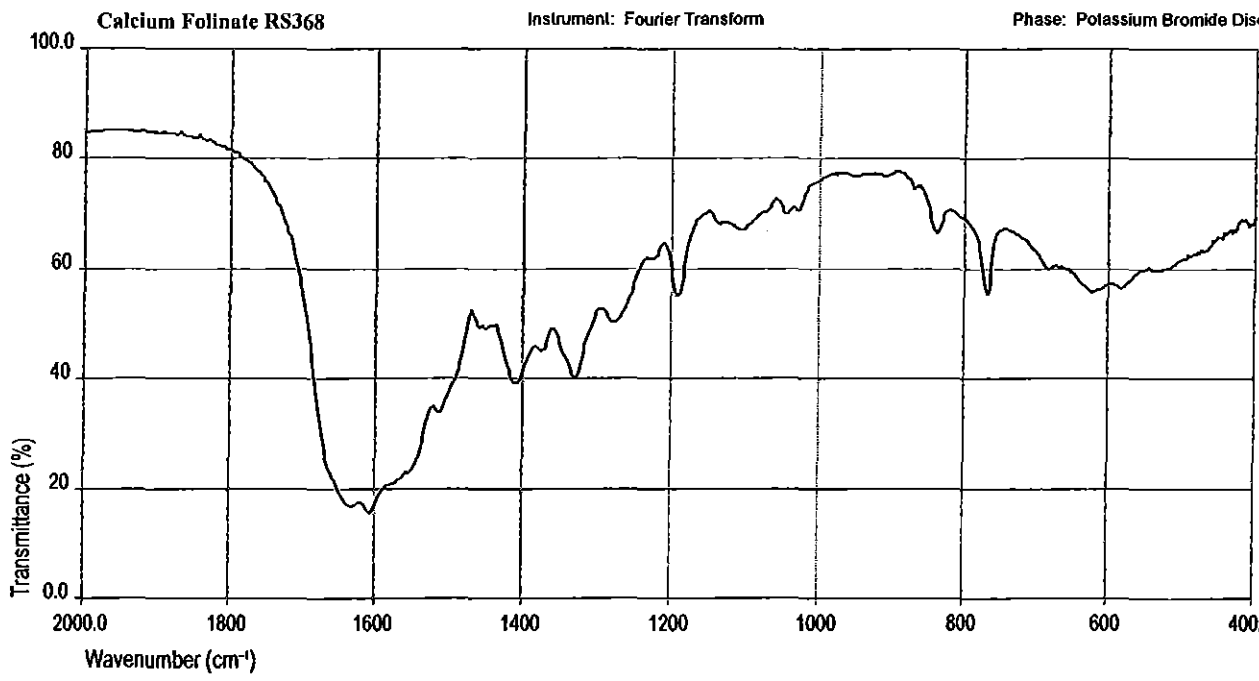
Phase: Potassium Bromide Disc

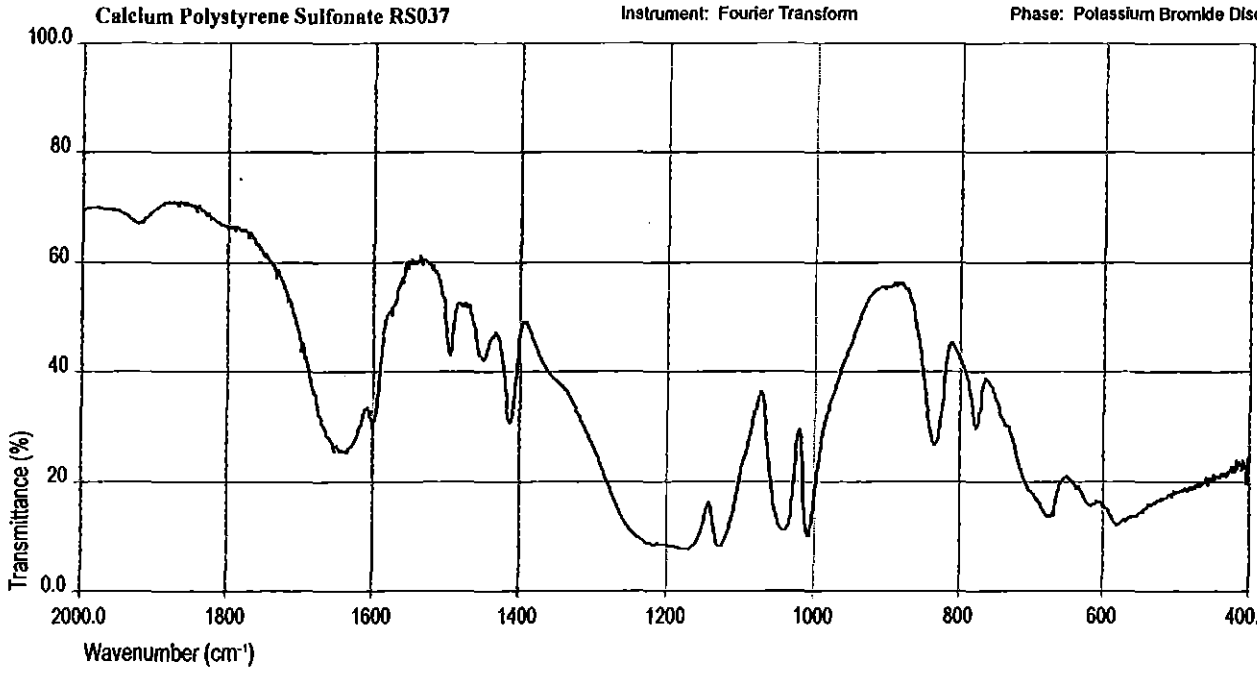


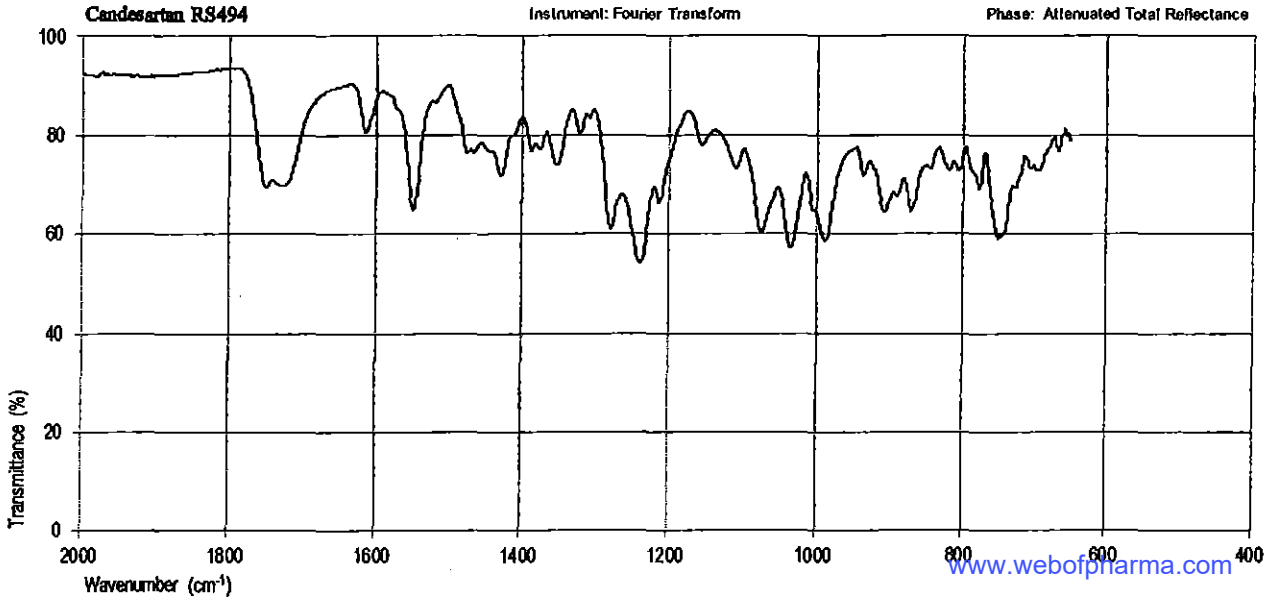


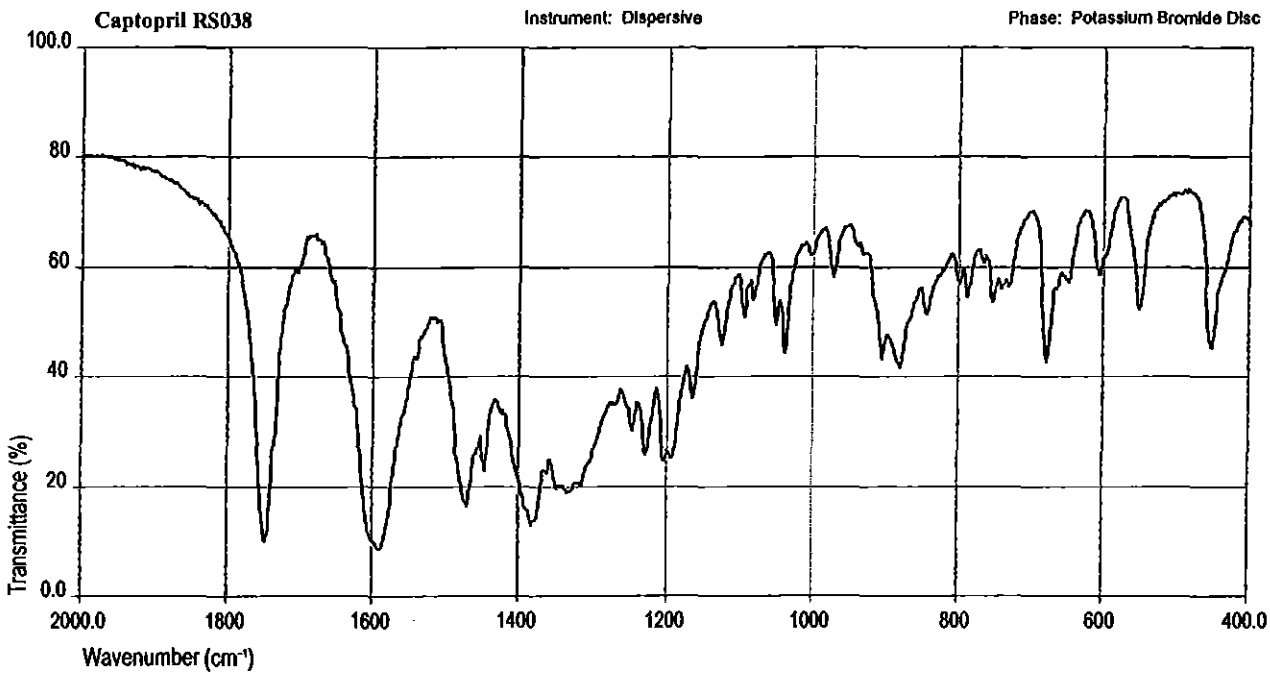


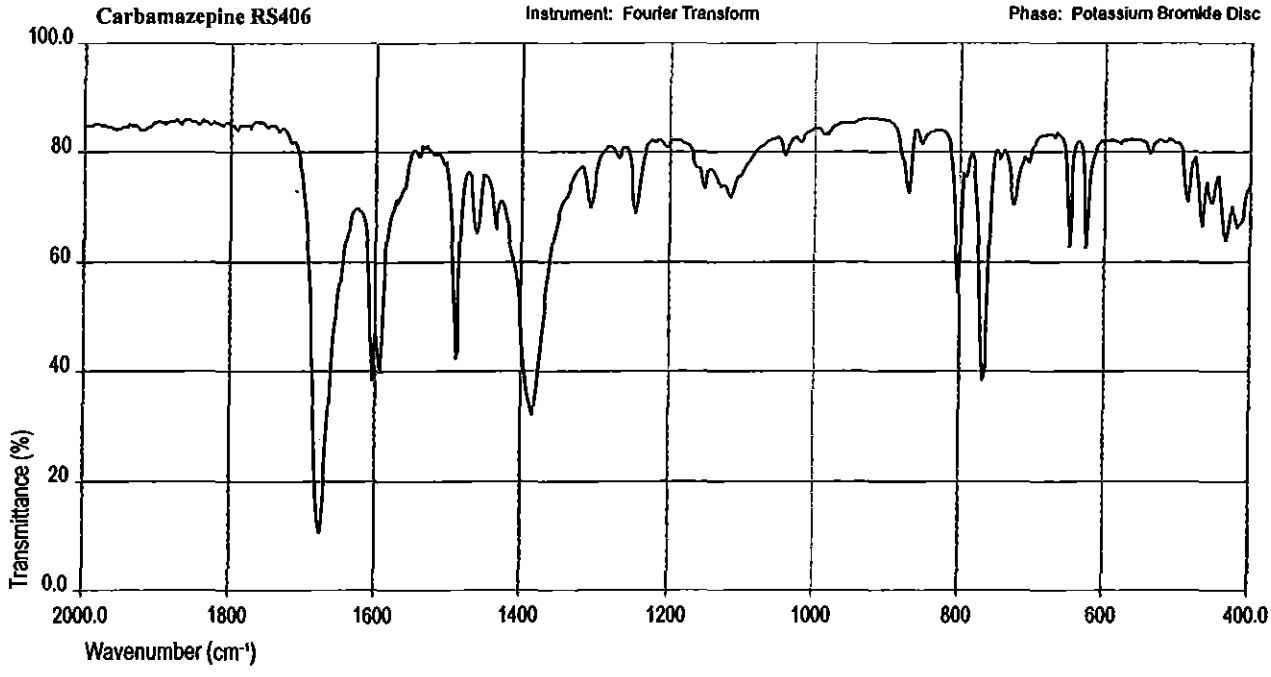


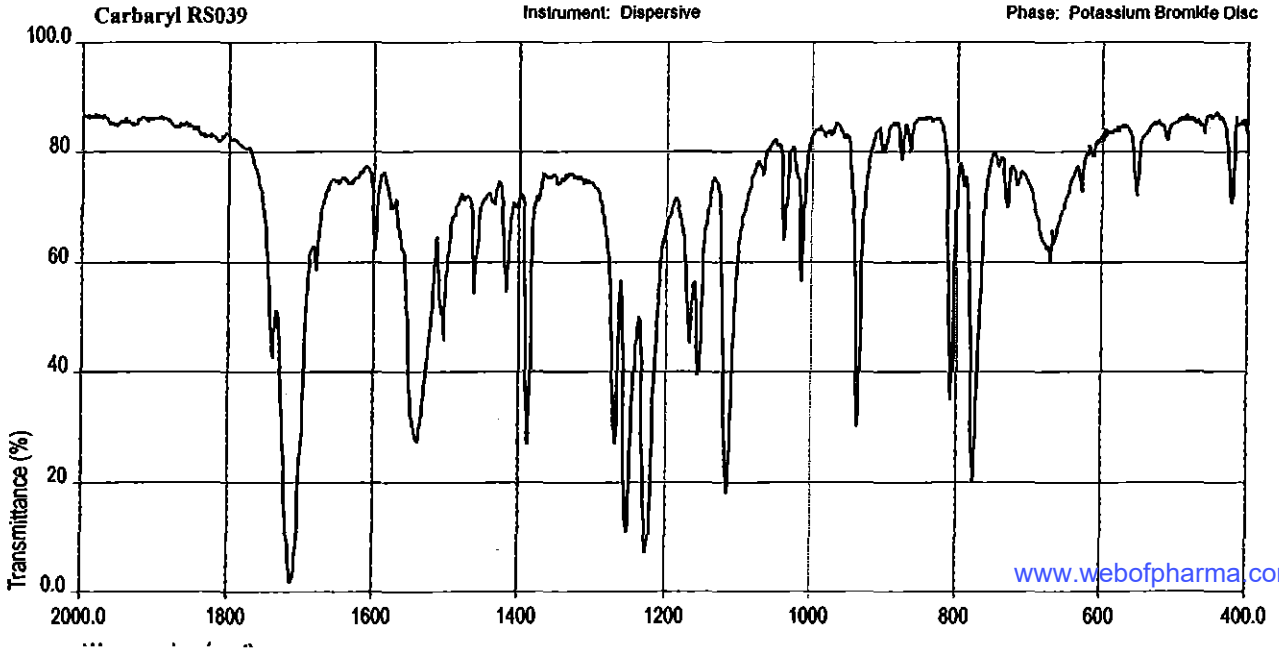




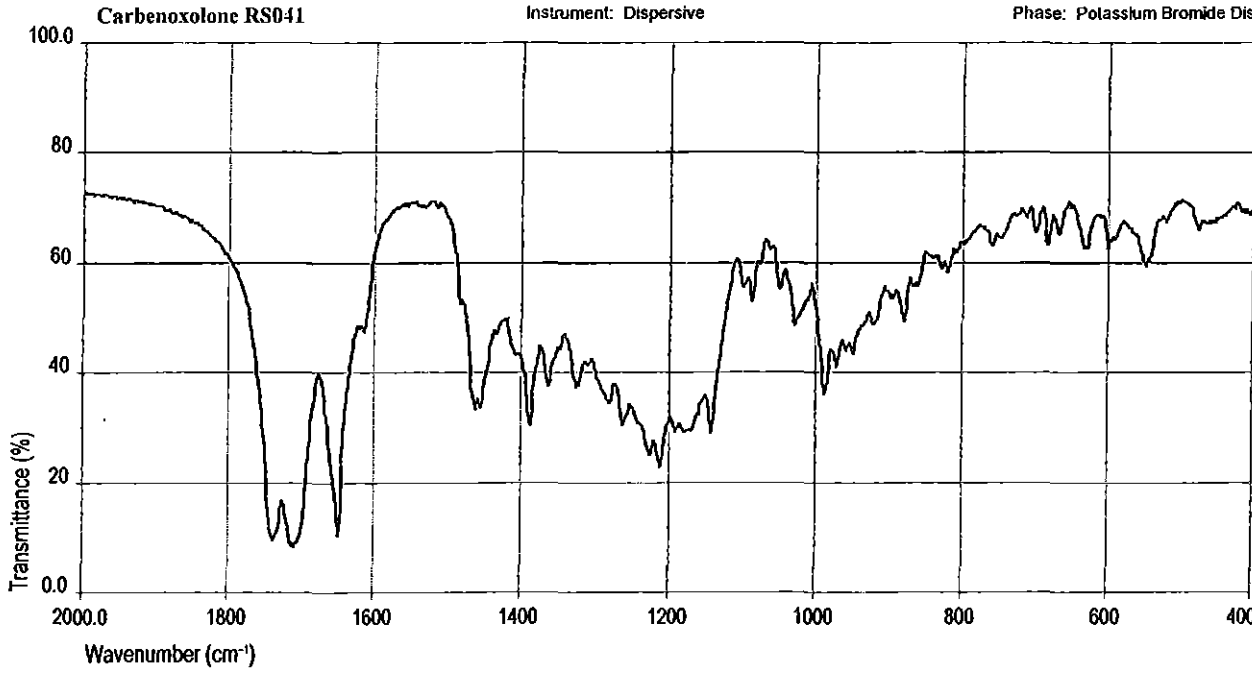




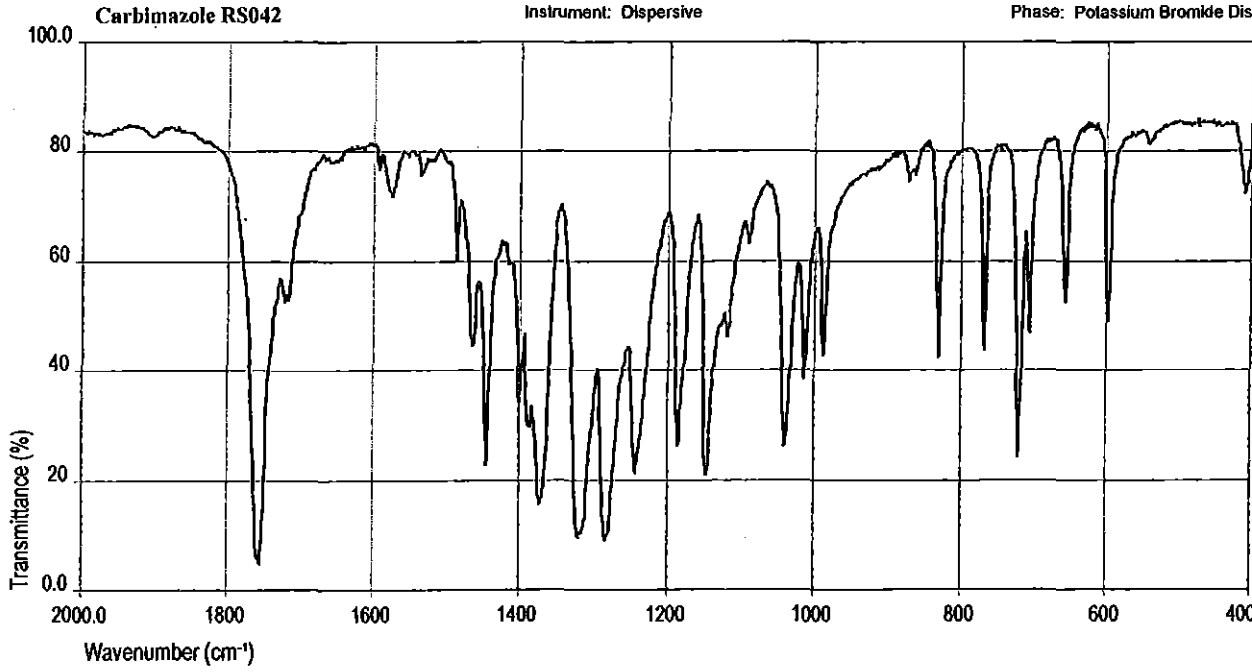




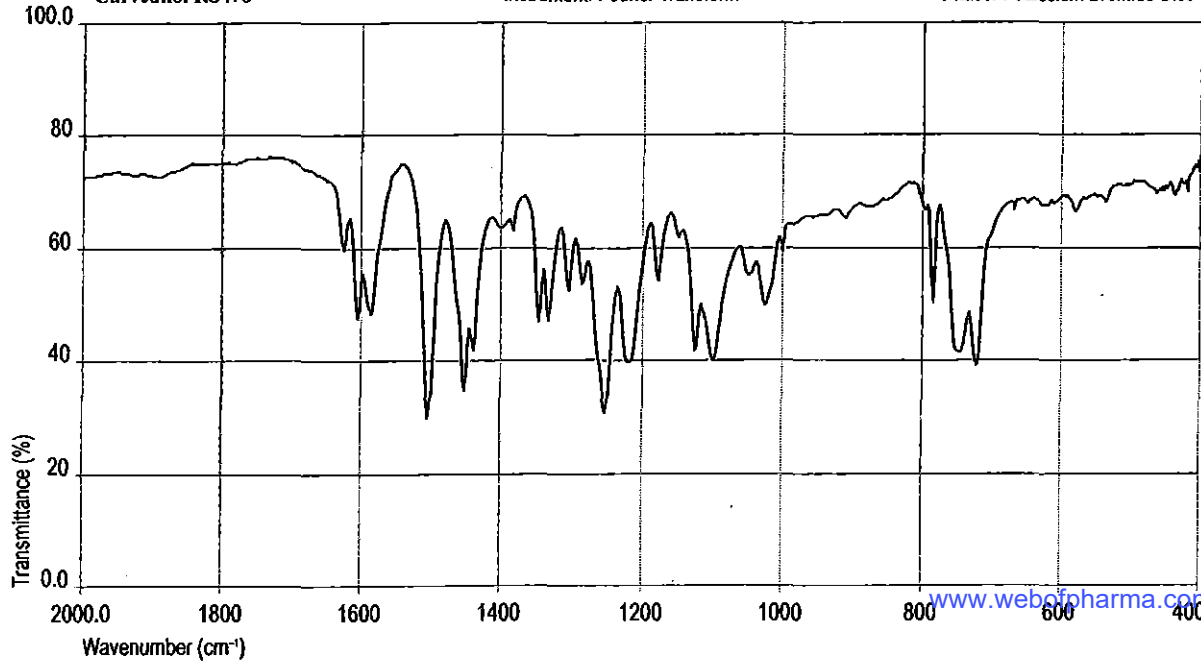
Carbenoxolone RS041 Instrument: Dispersive Phase: Potassium Bromide Disc

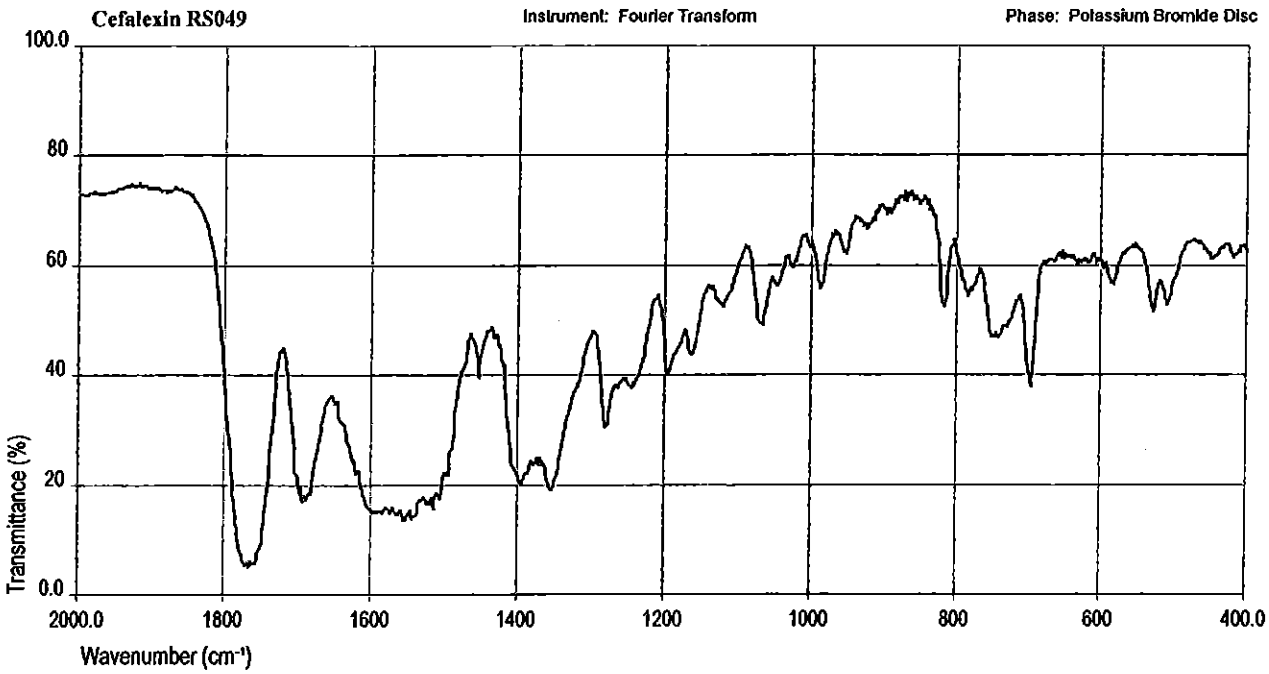


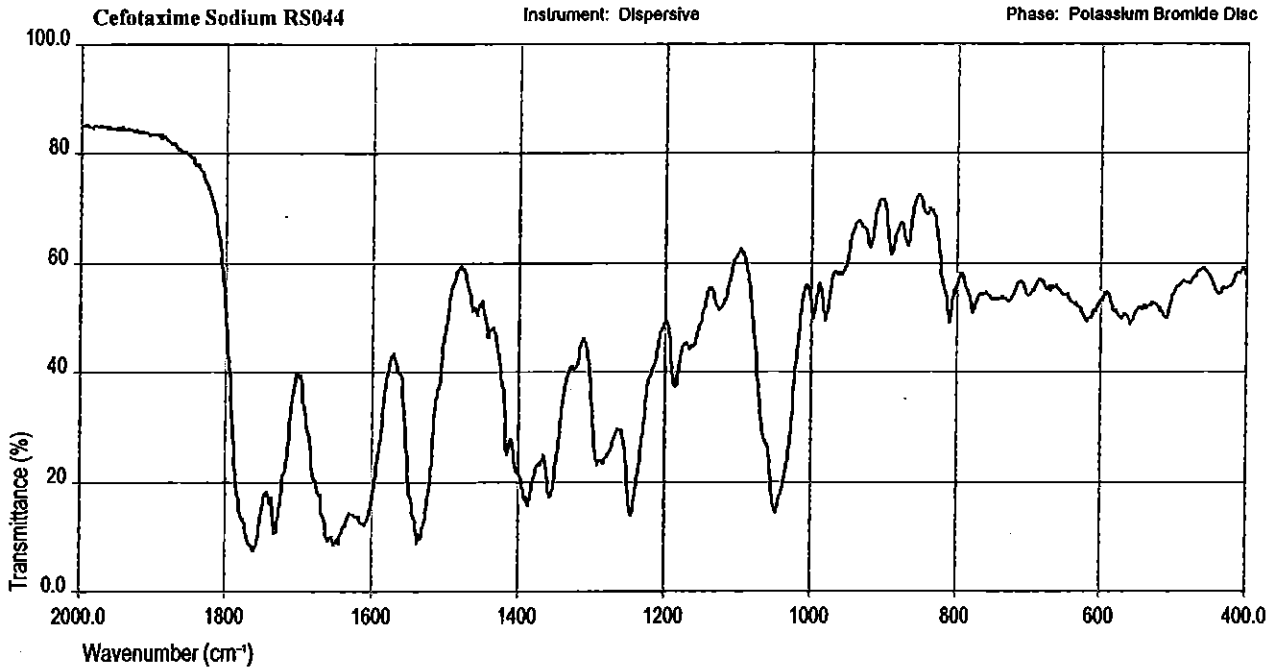
Carbimazole RS042 Instrument: Dispersive Phase: Potassium Bromide Disc

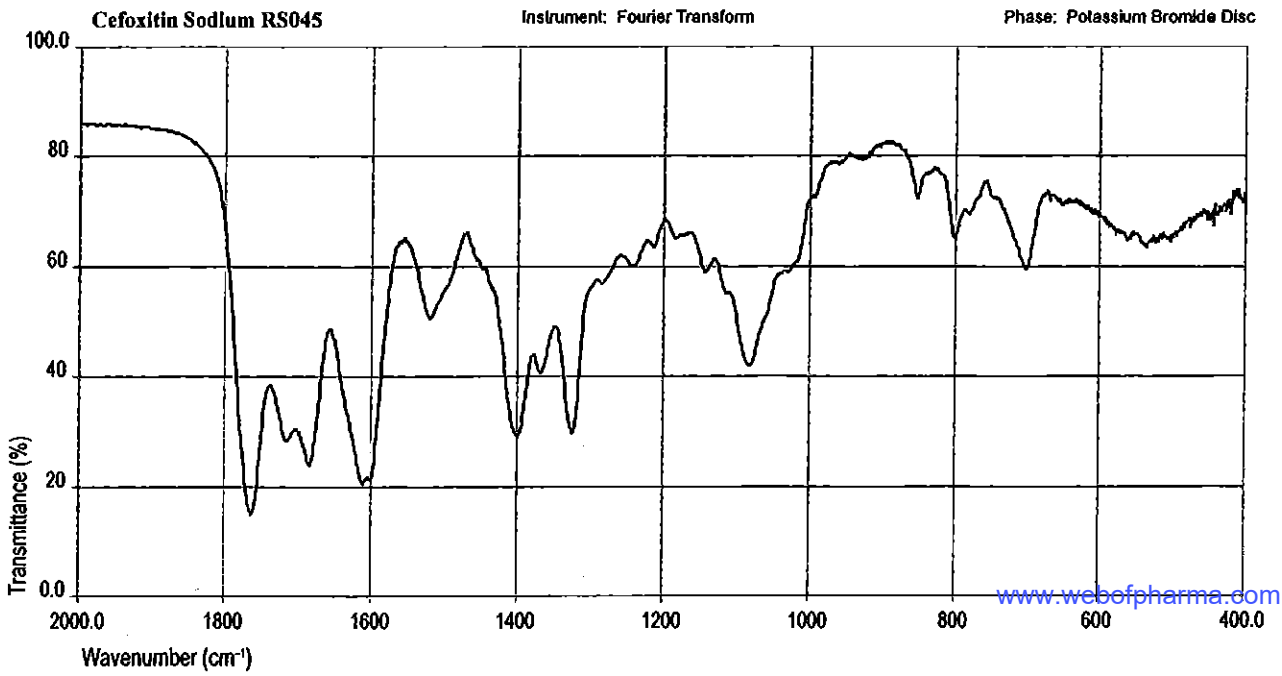


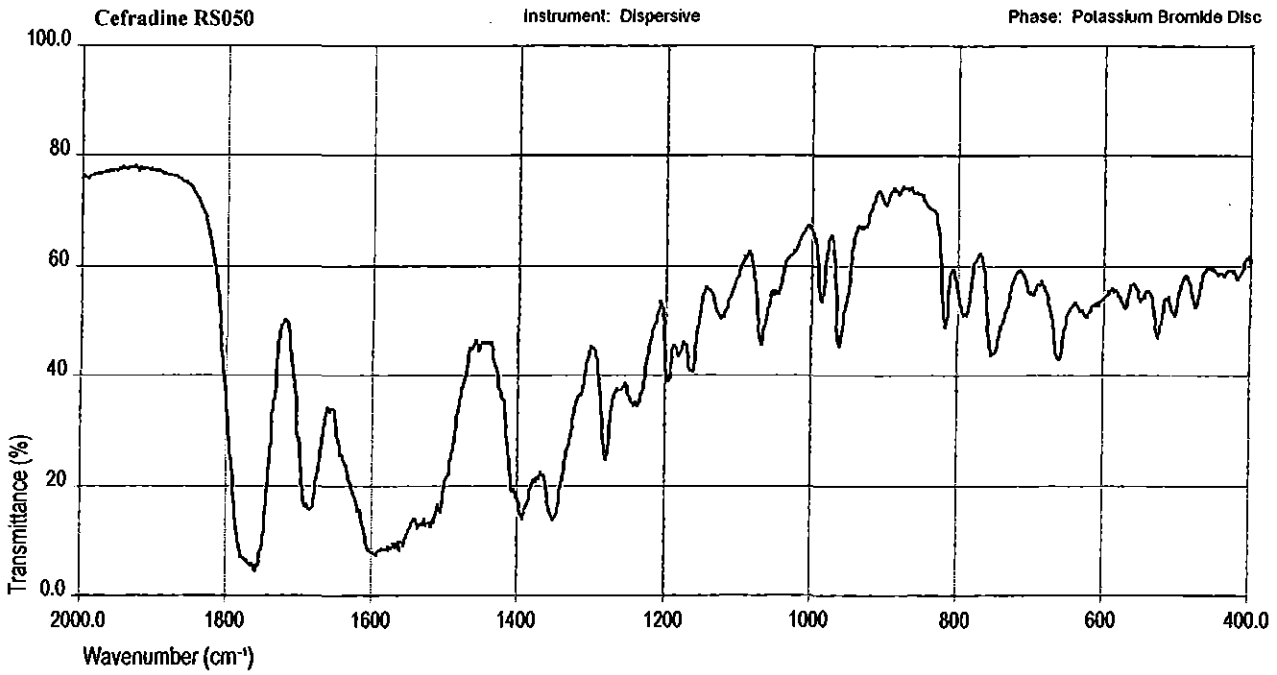
Carvedilol RS476 Instrument: Fourier Transform Phase: Potassium Bromide Disc

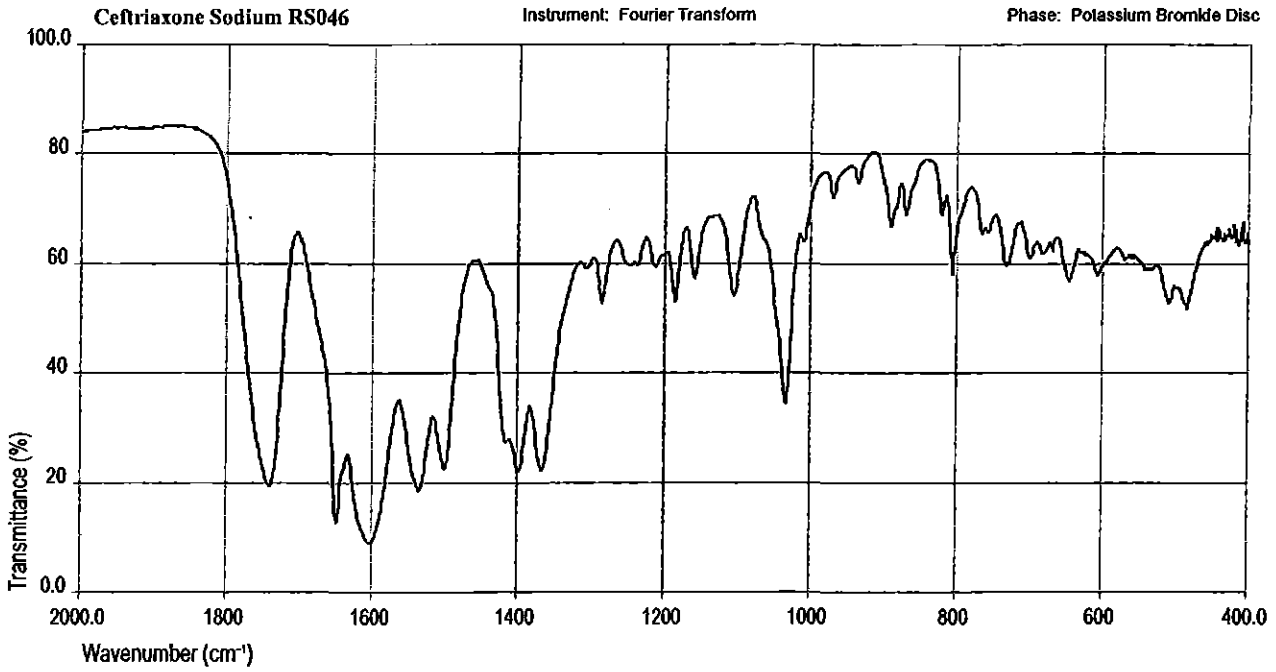


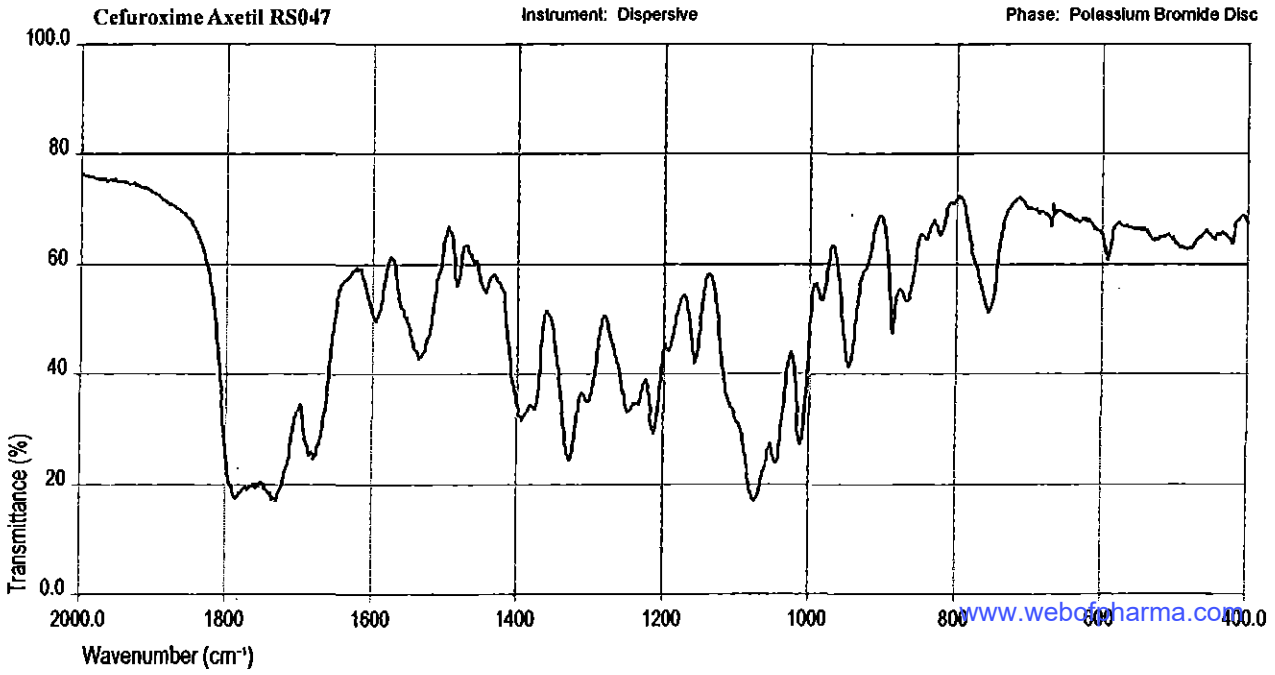


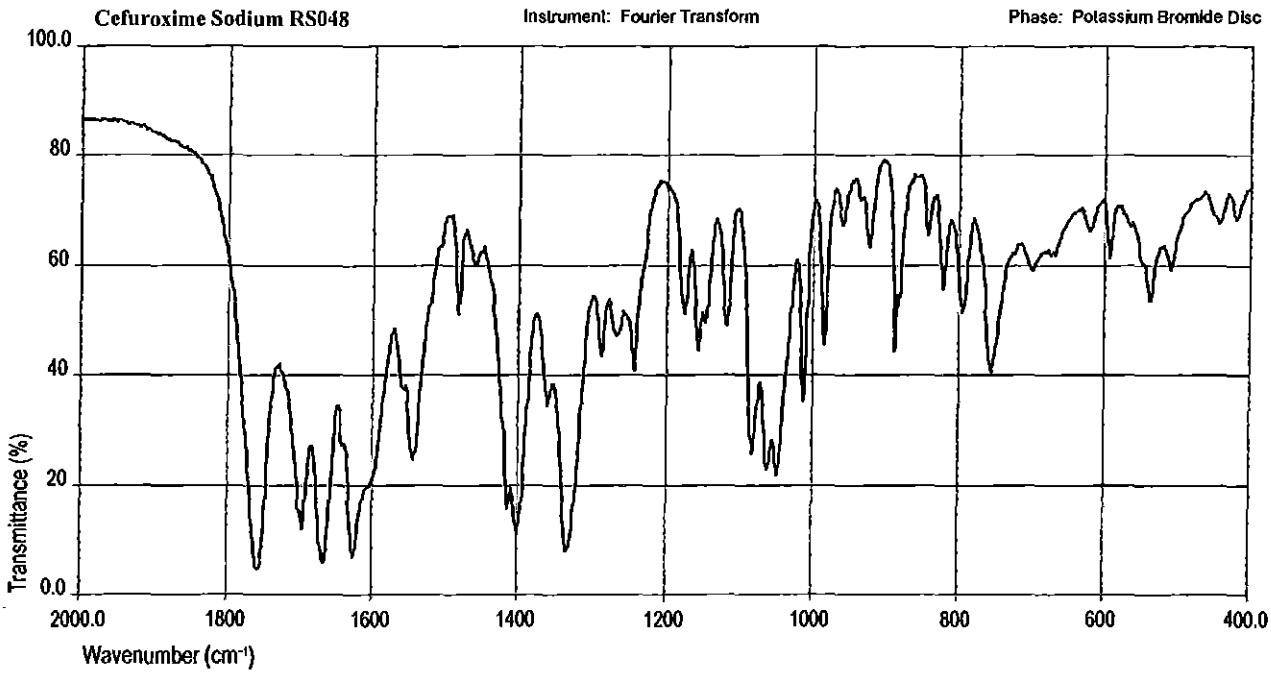


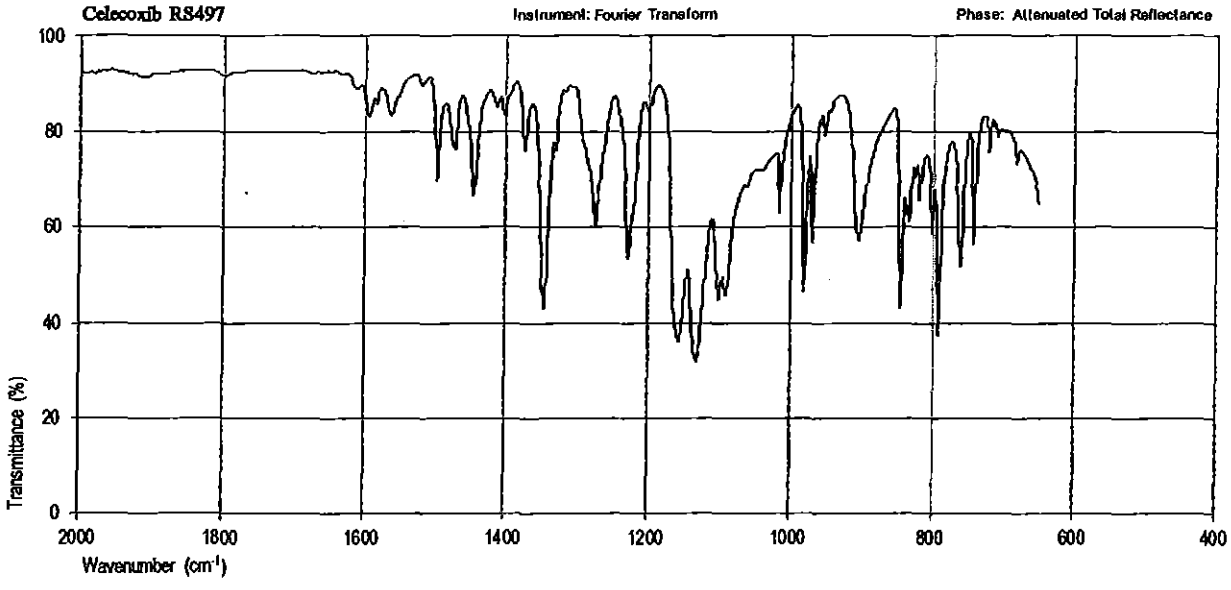


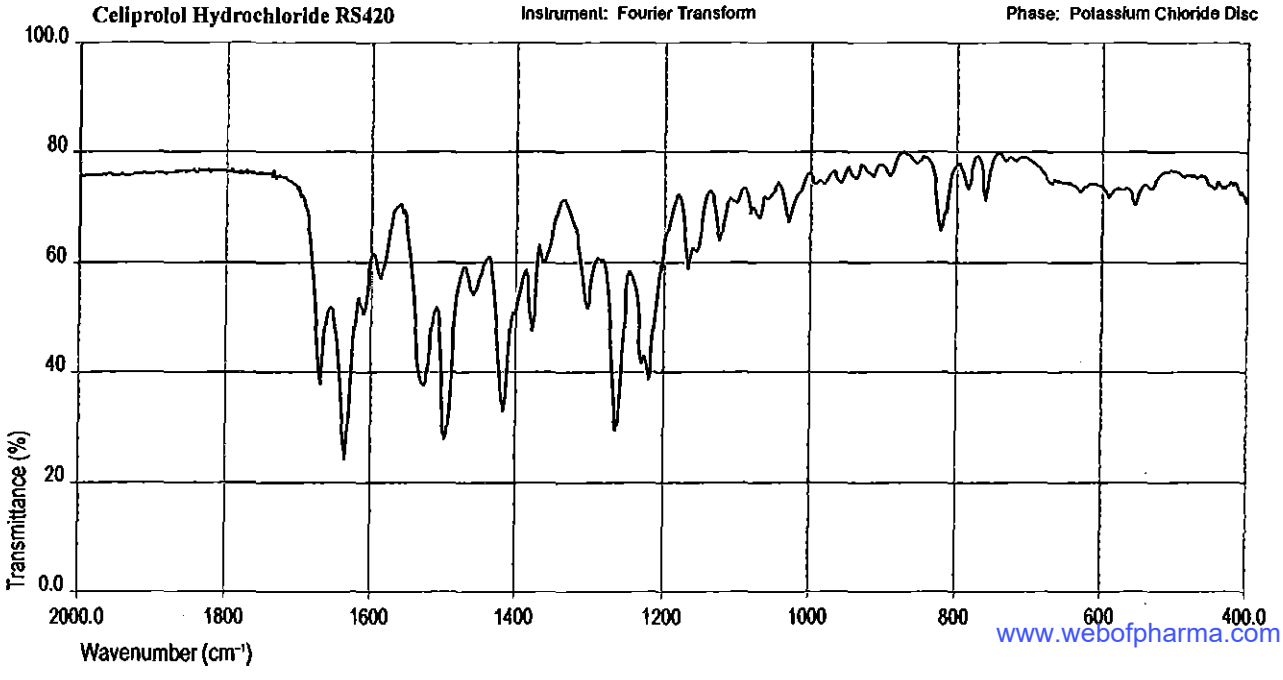




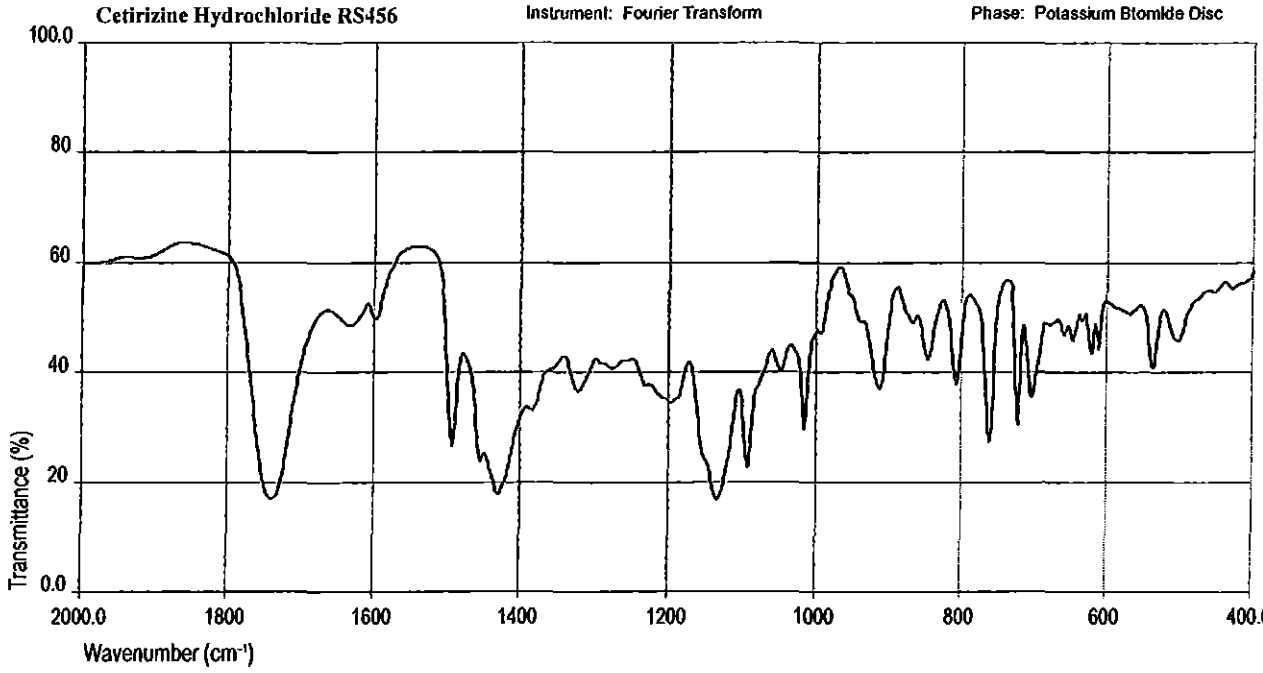




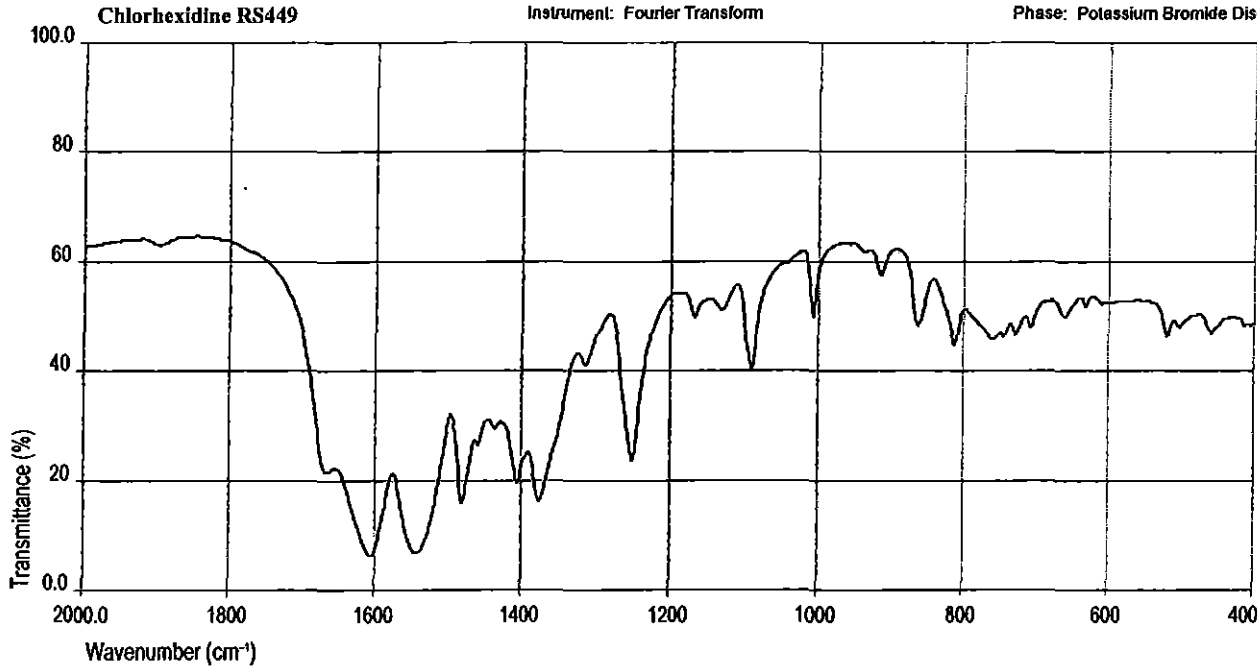




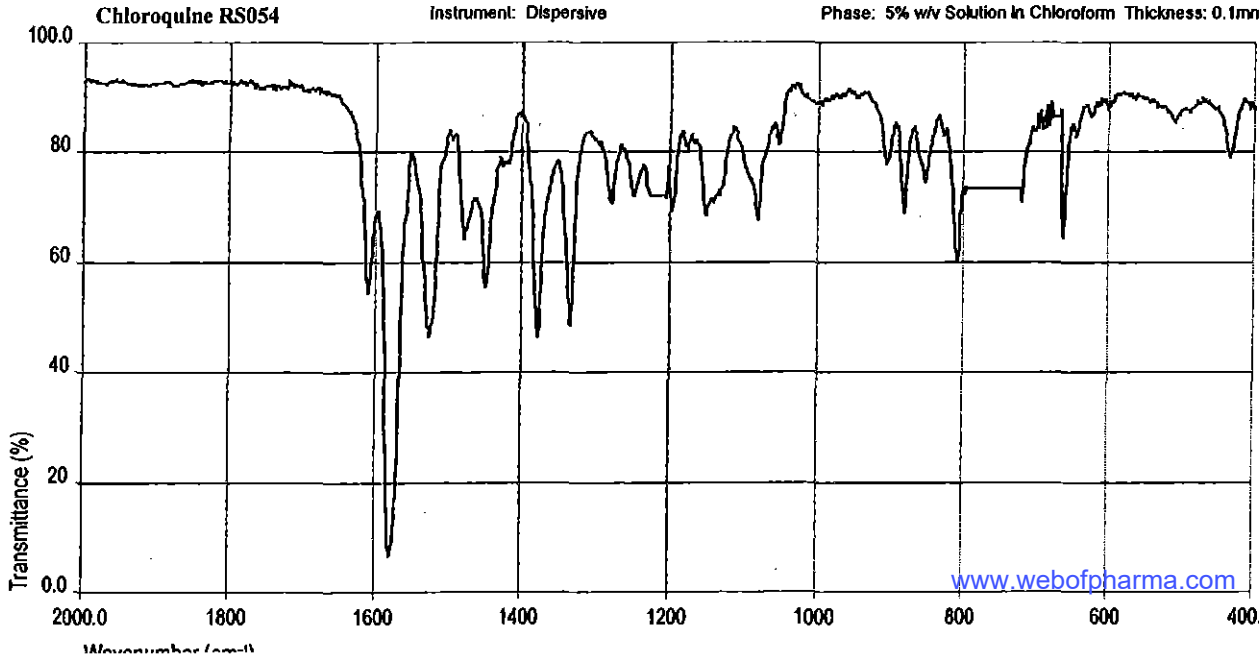
Cetirizine Hydrochloride RS456 Instrument: Fourier Transform Phase: Potassium Bromide Disc

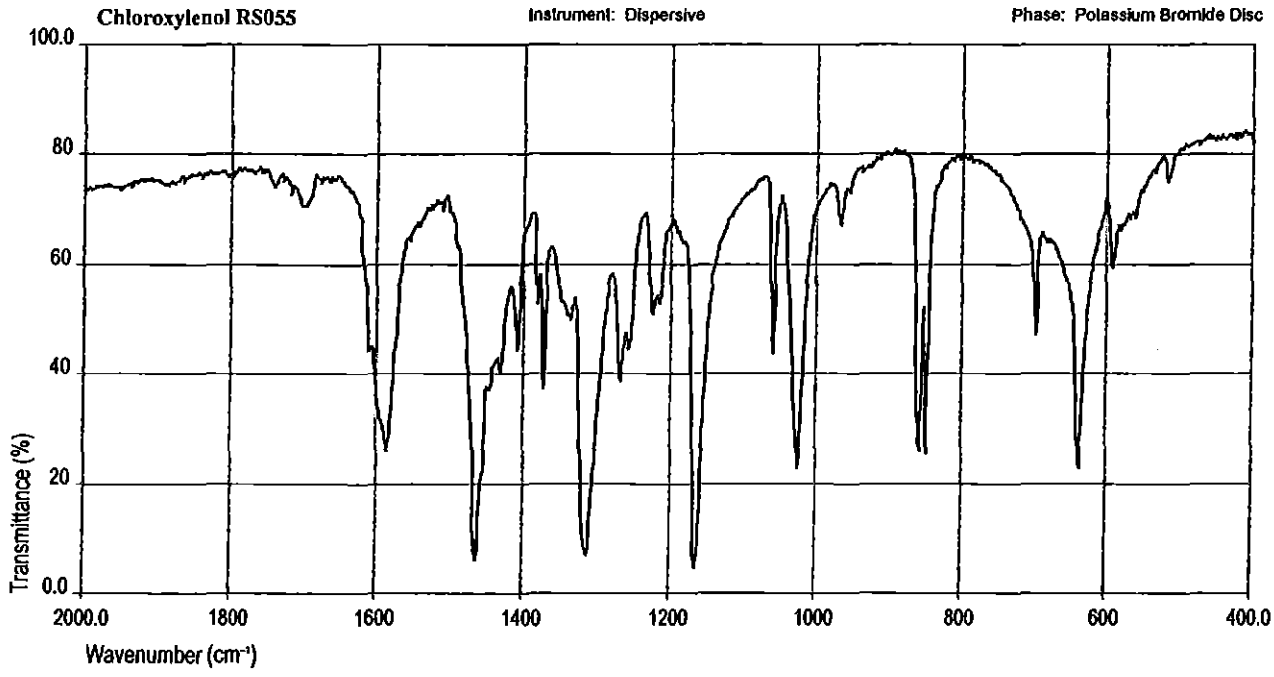


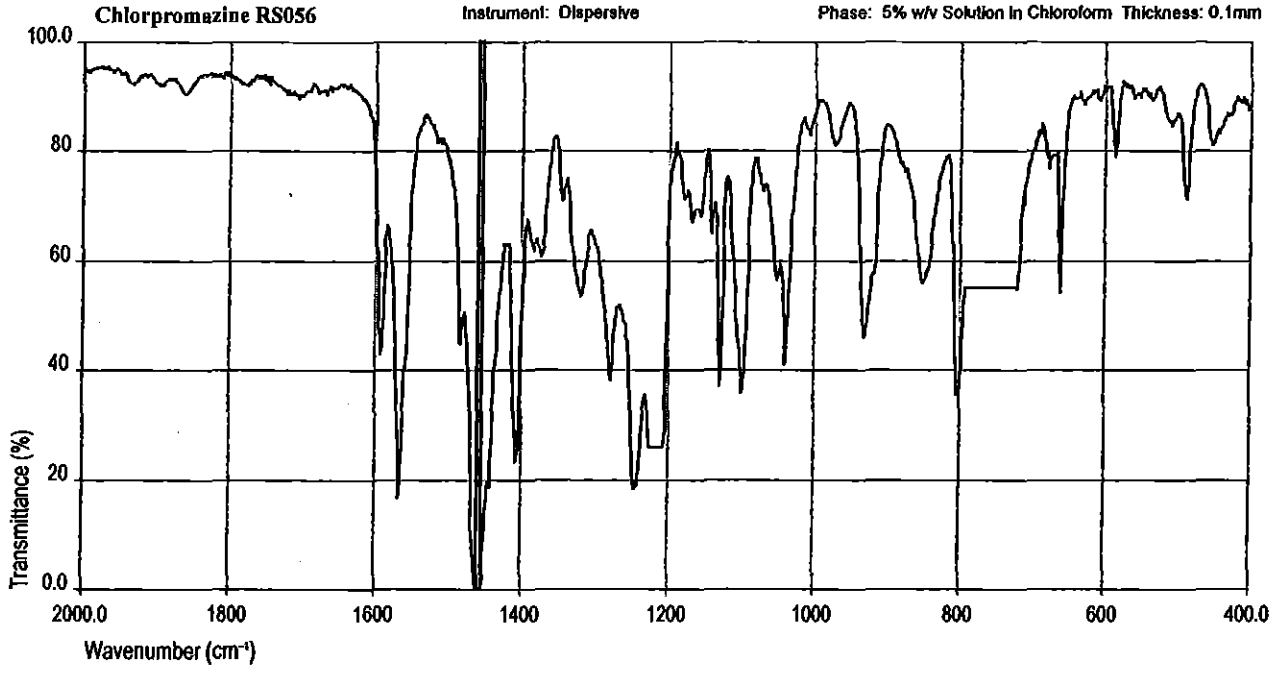
Chlorhexidine RS449 Instrument: Fourier Transform Phase: Potassium Bromide Disc

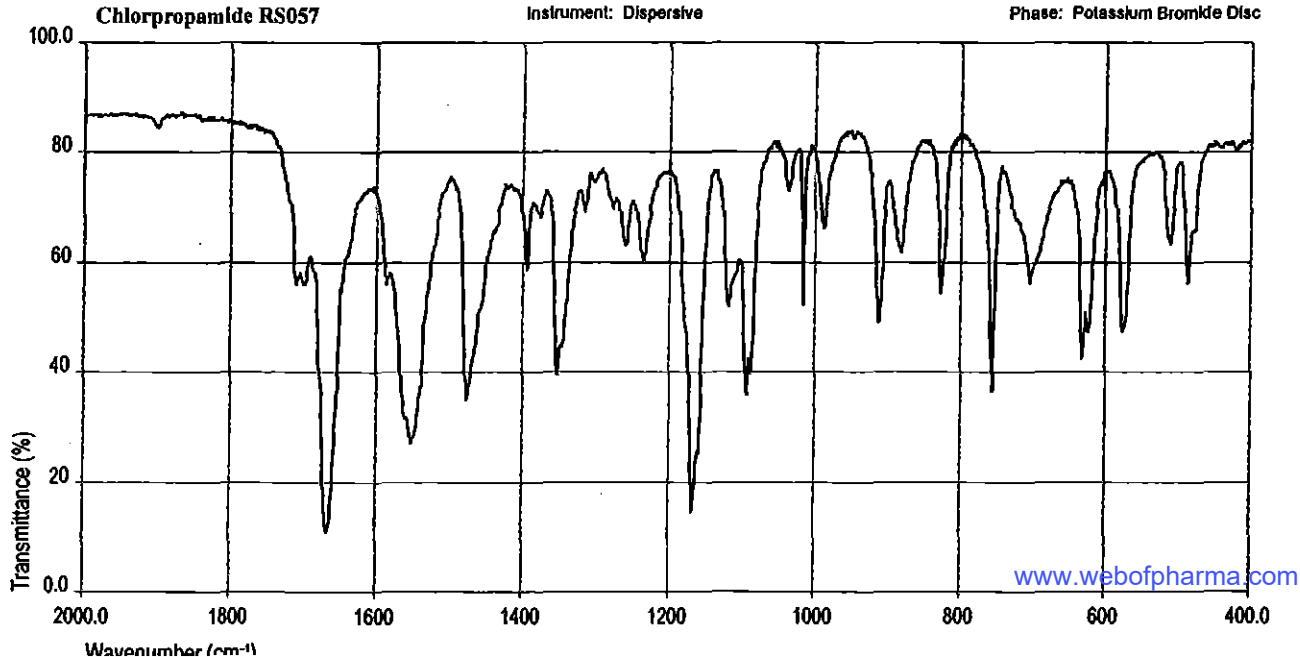


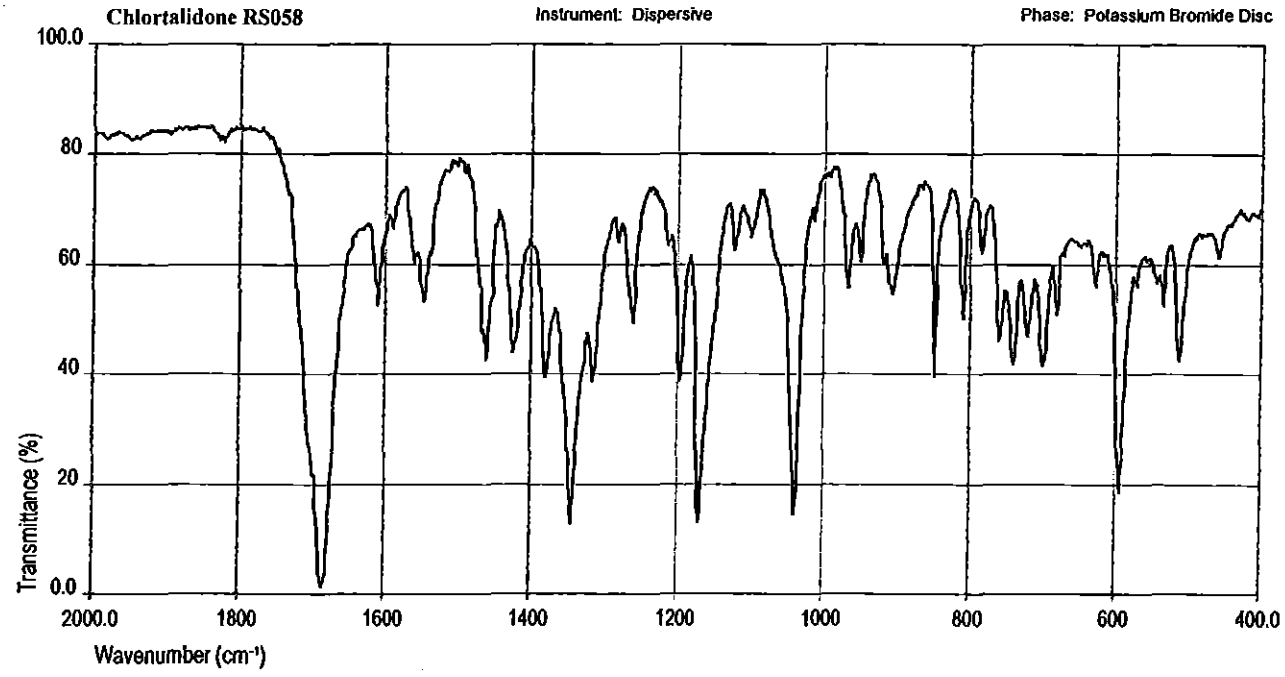
Chloroquine RS054 Instrument: Dispersive Phase: 5% w/v Solution in Chloroform Thickness: 0.1mm

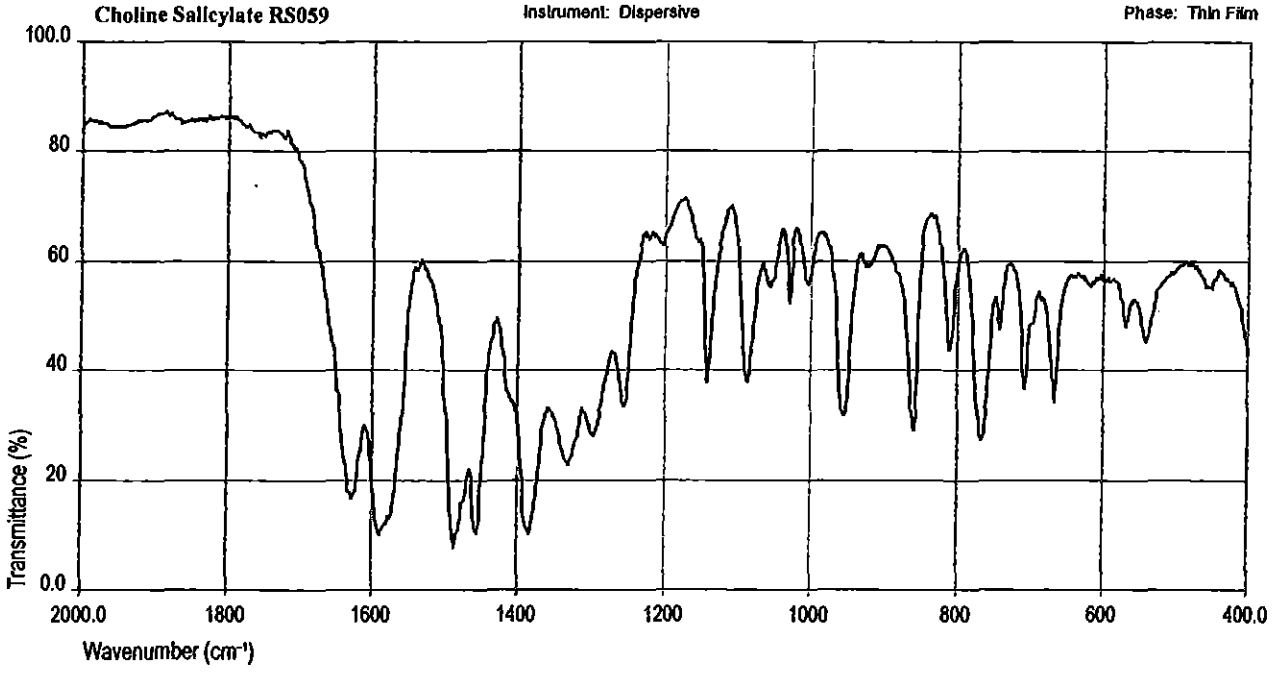


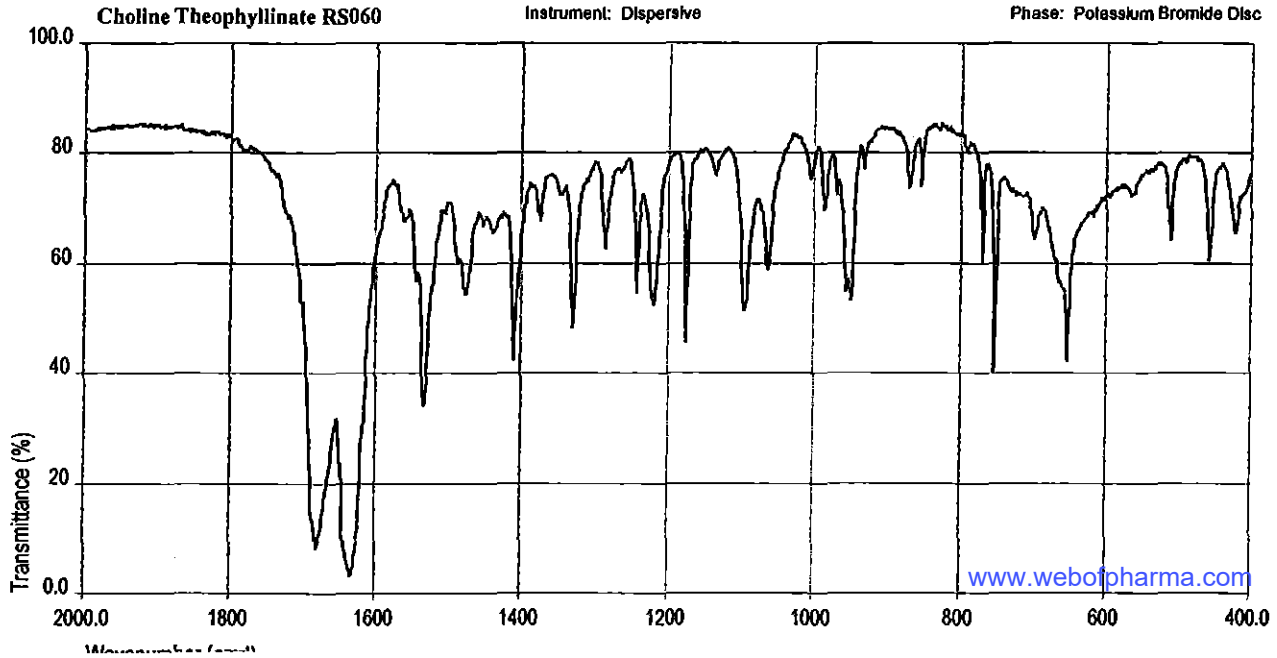


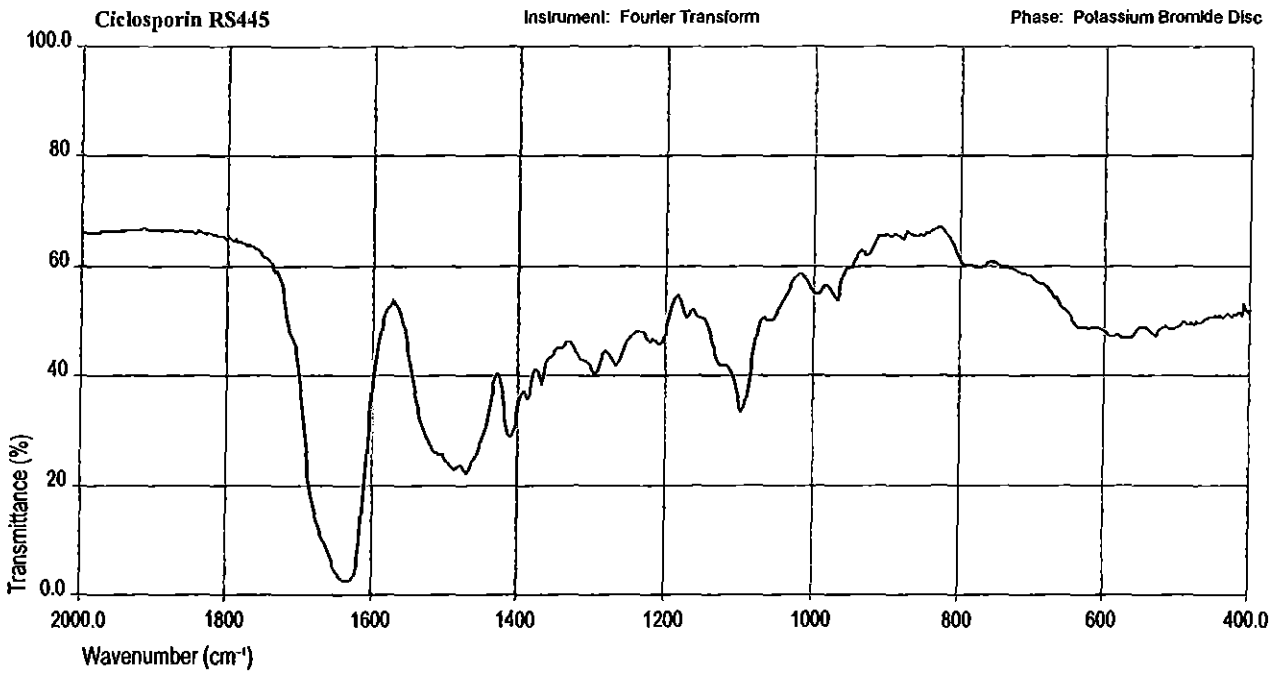


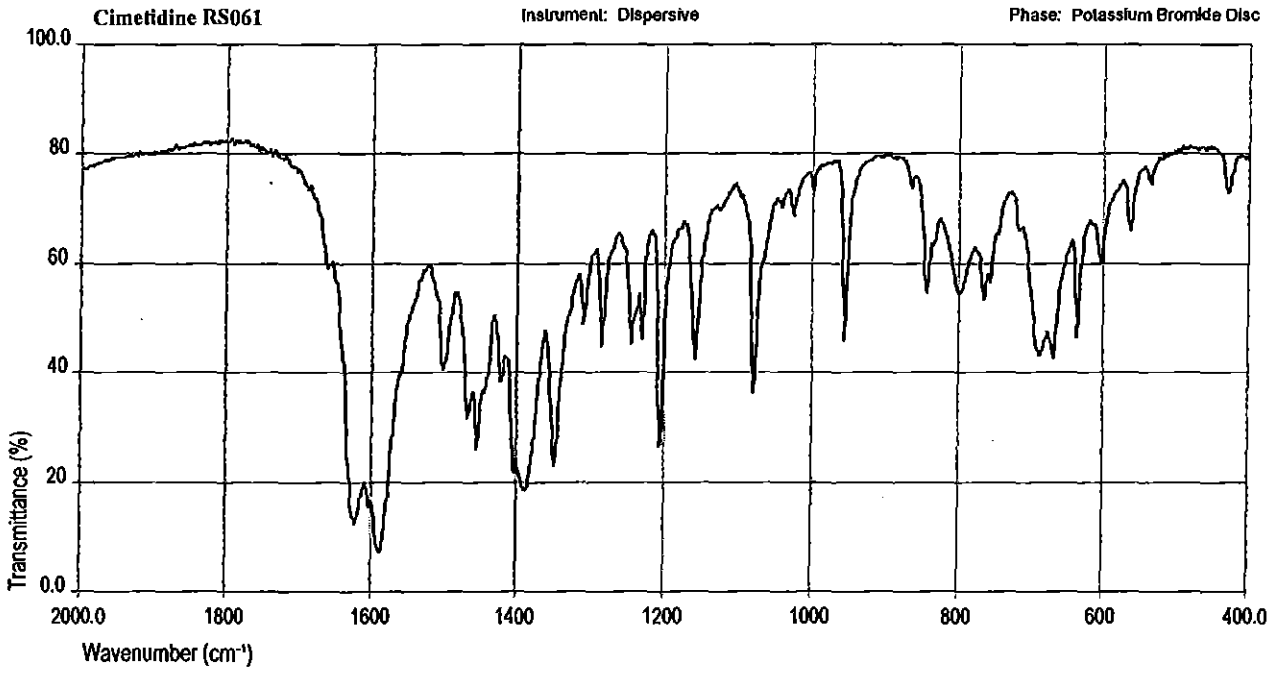


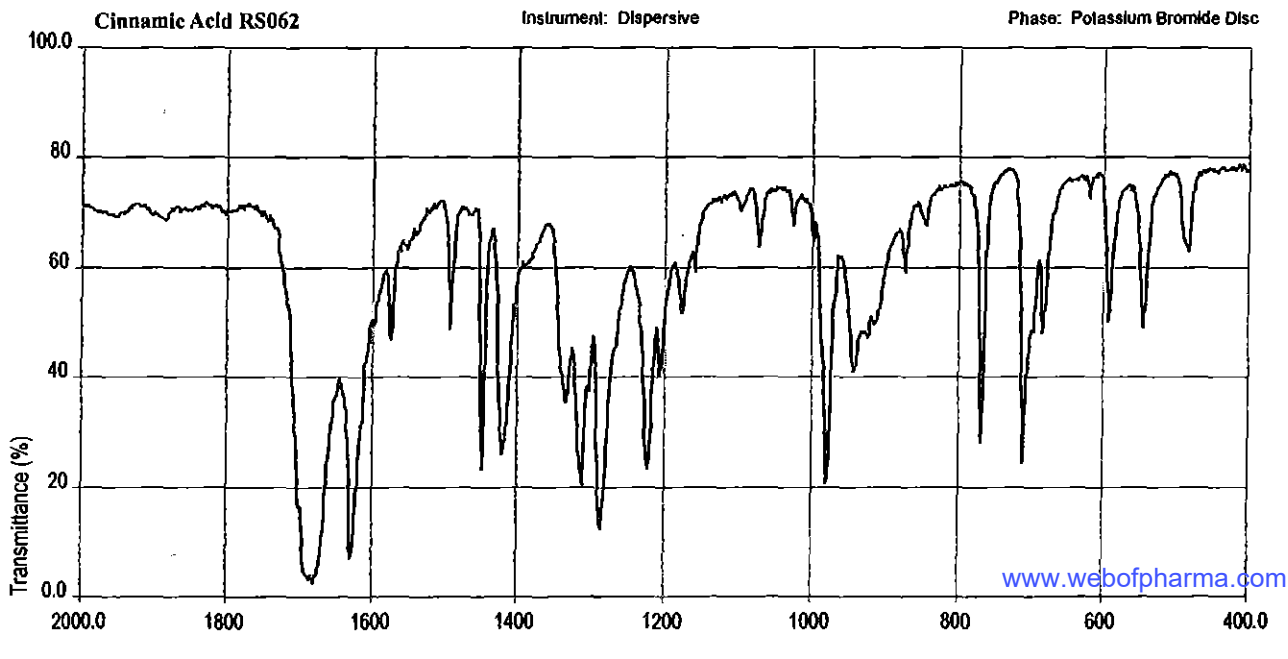


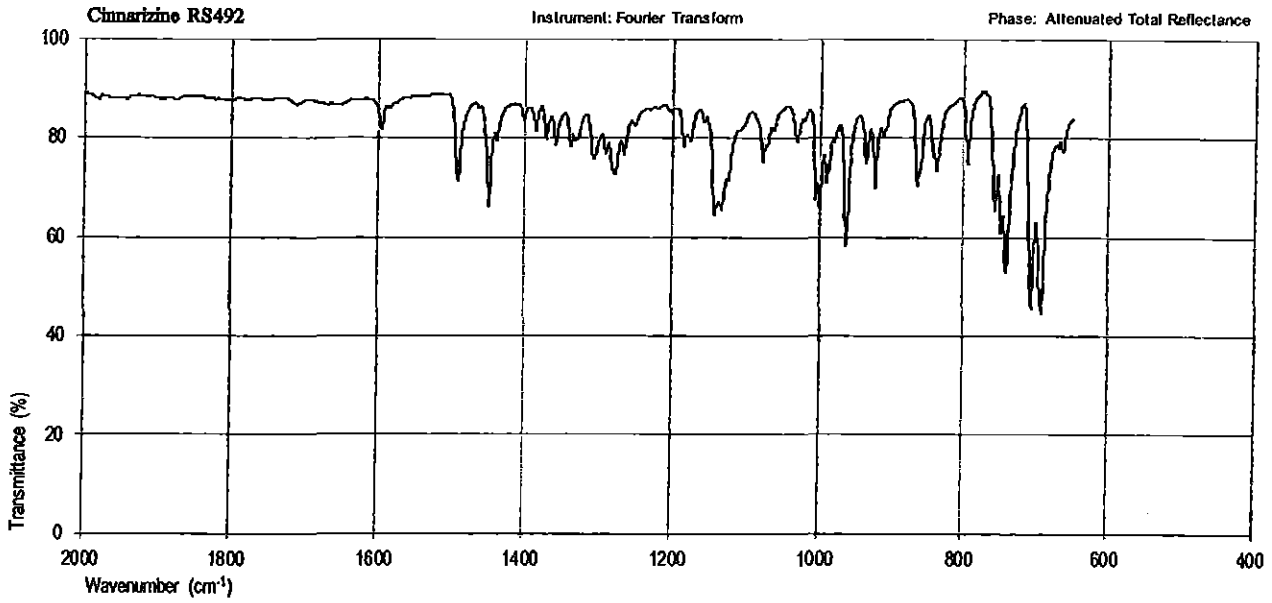


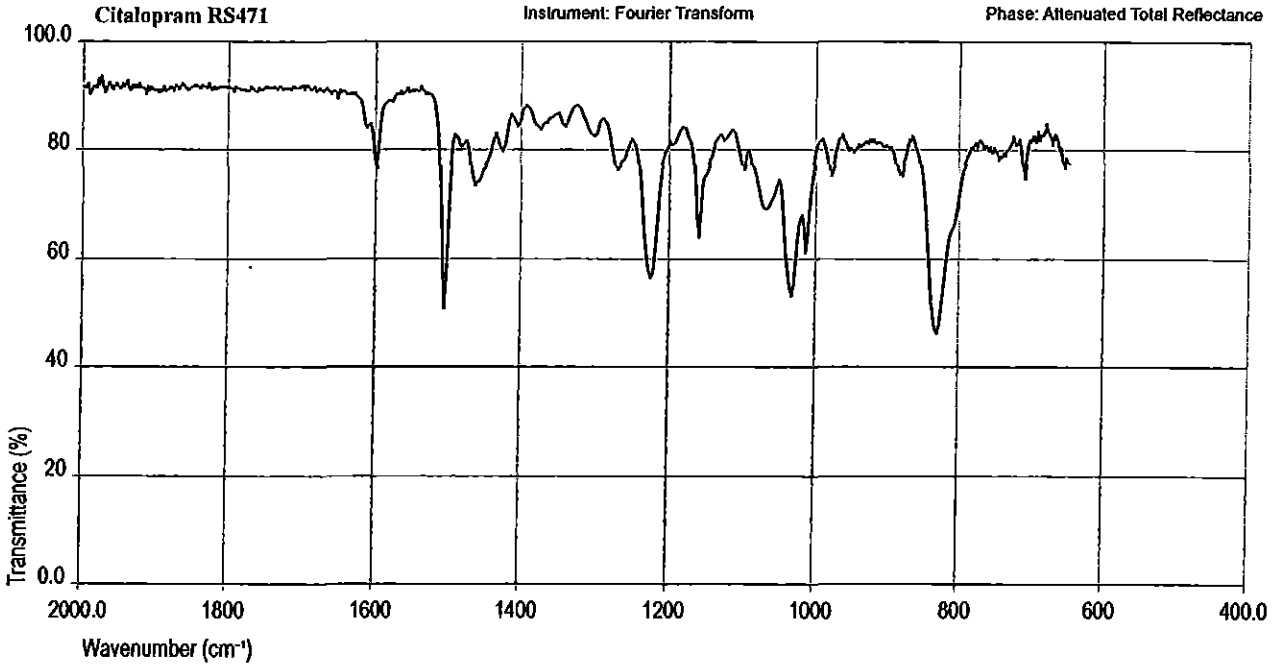


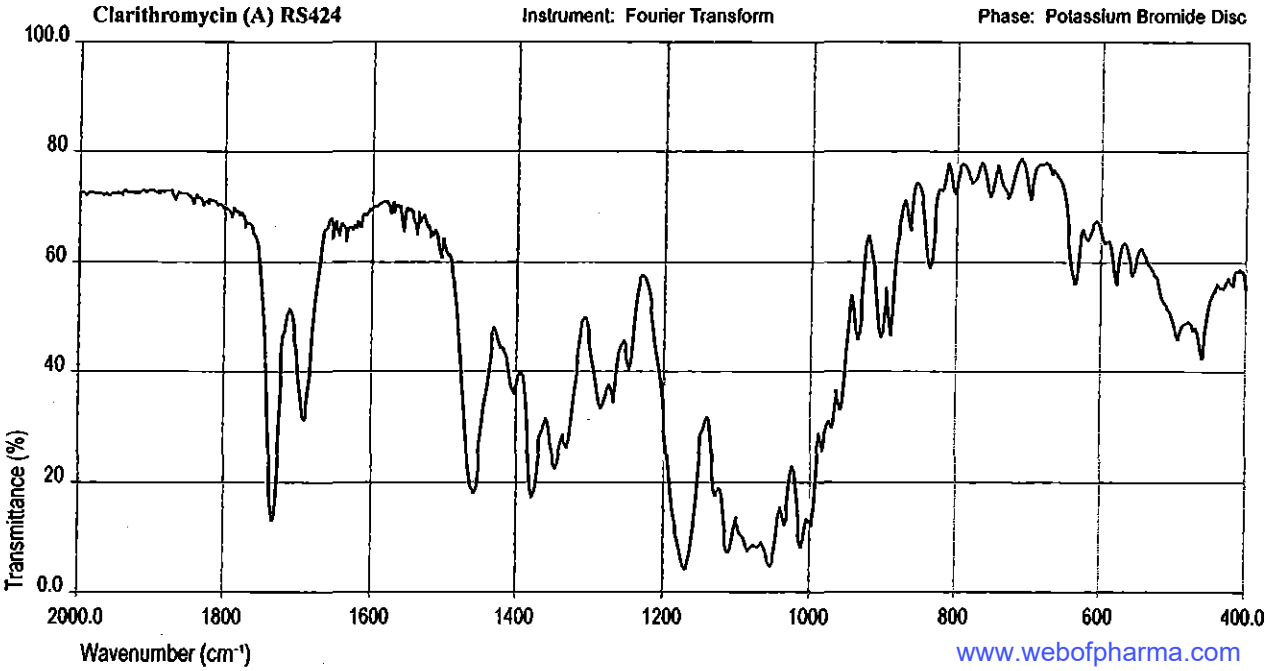


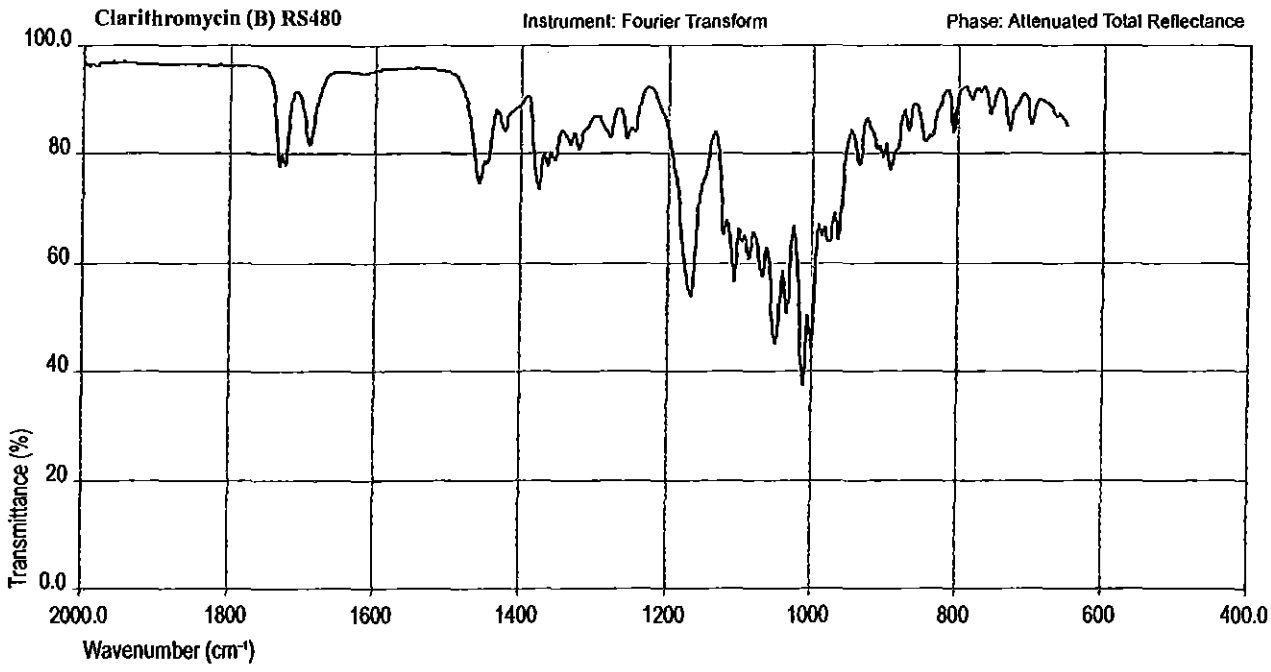


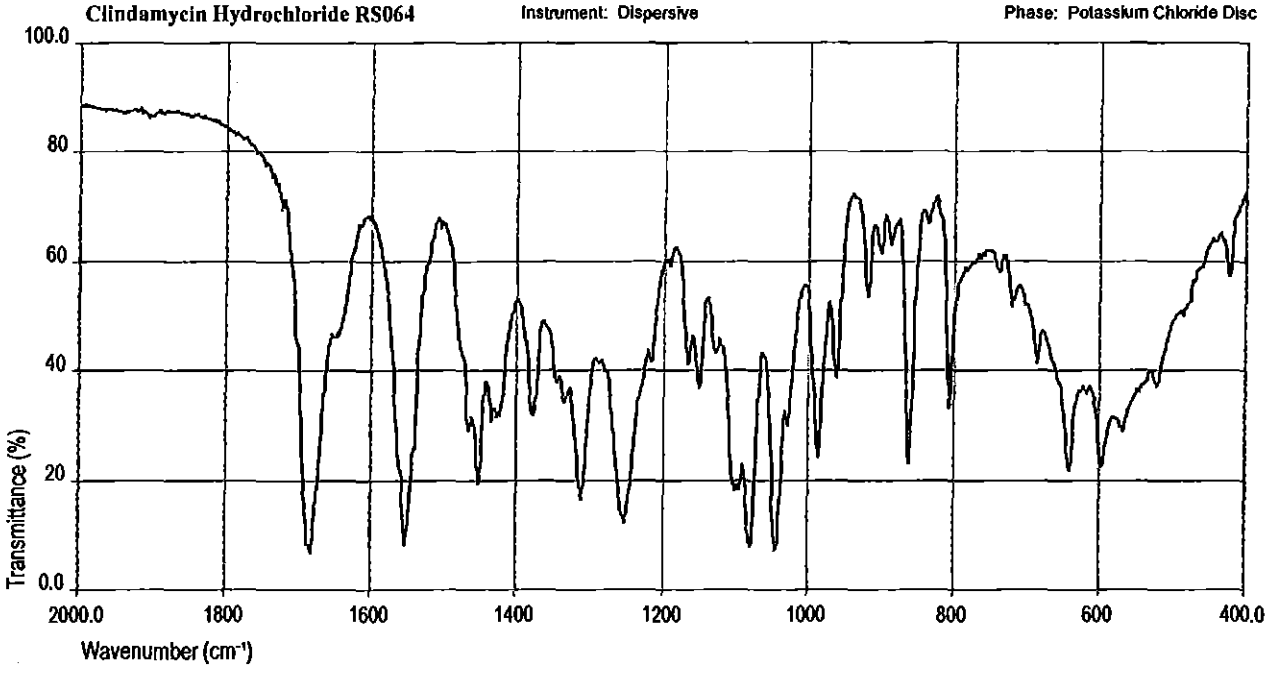


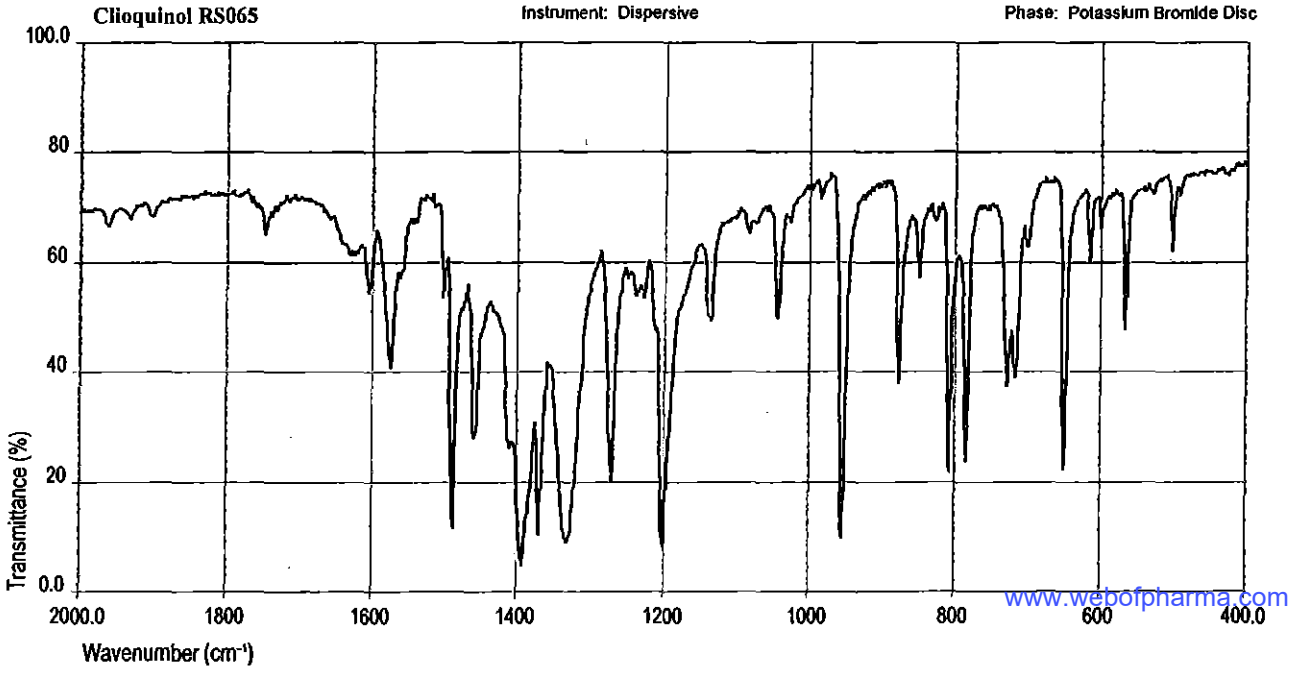


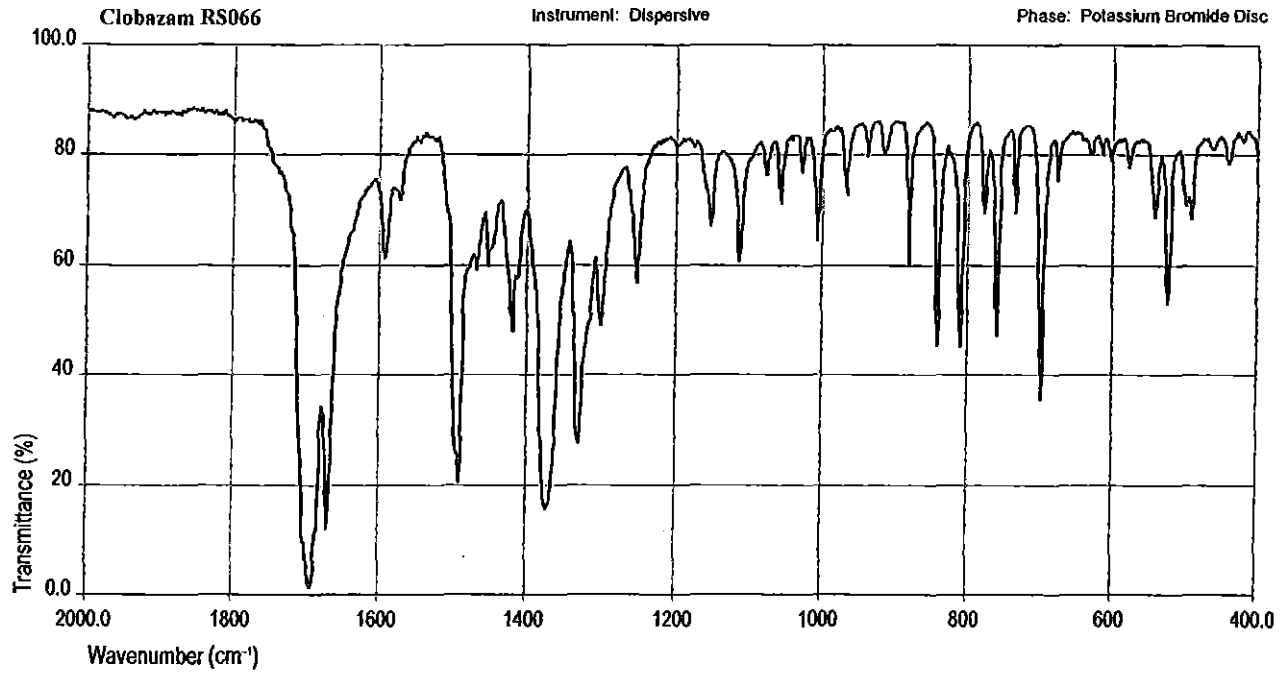


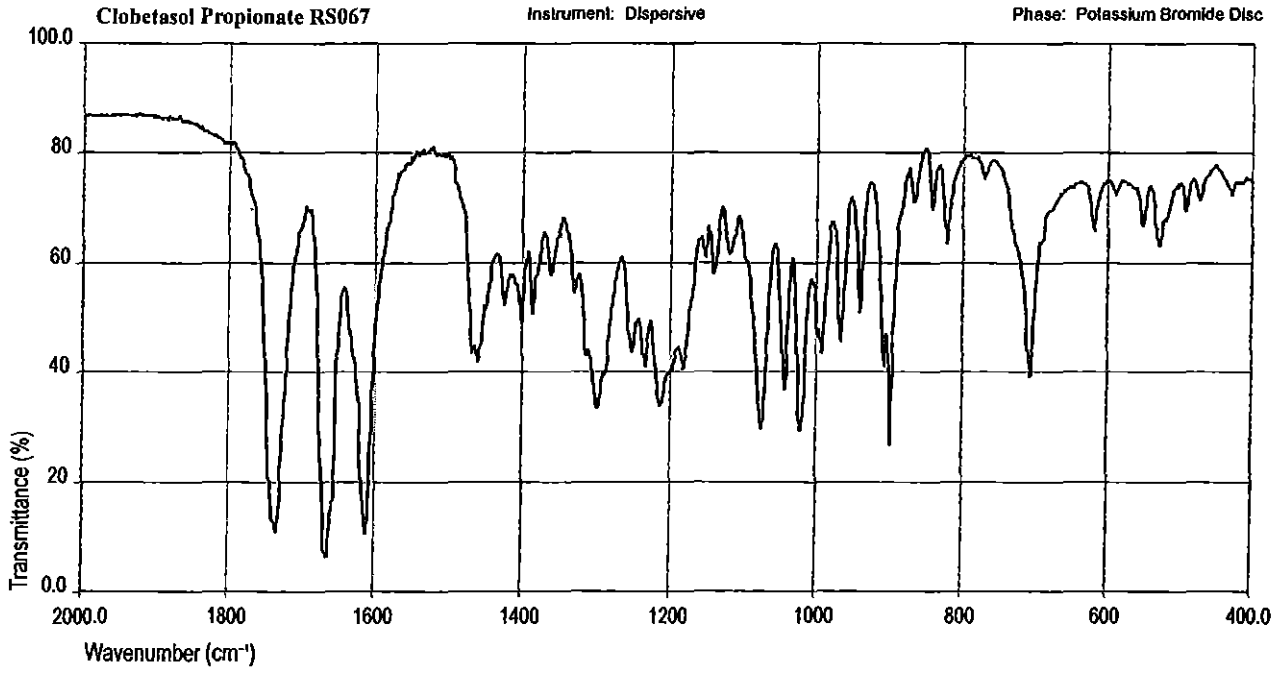


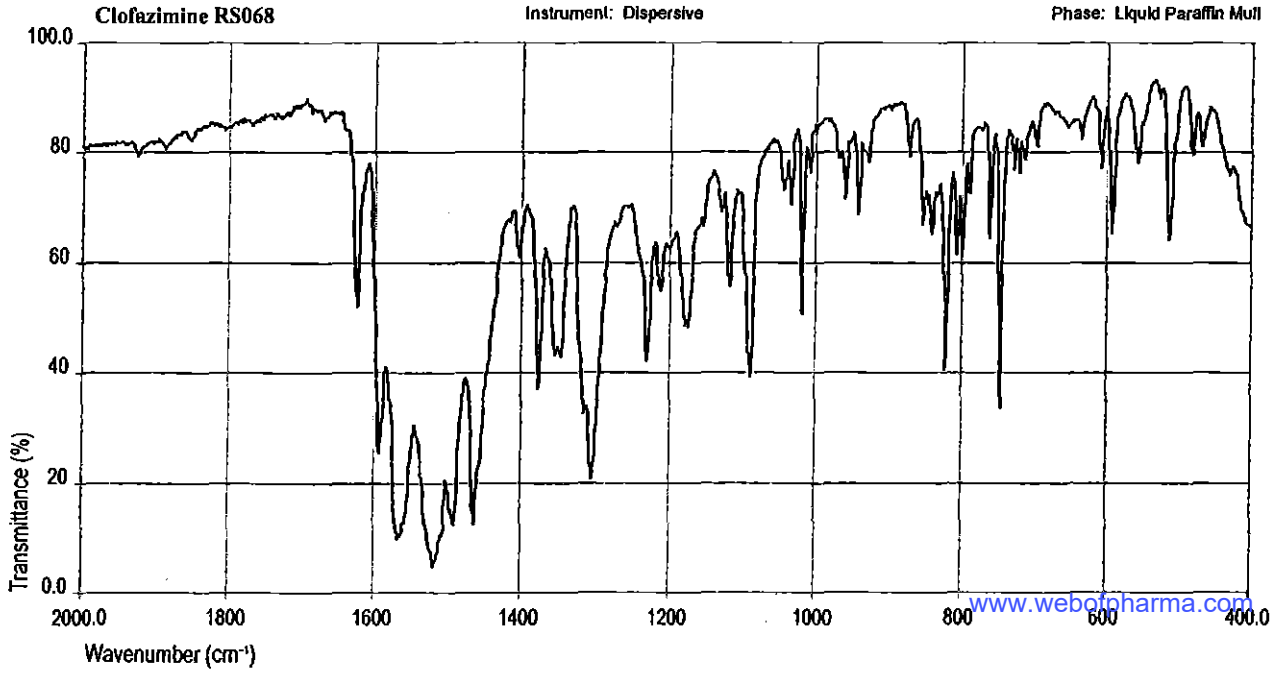


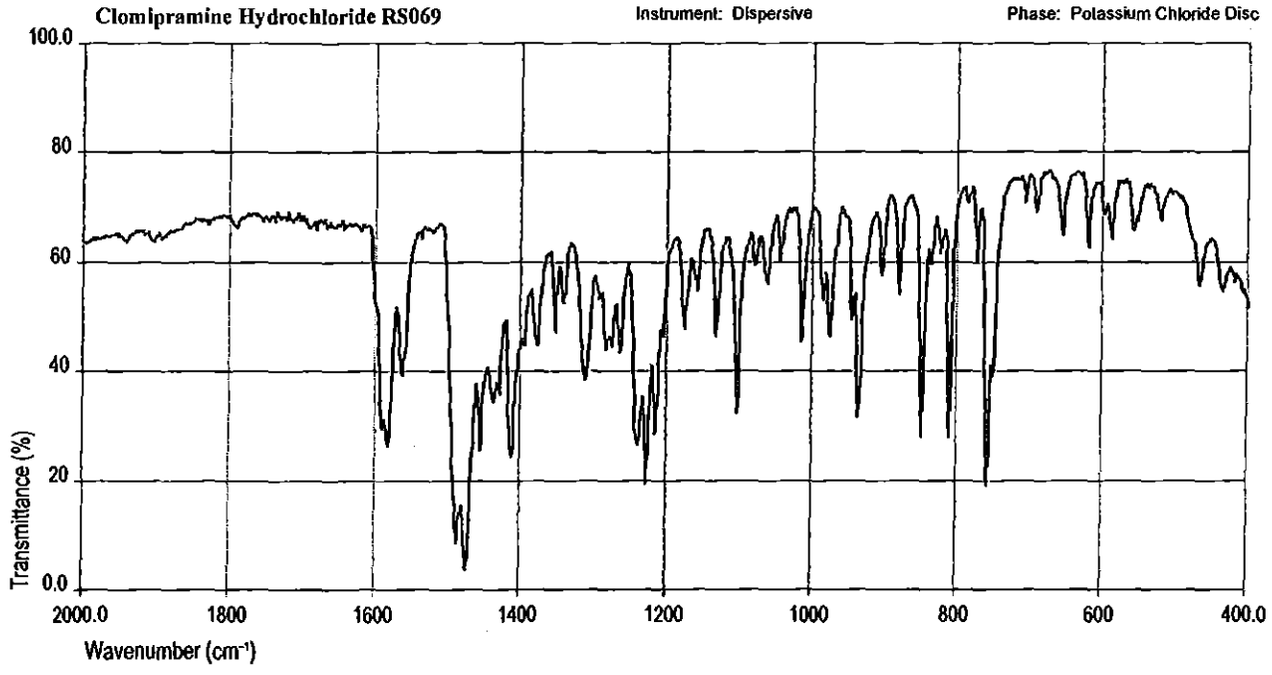


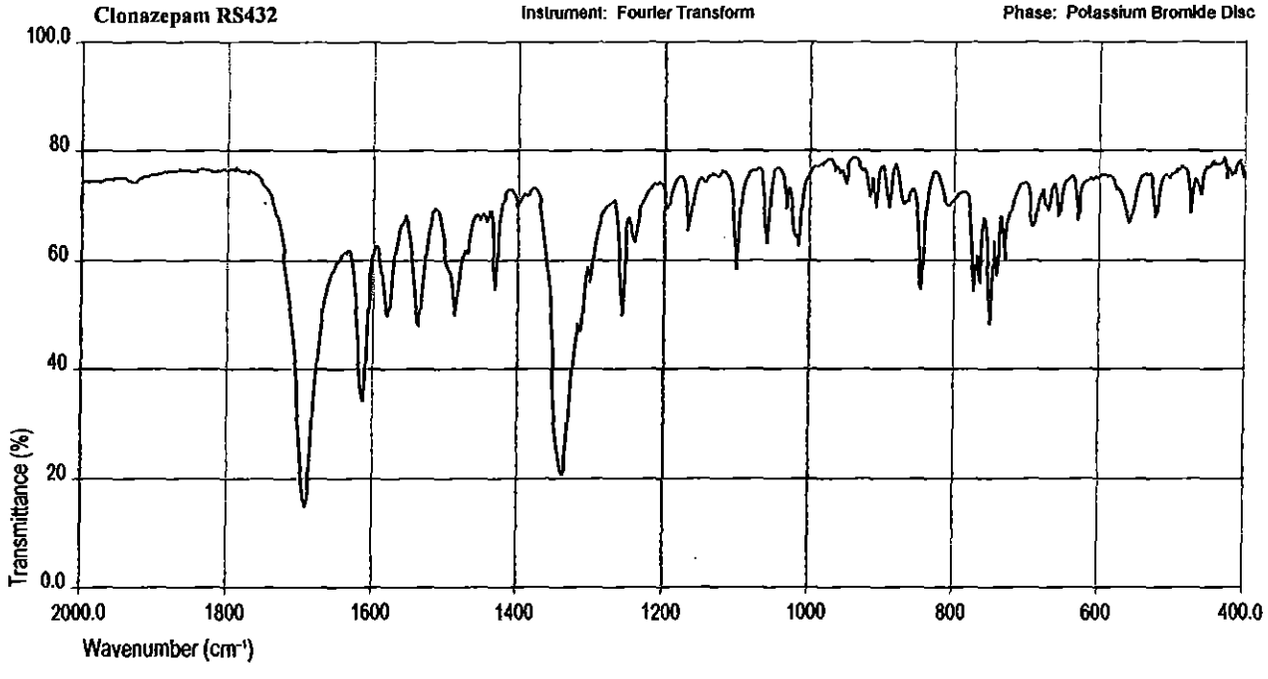


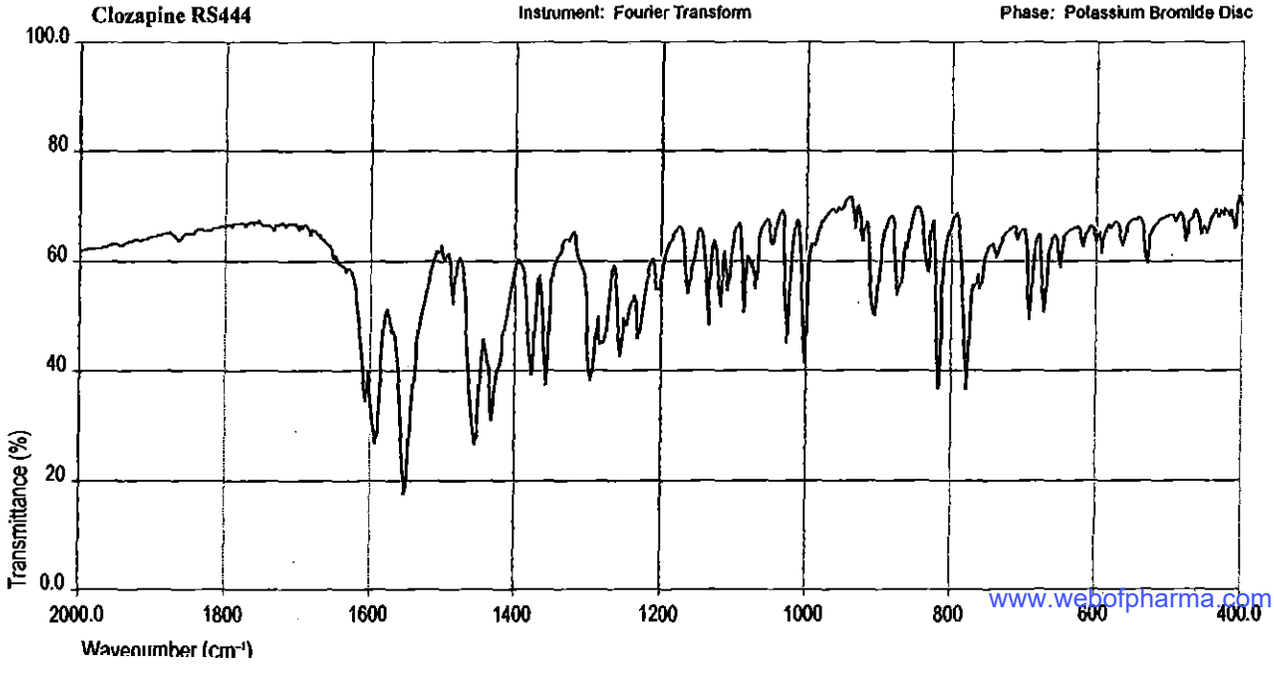


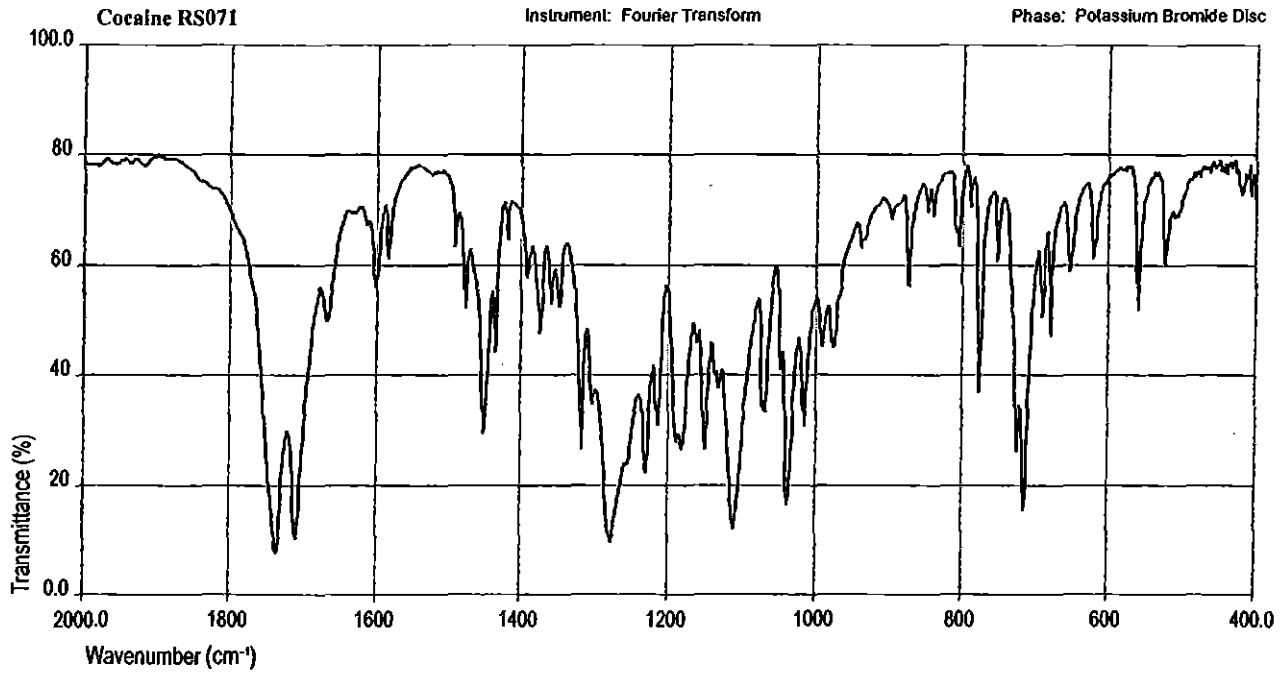


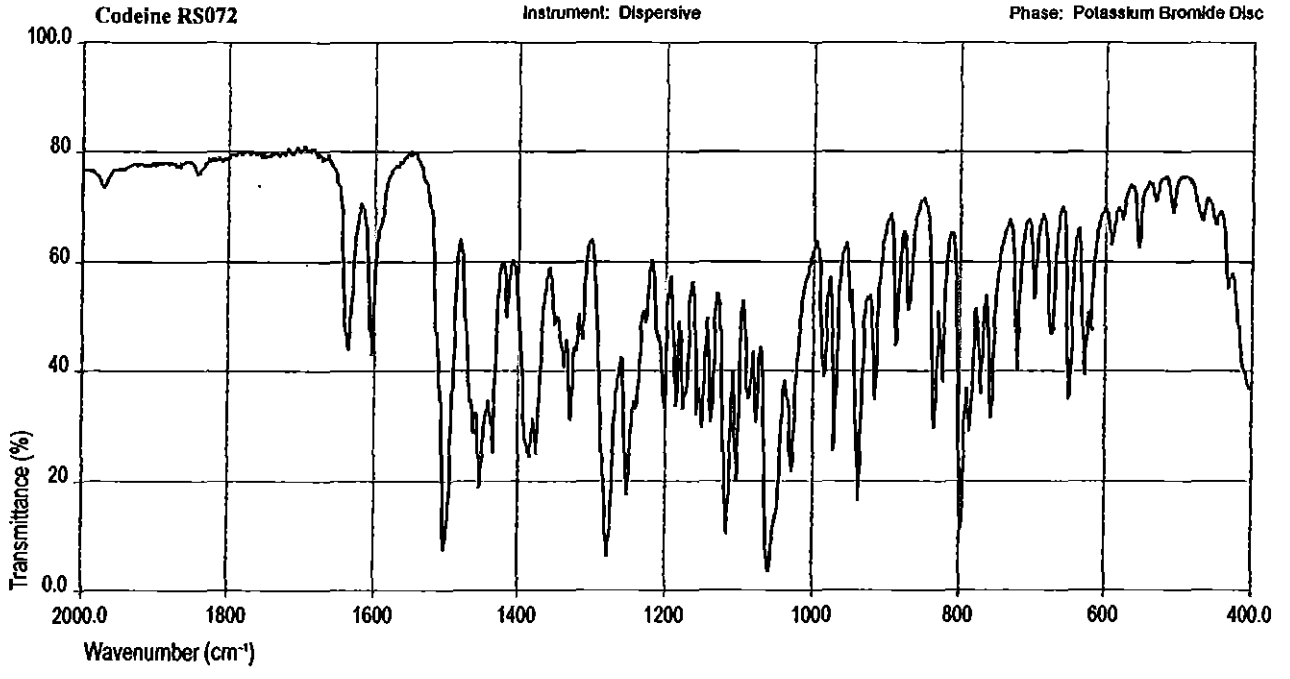


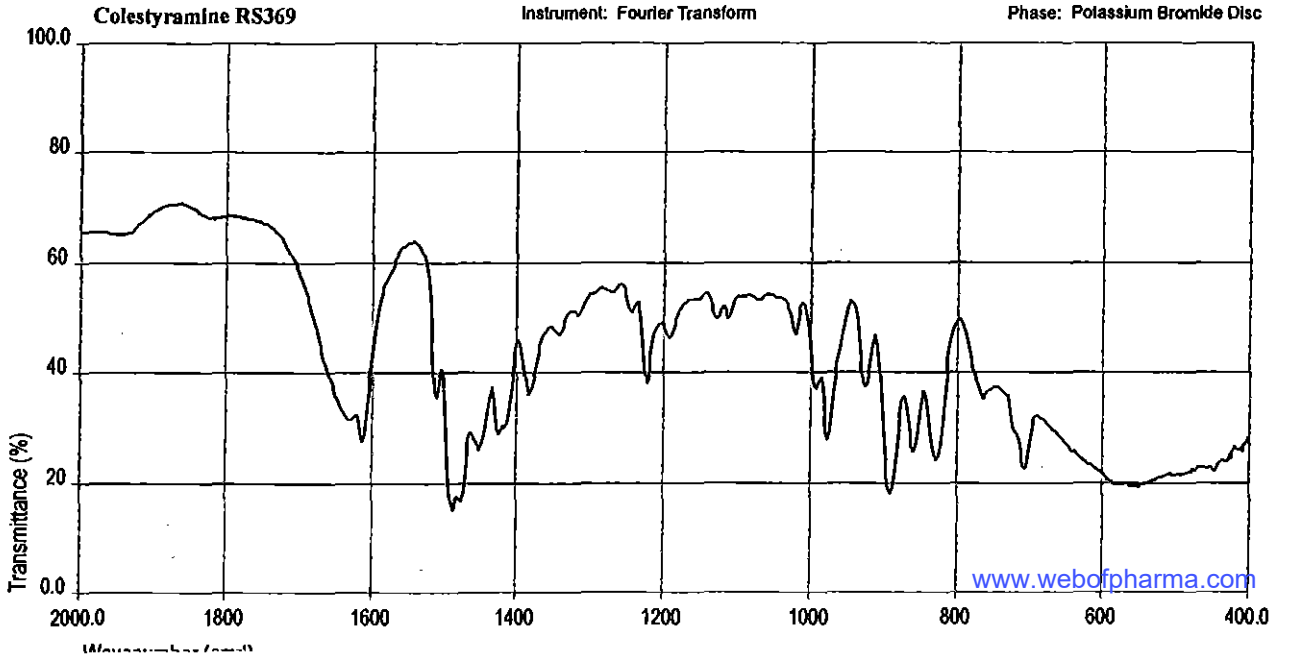


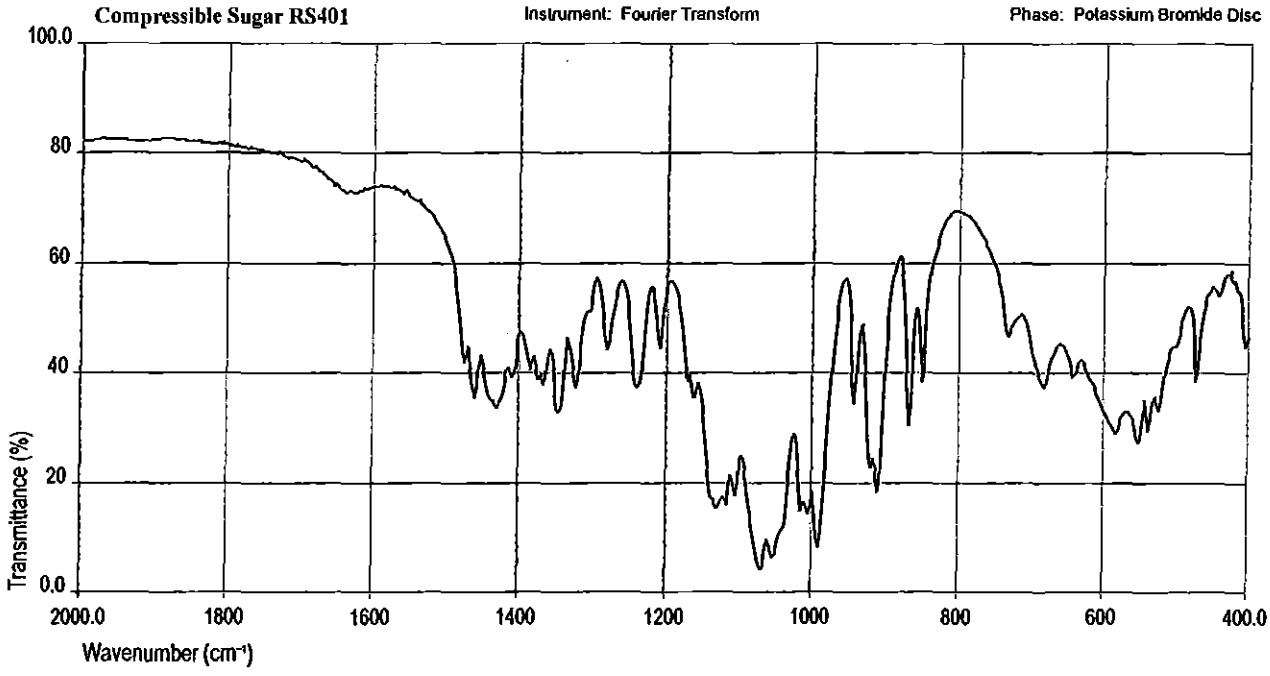


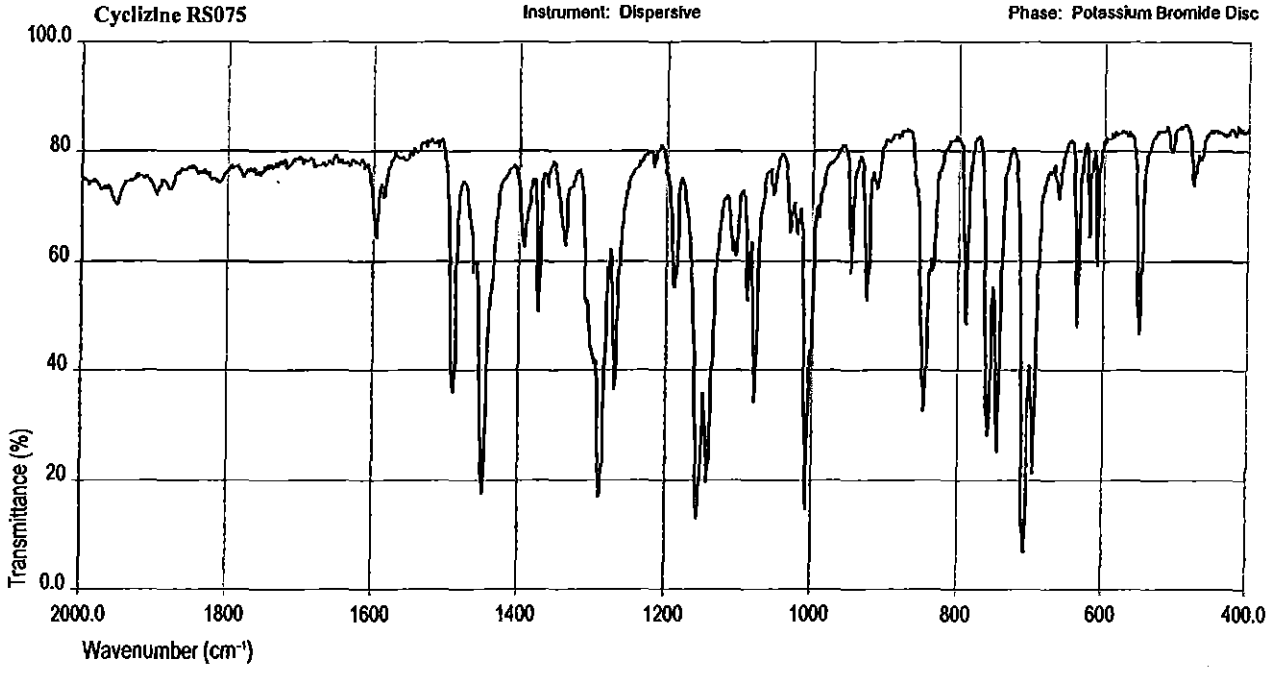


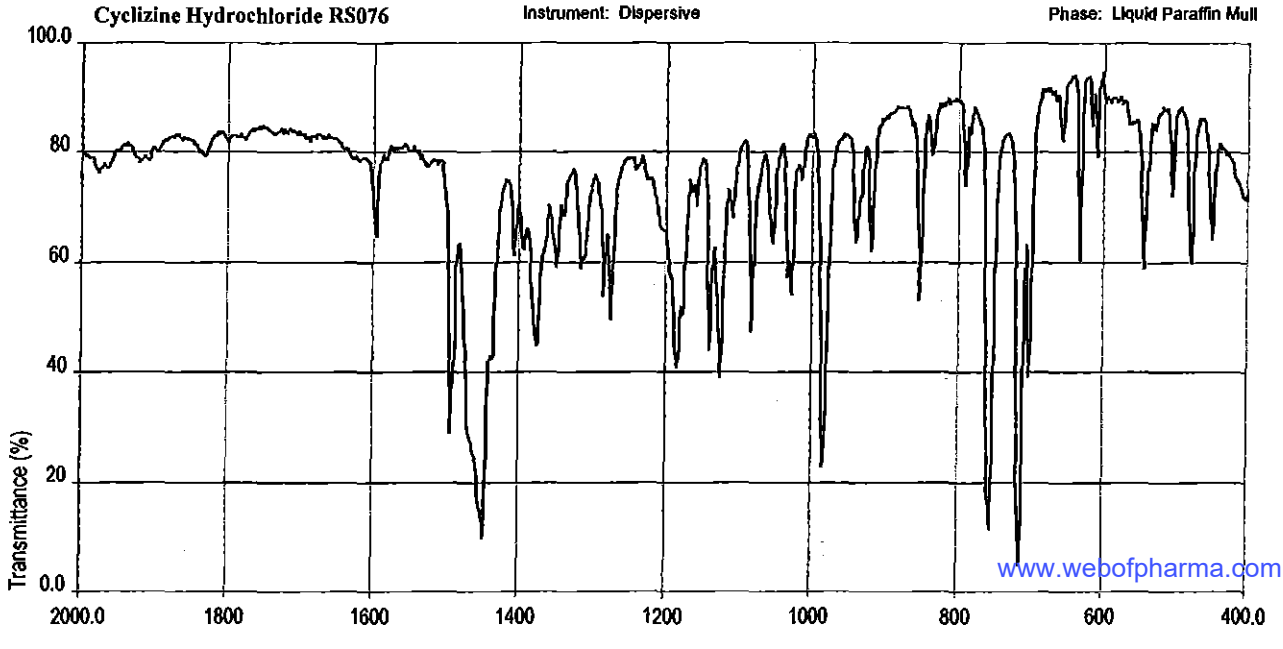


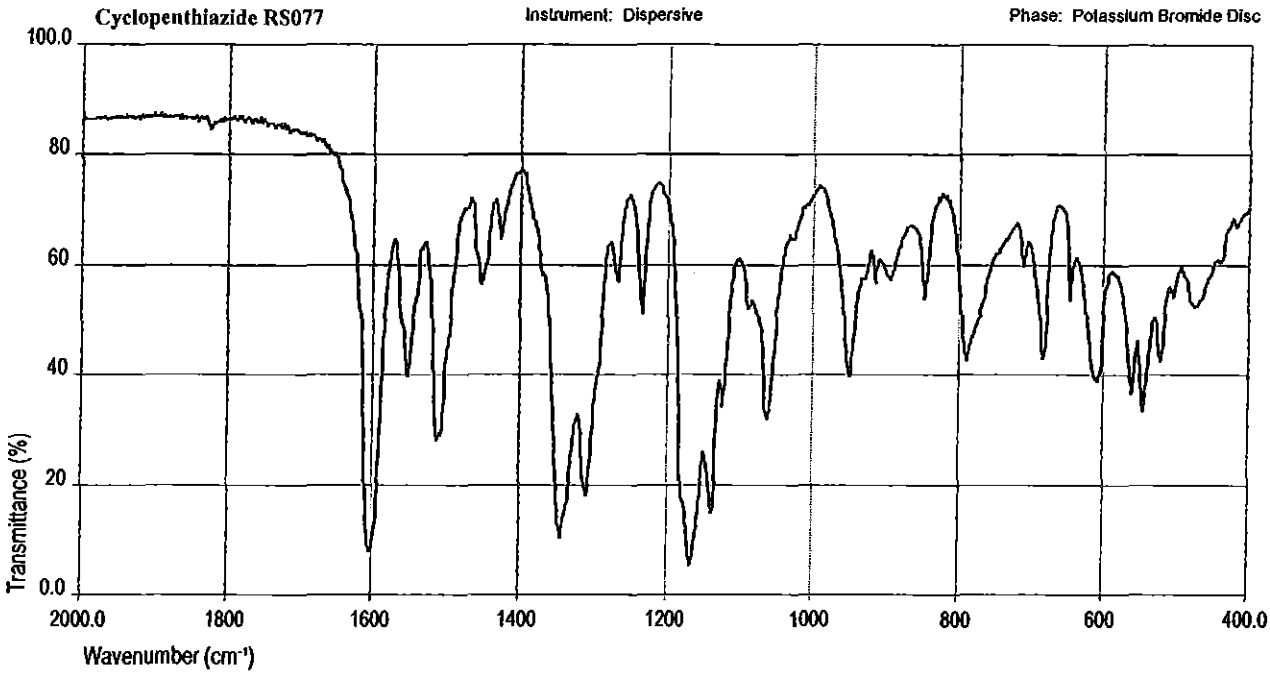


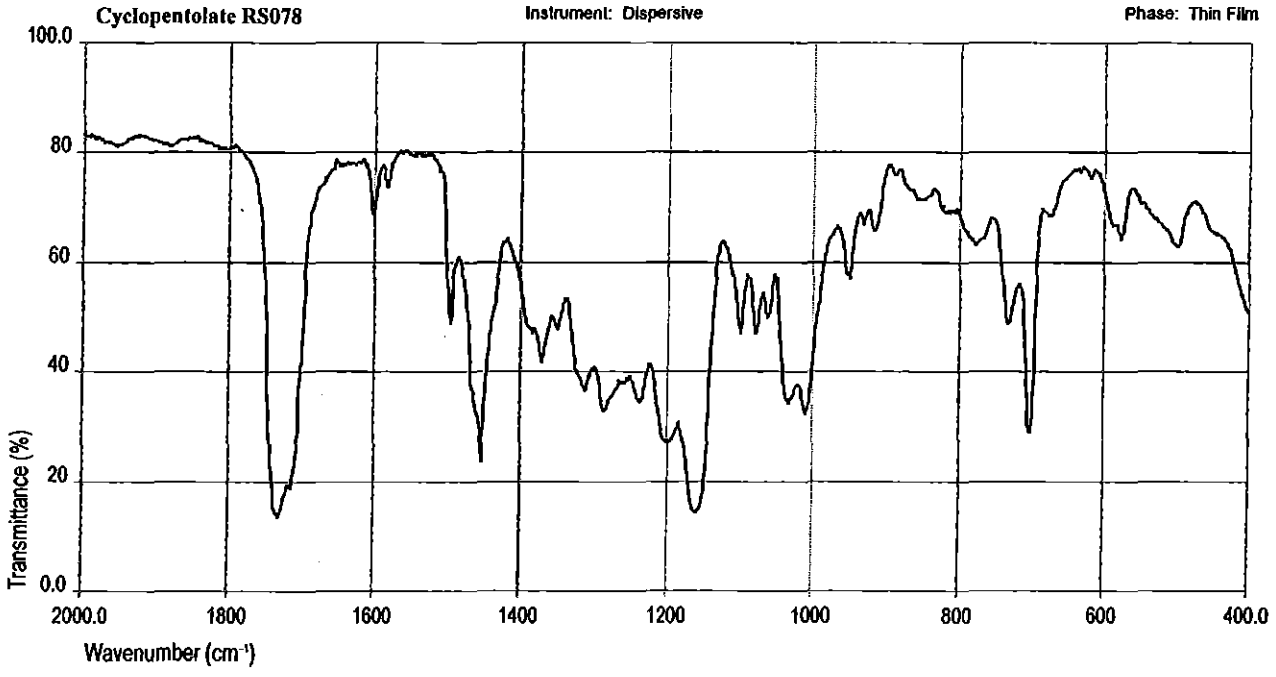


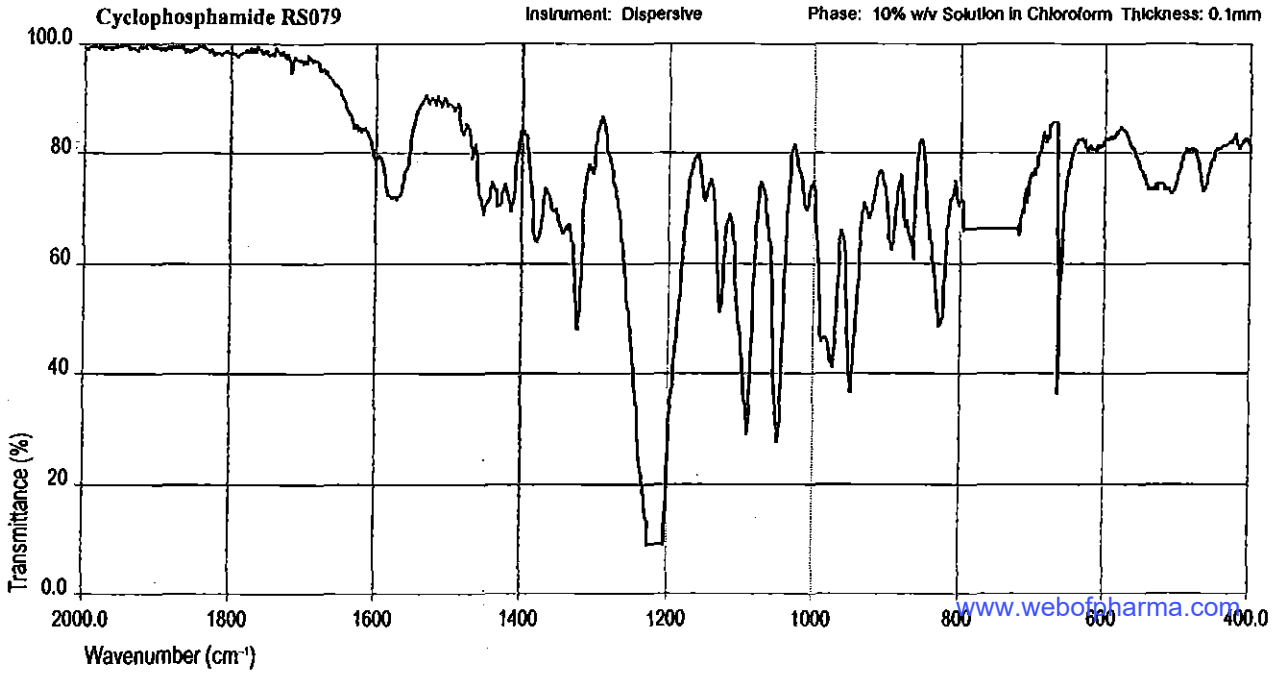


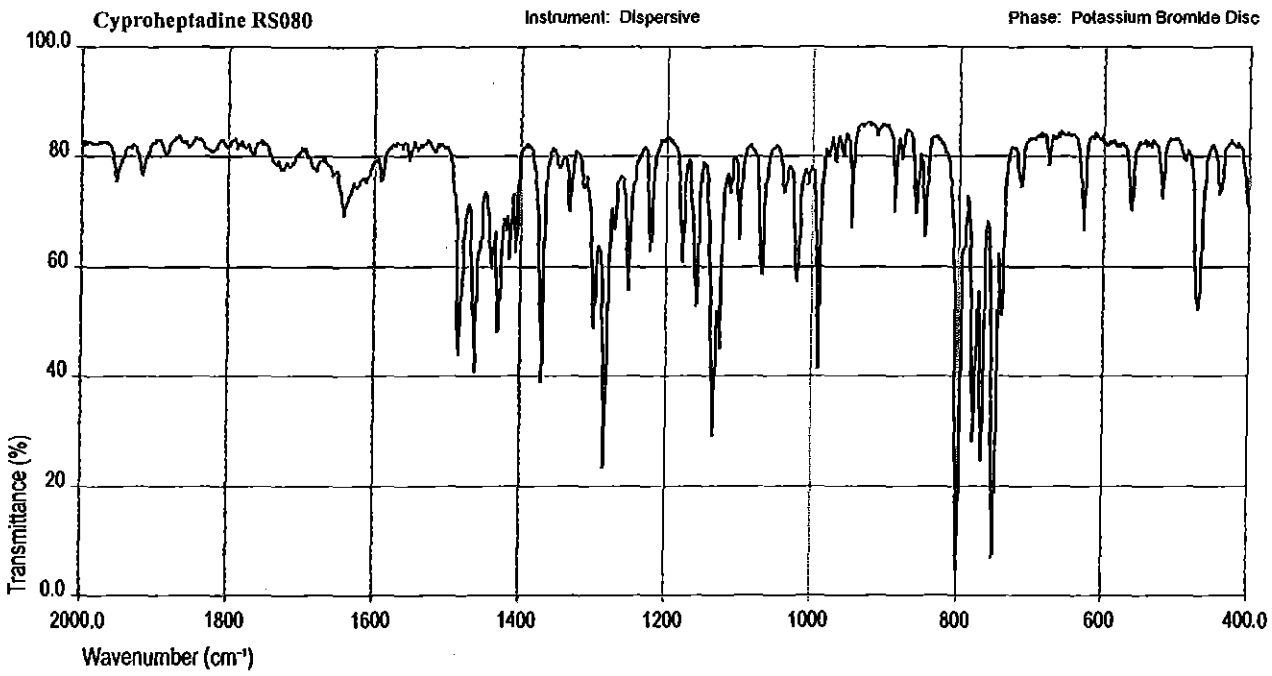


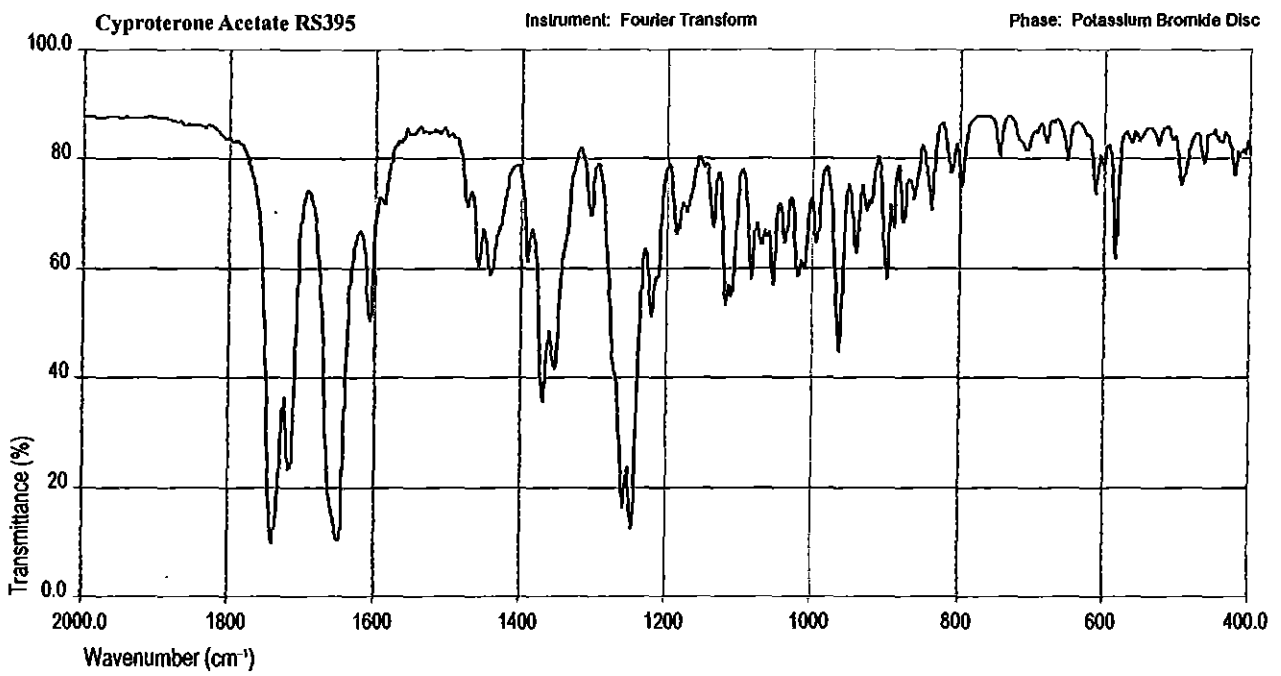


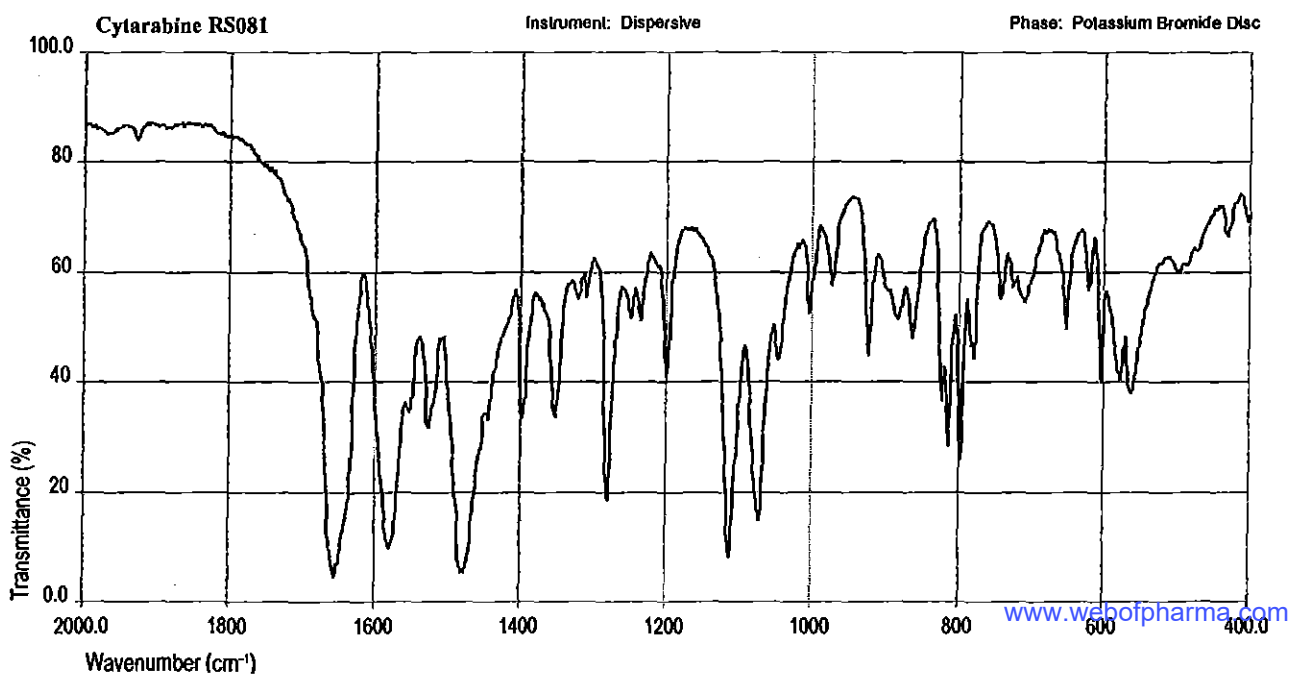




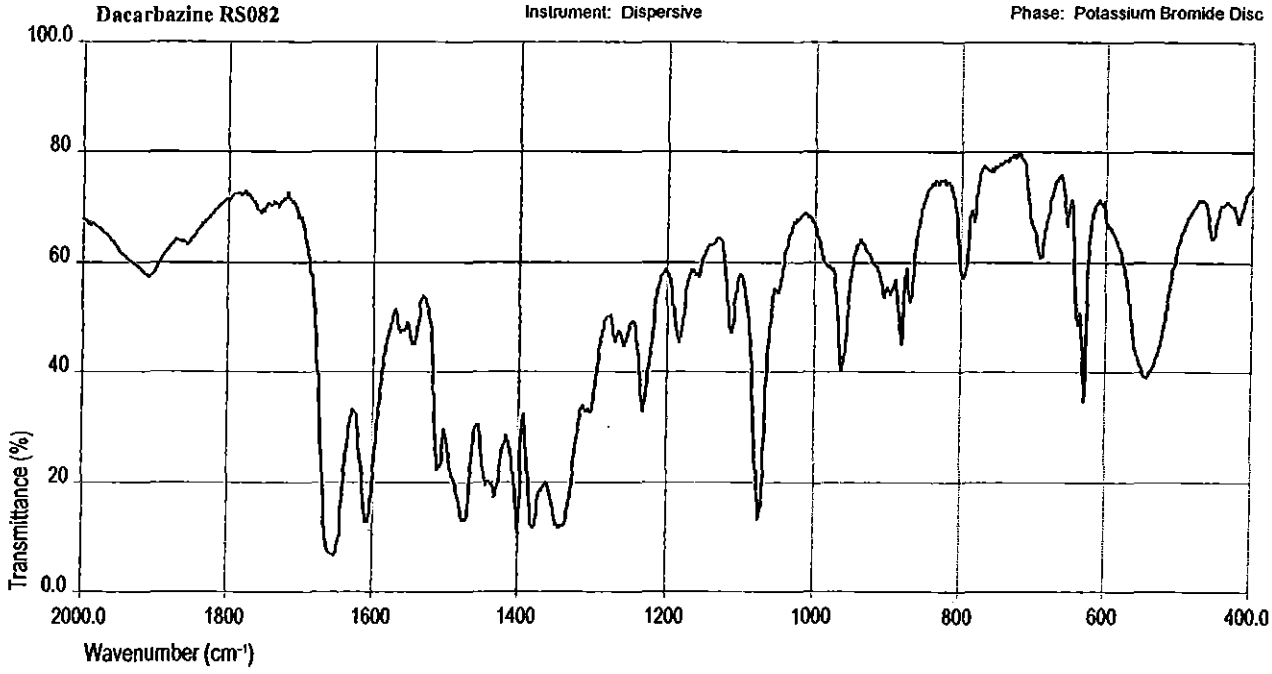




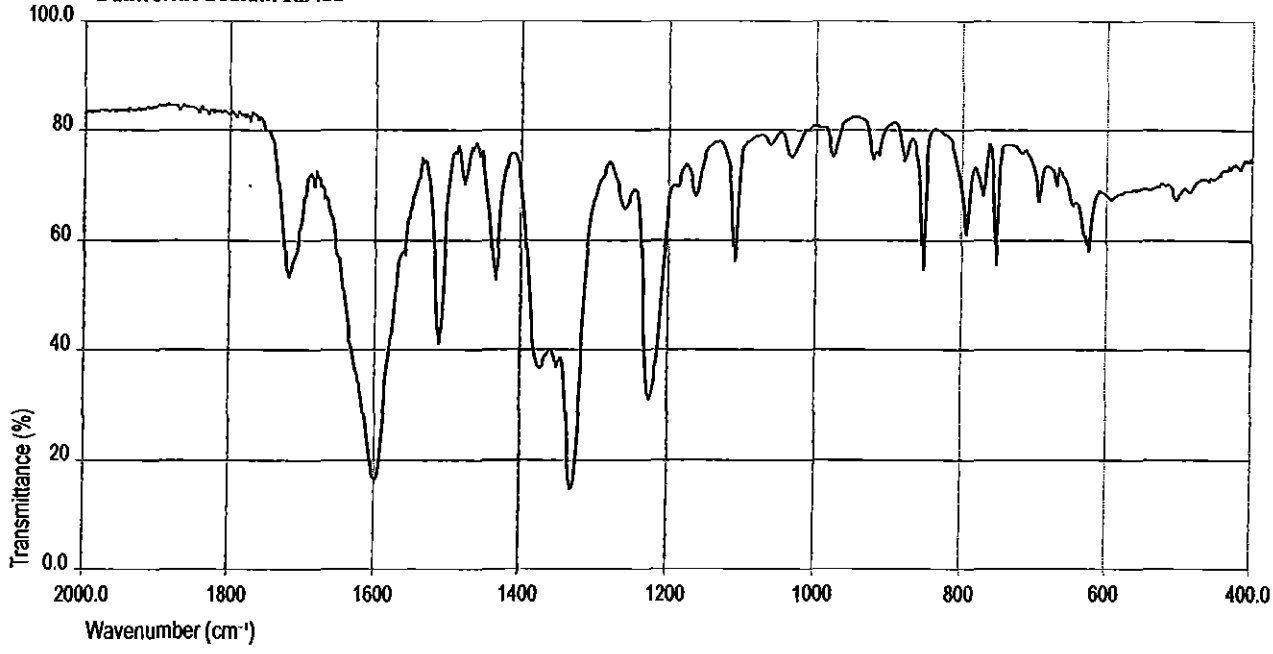




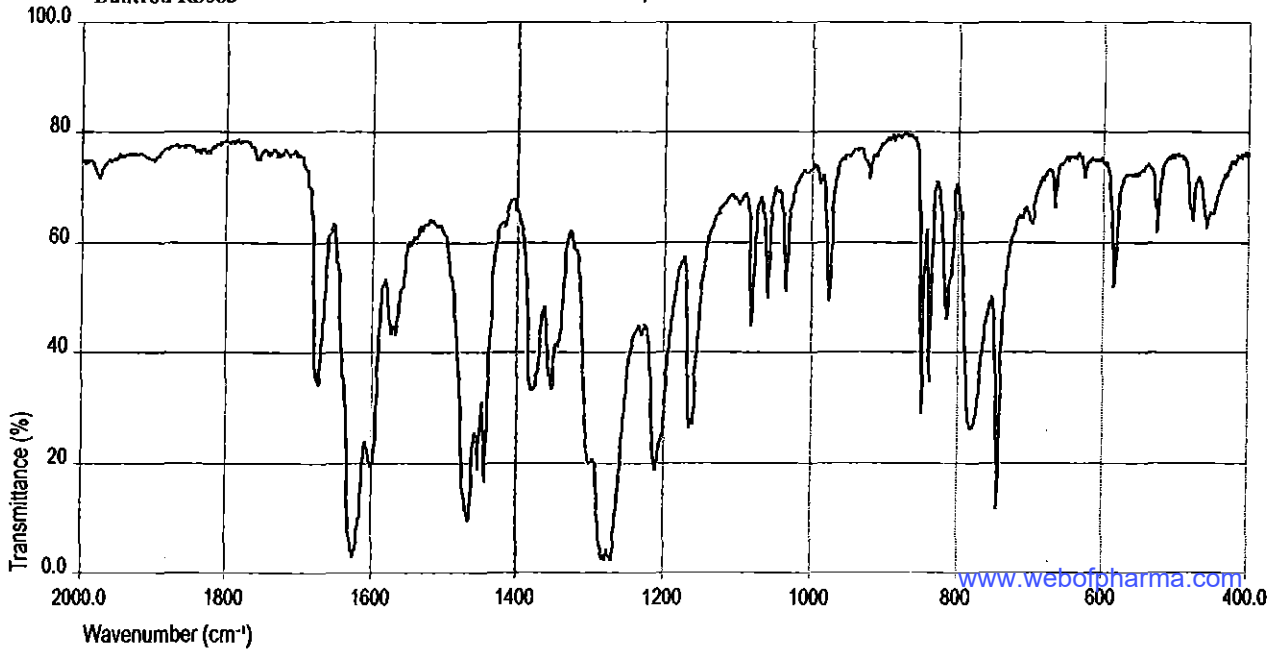
Dacarbazine RS082 Instrument: Dispersive Phase: Potassium Bromide Disc

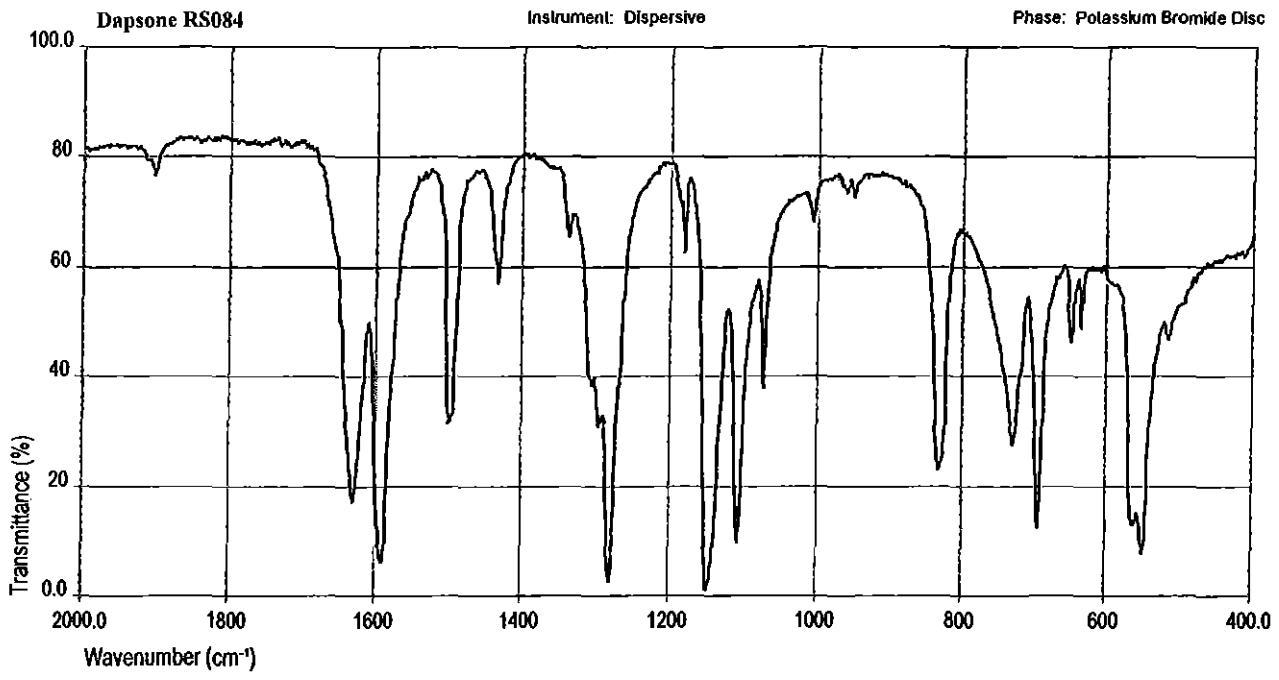


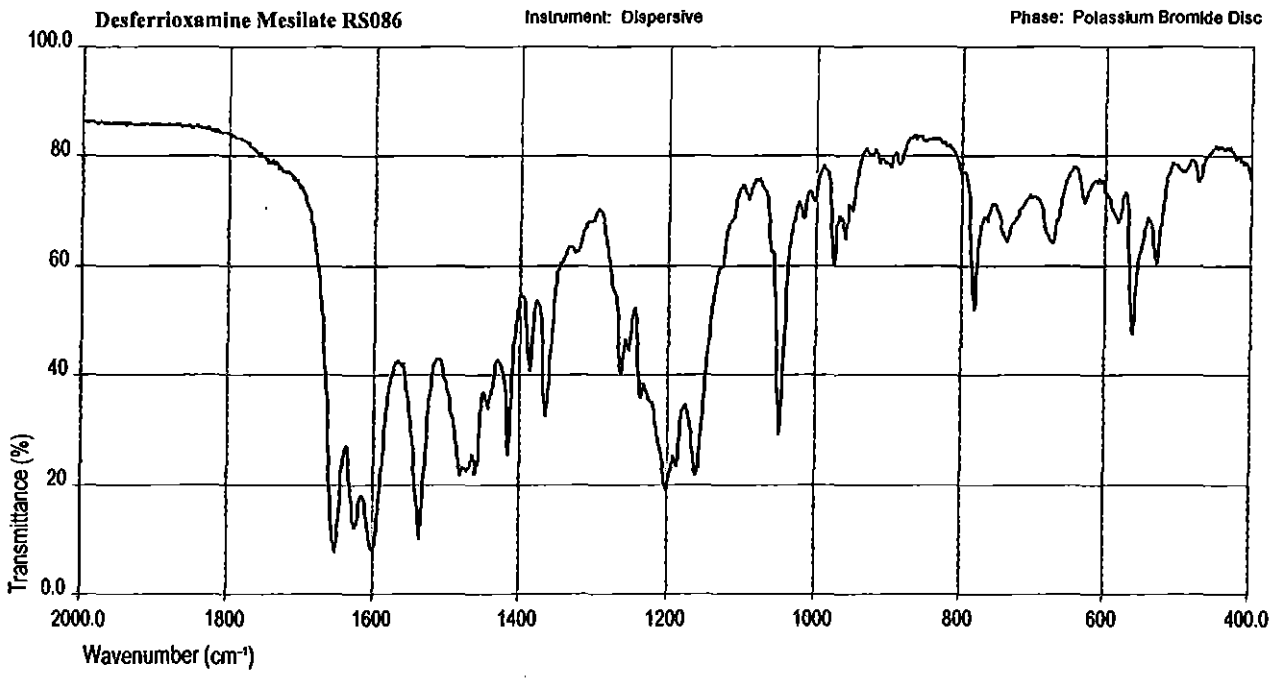
Dantrolene Sodium RS422 Instrument: Fourier Transform Phase: Potassium Bromide Disc

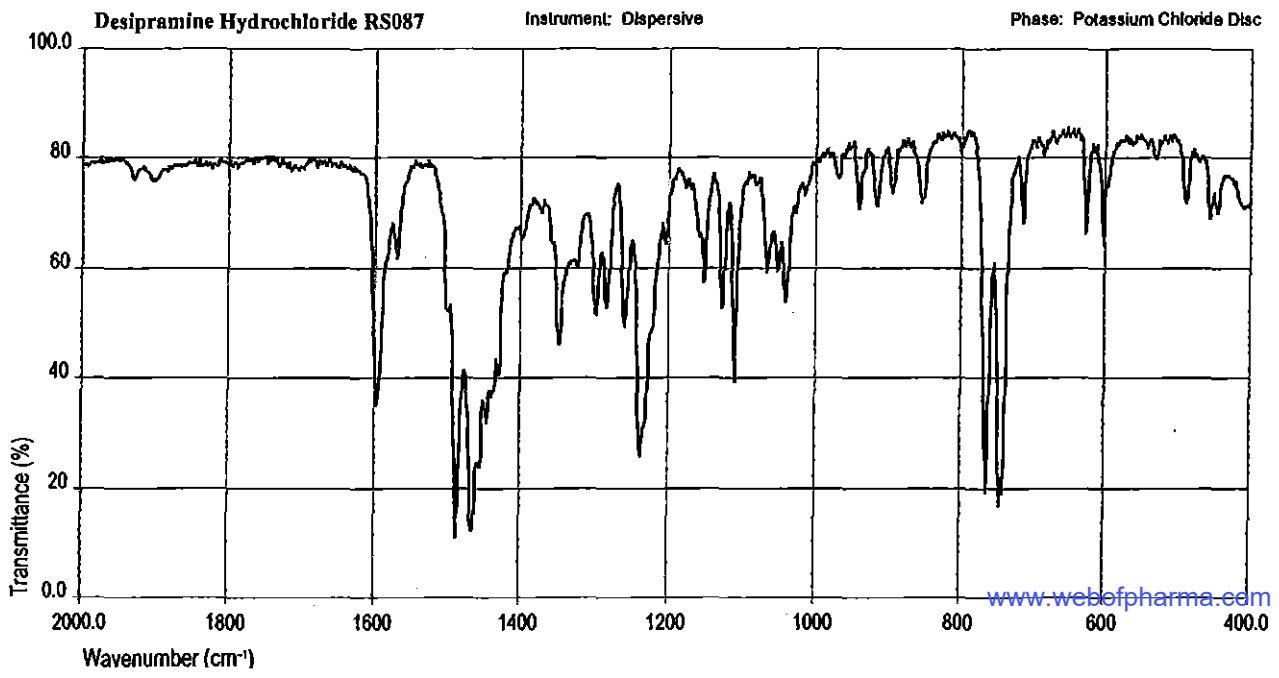


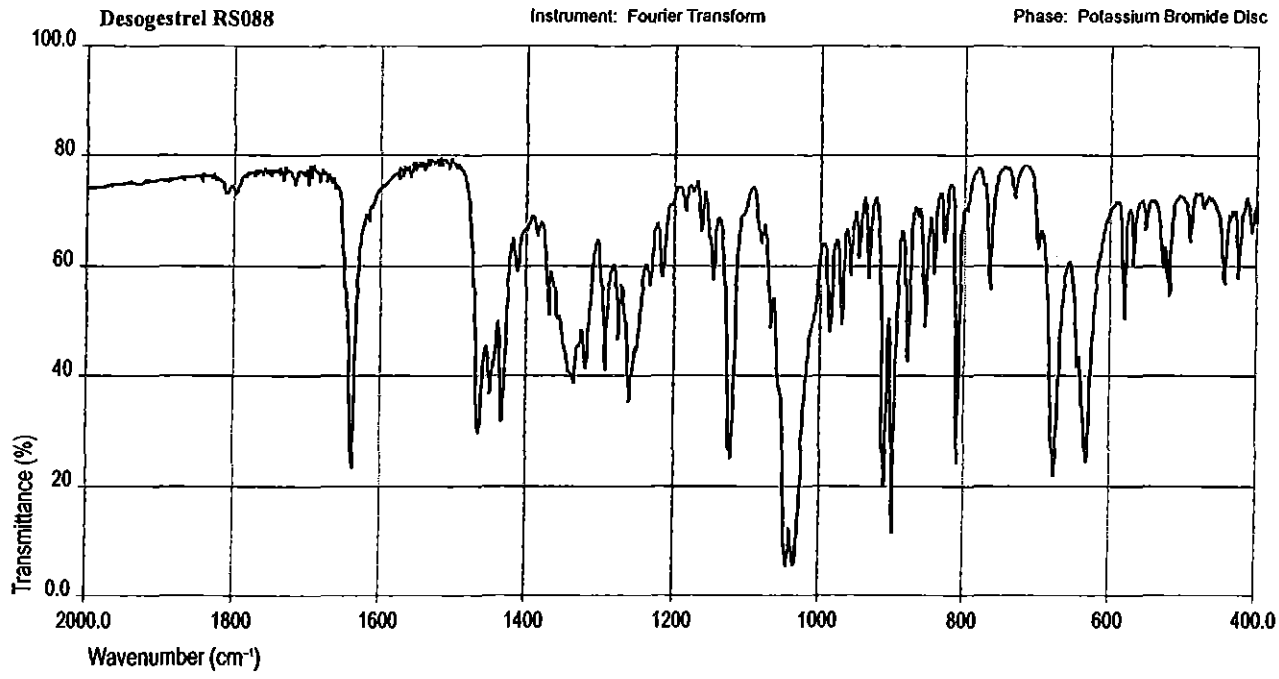
Dantron RS083 Instrument: Dispersive Phase: Potassium Bromide Disc

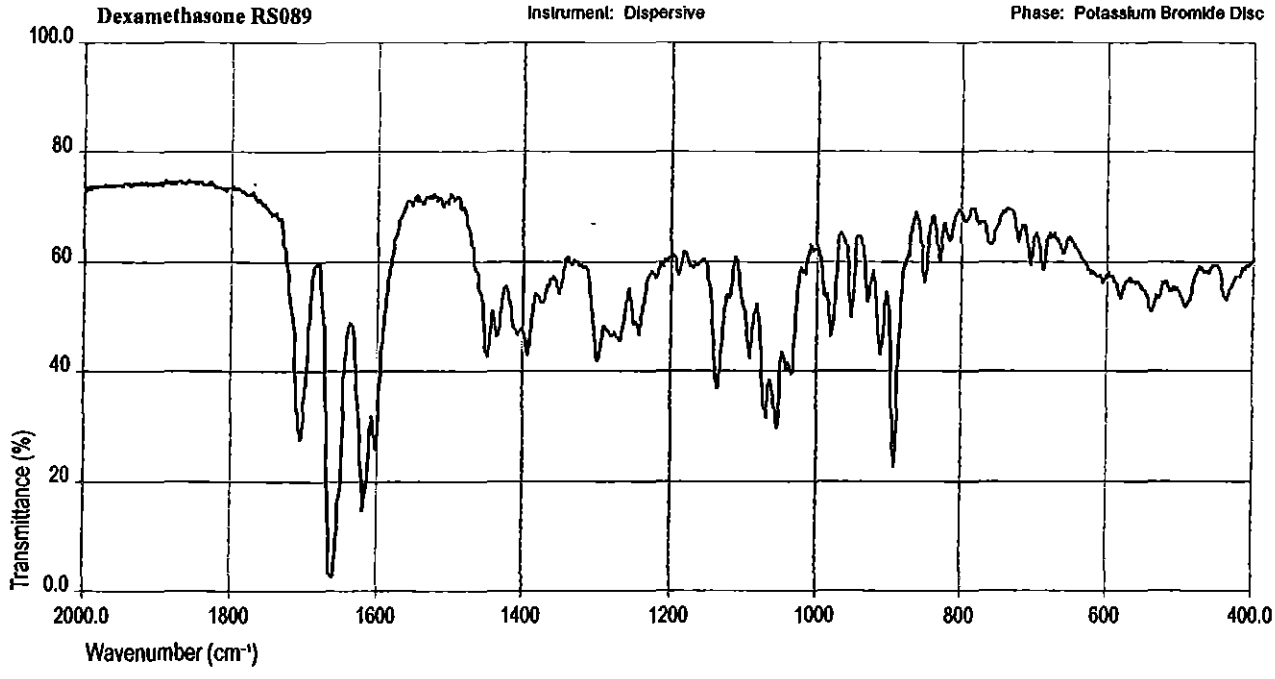


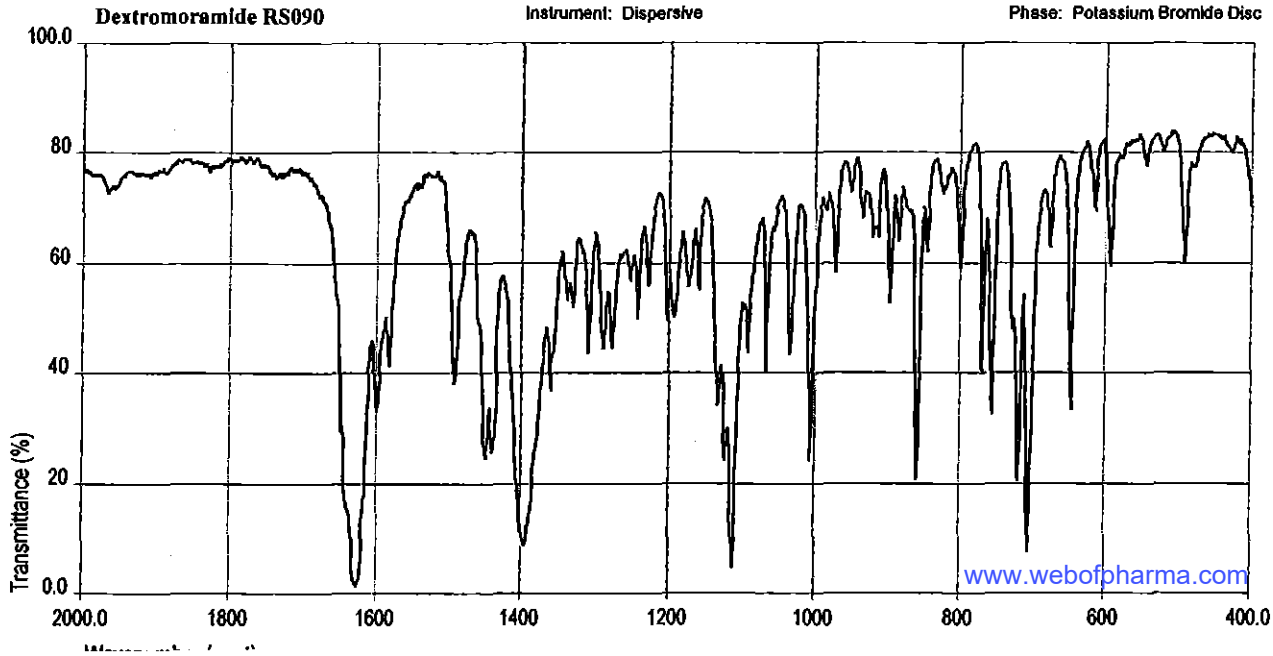


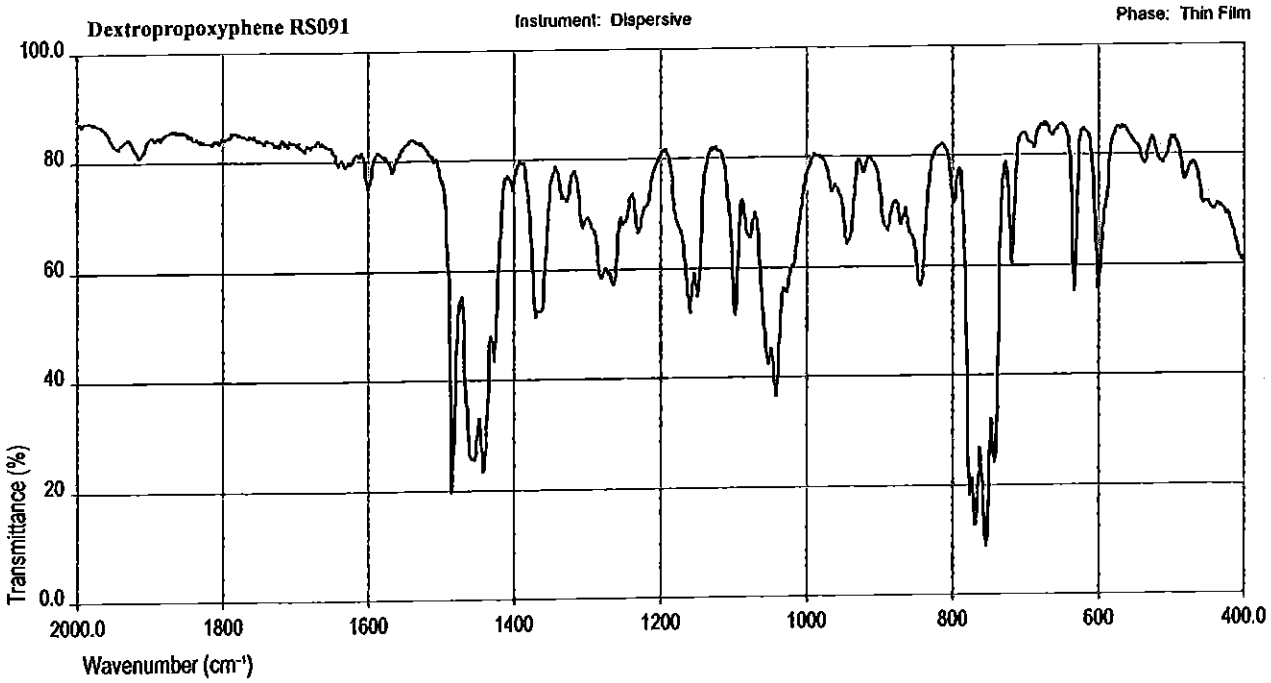


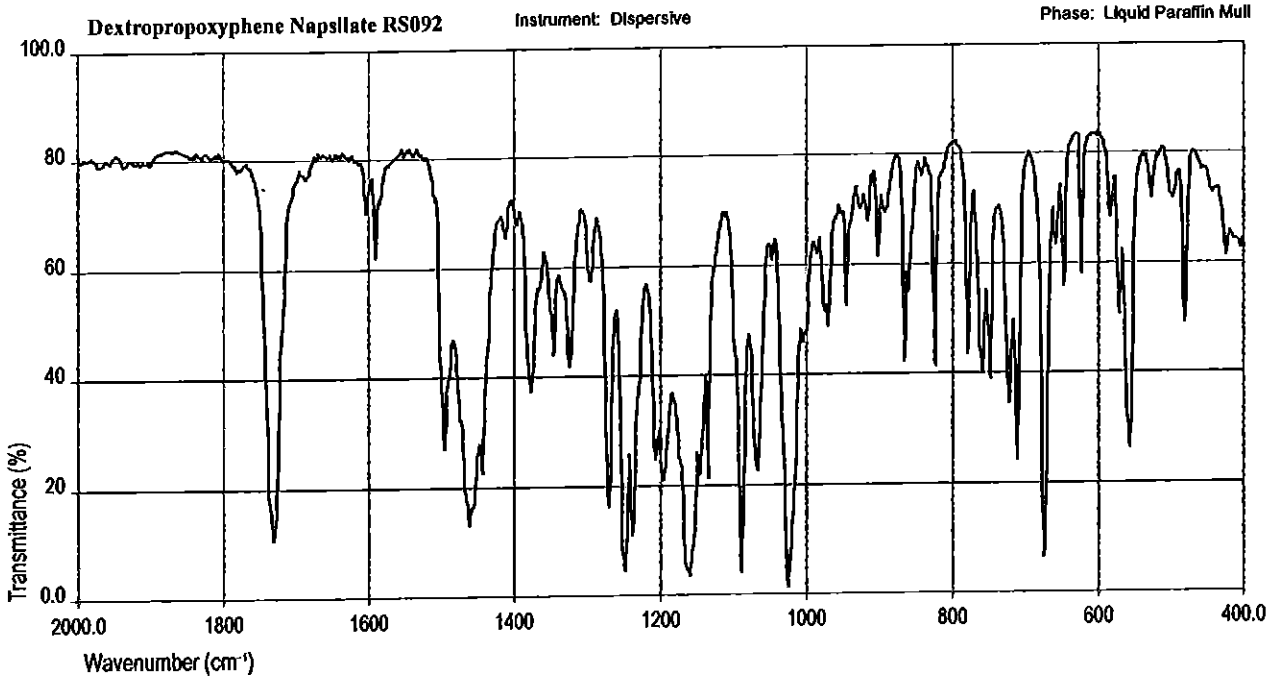


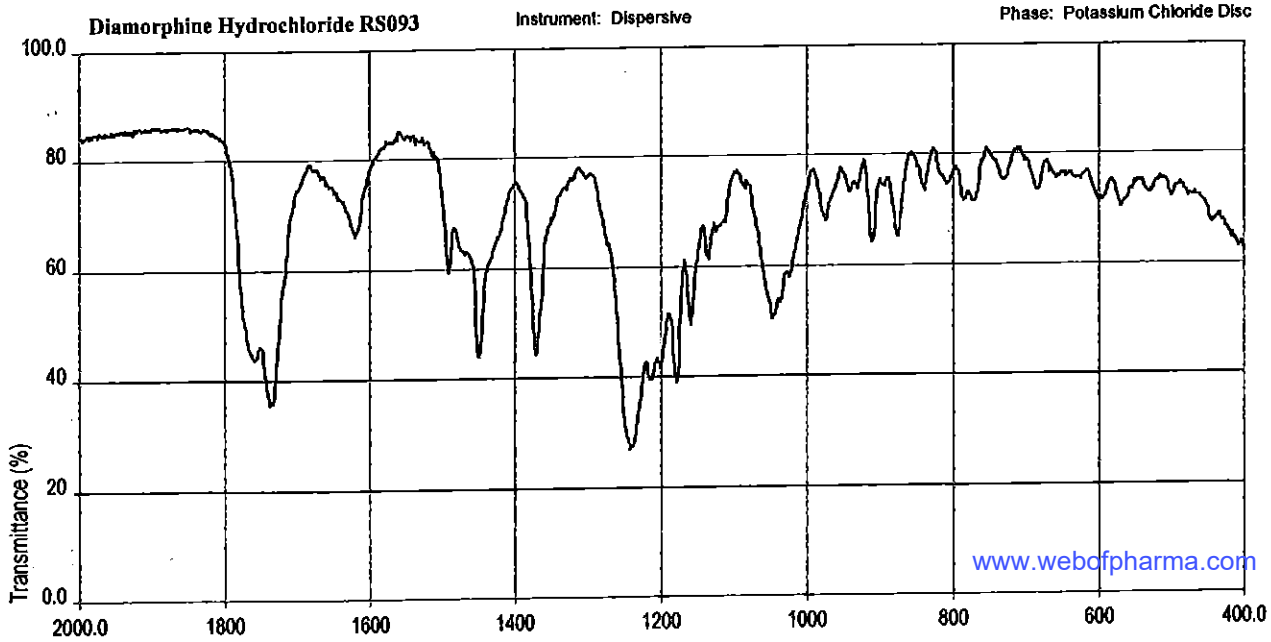


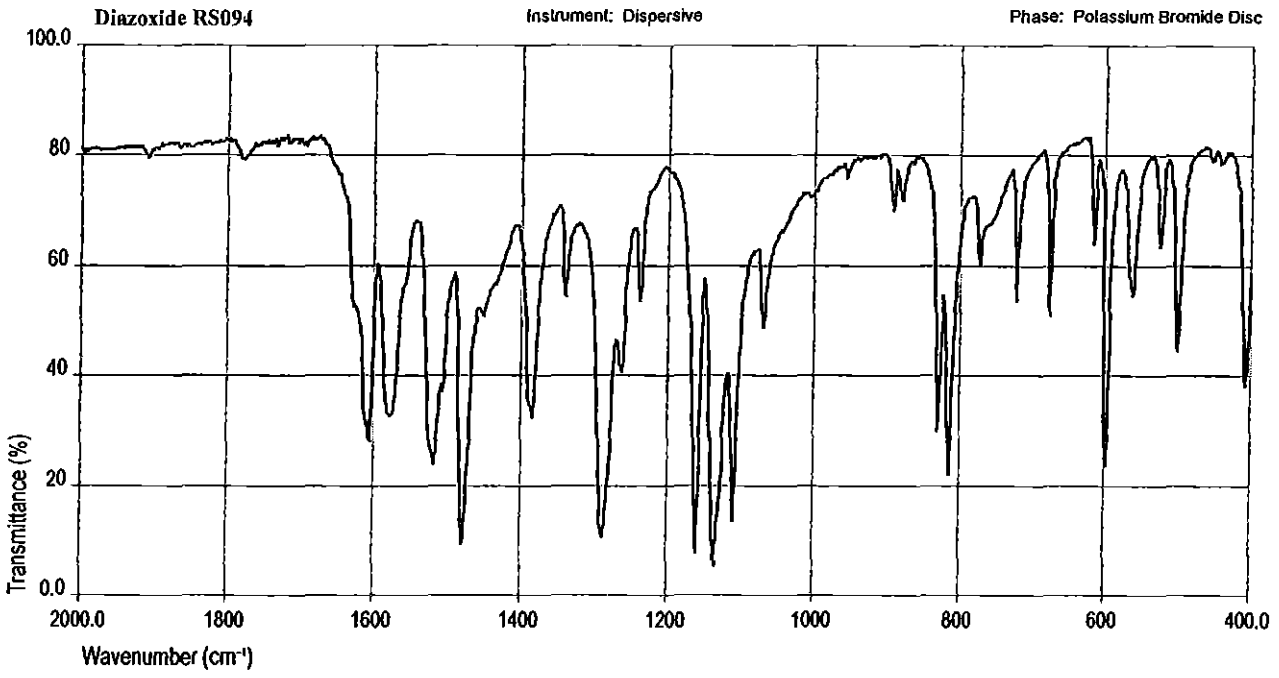


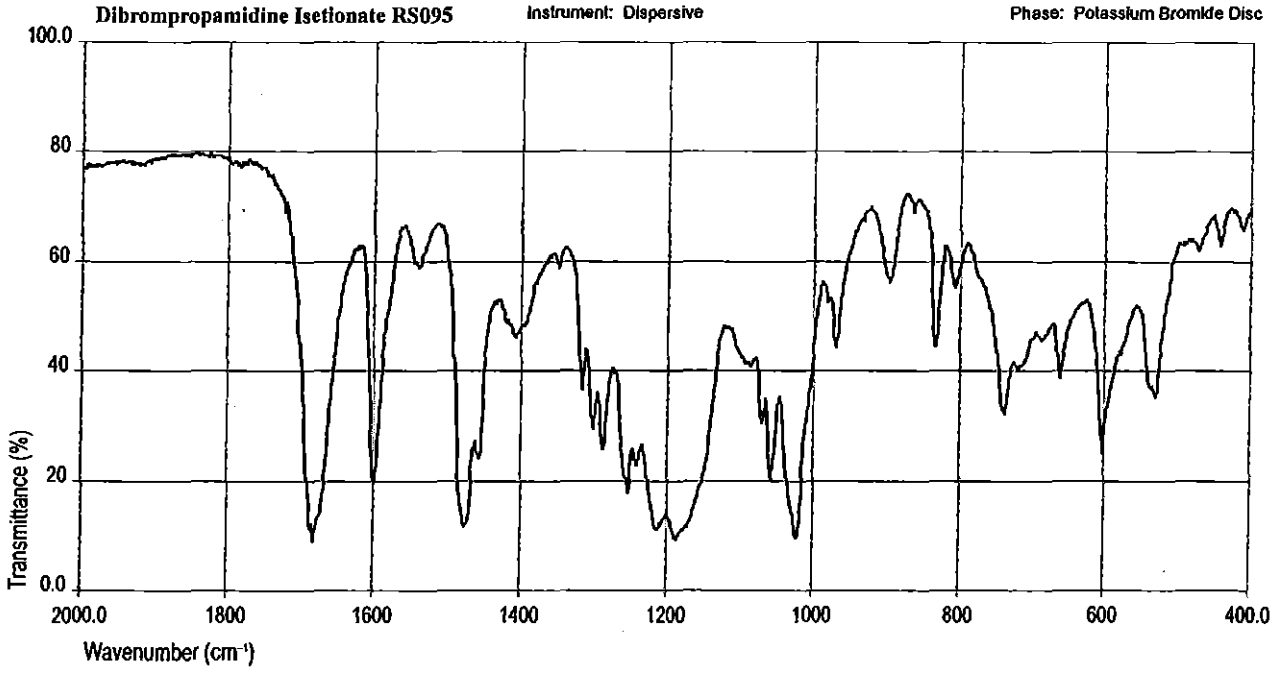


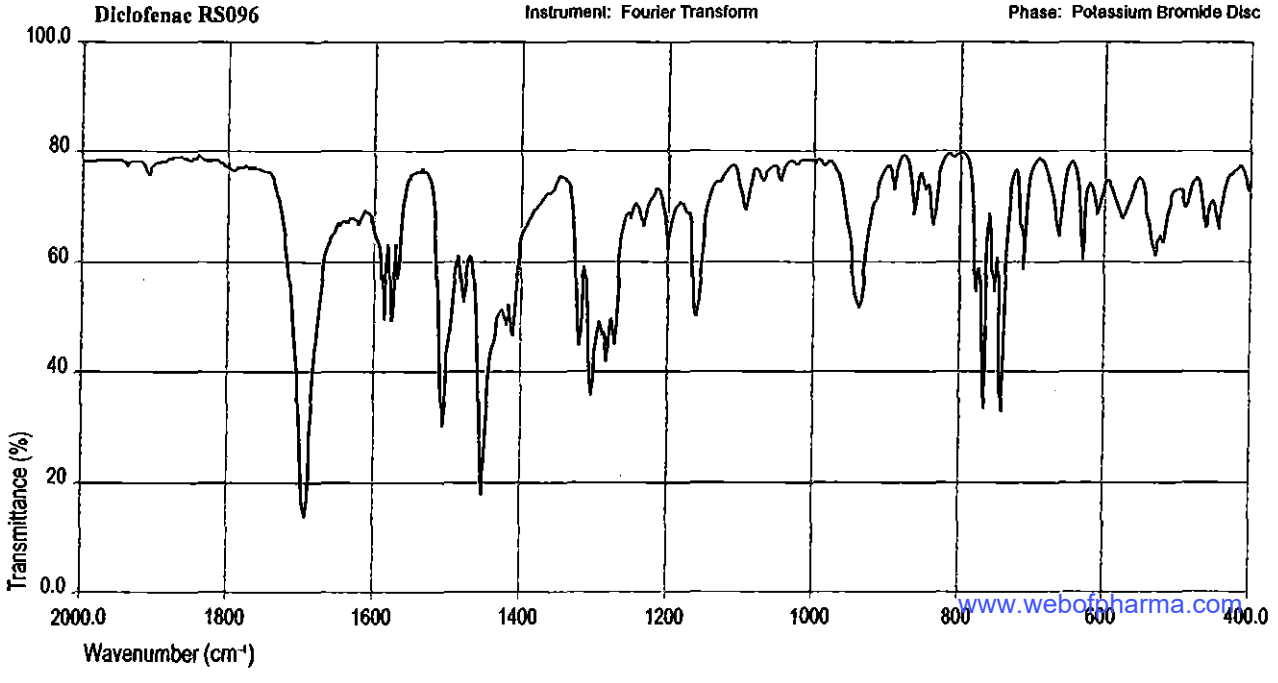


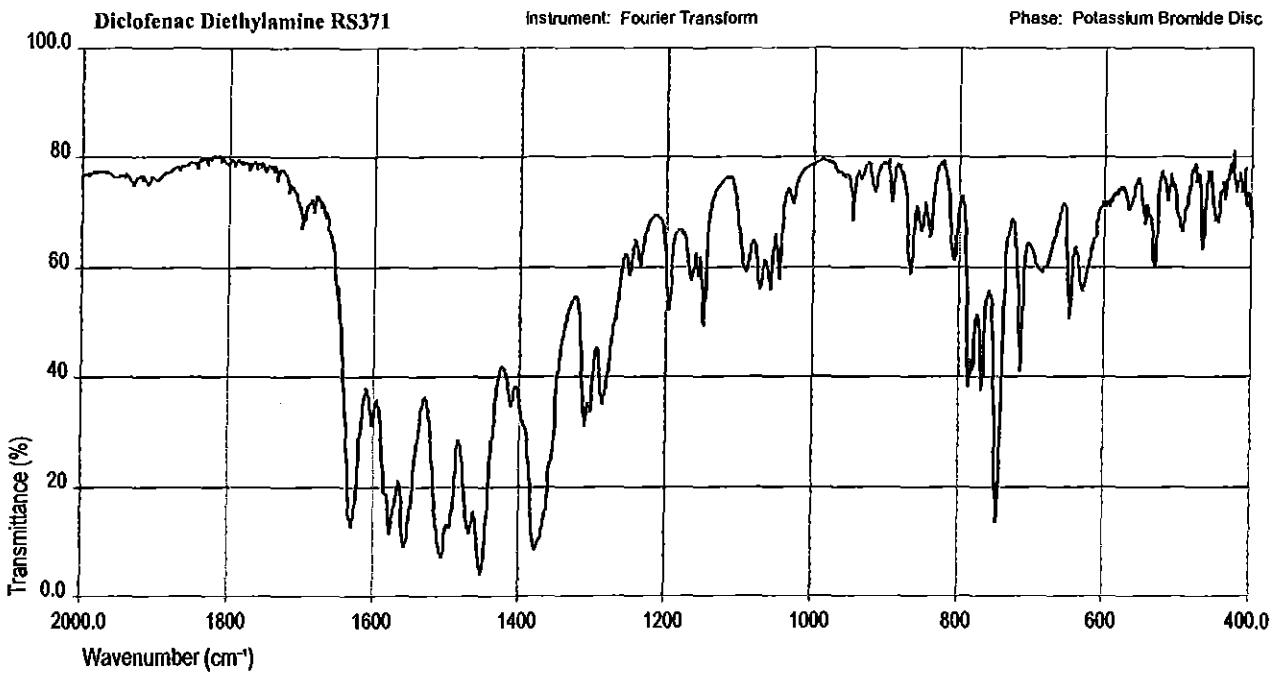


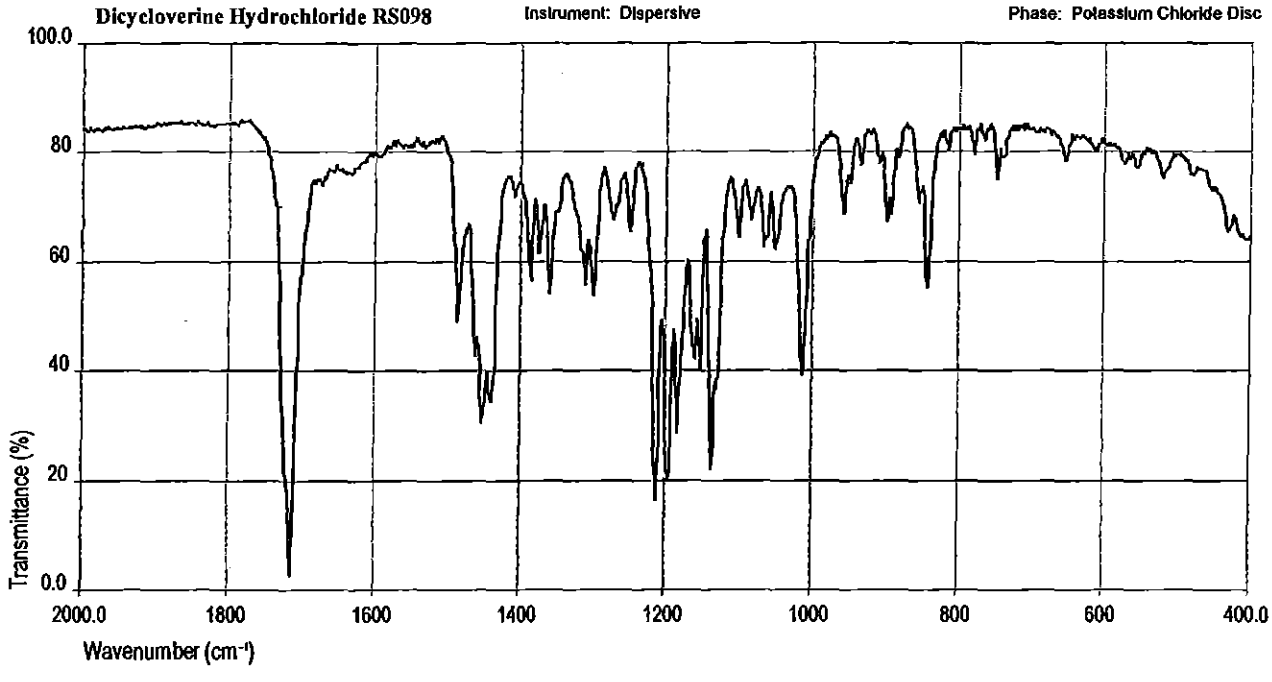


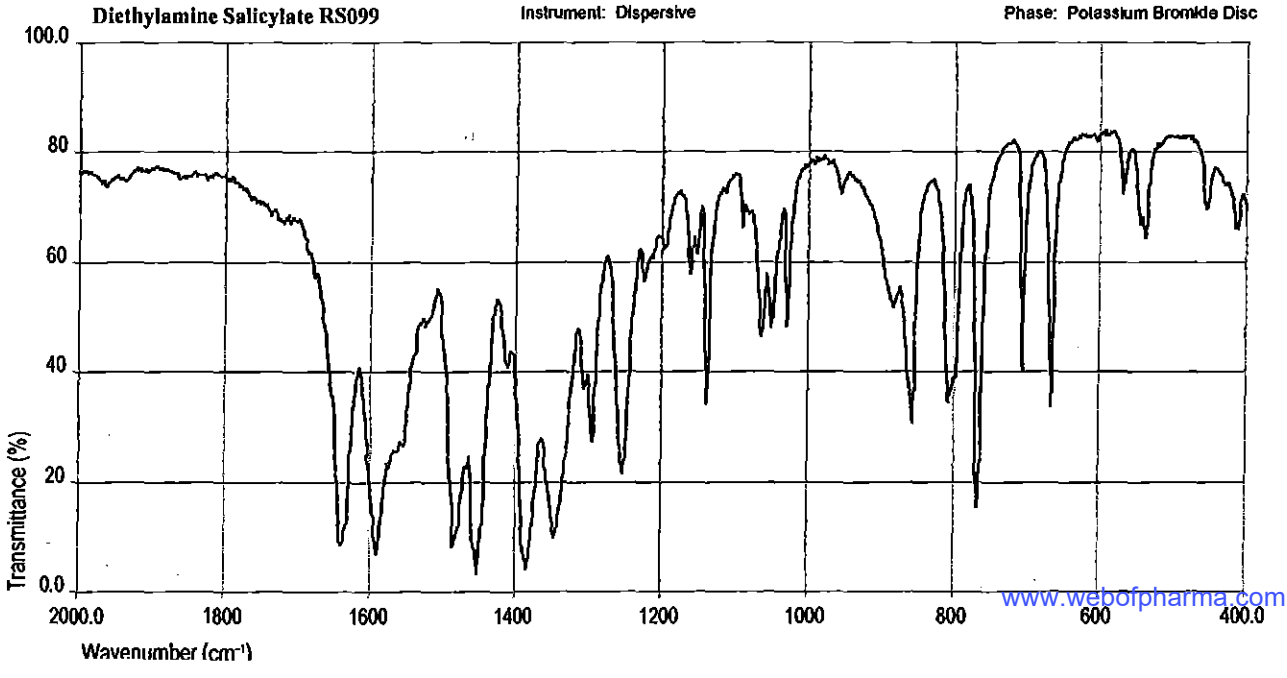




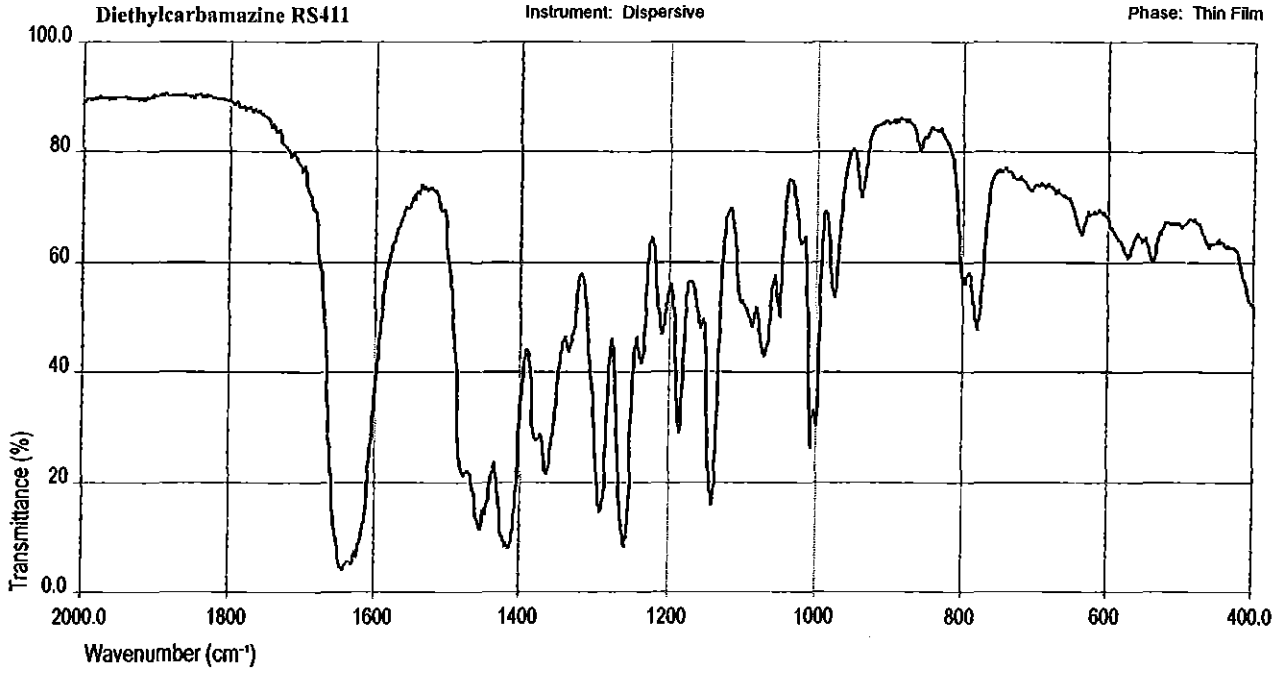




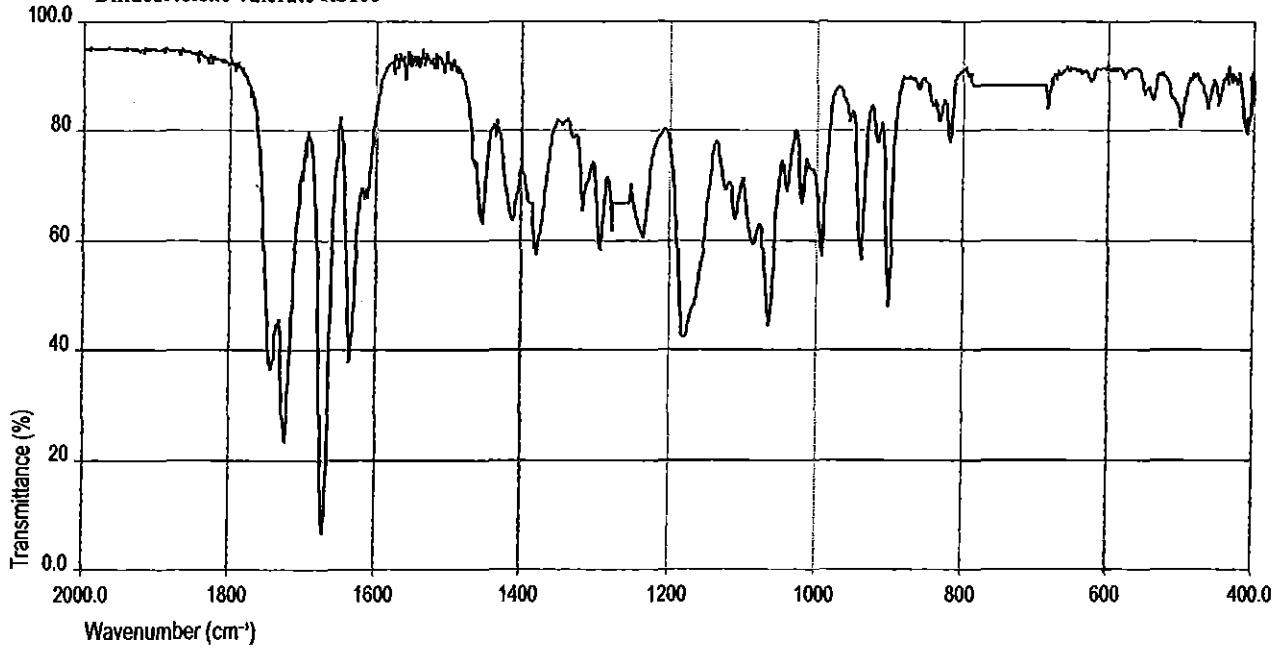




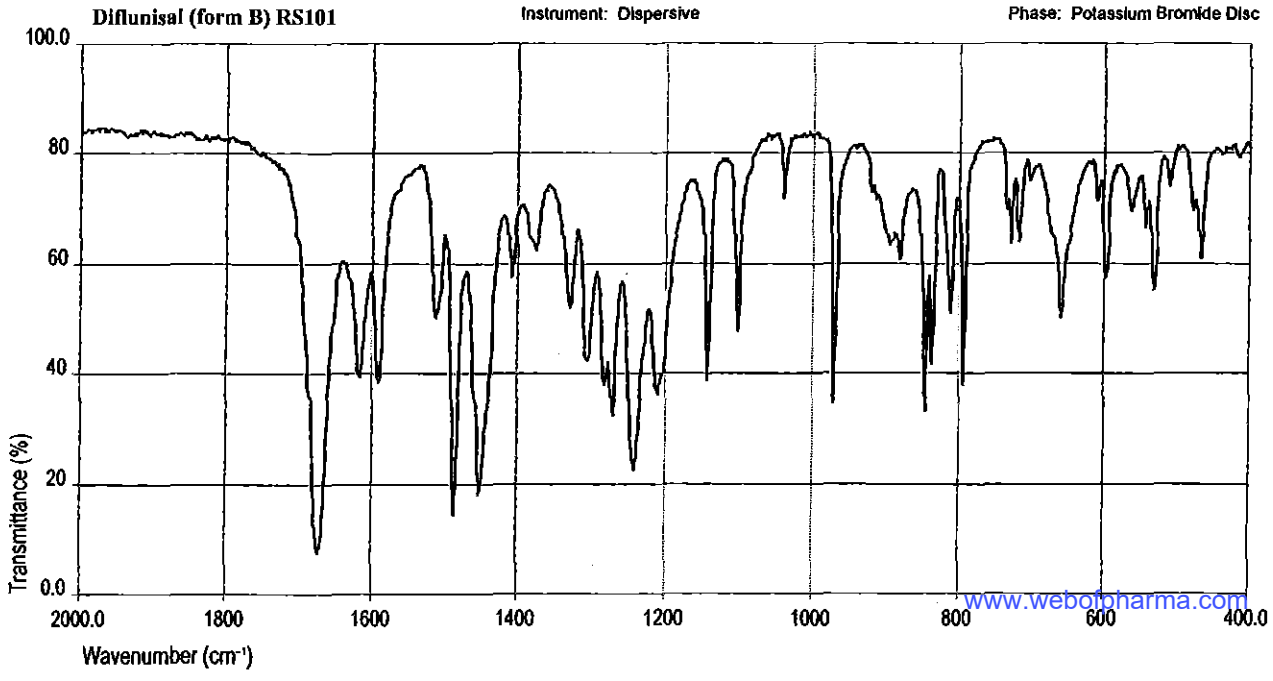
Diethylcarbamazine RS411 Instrument: Dispersive Phase: Thin Film

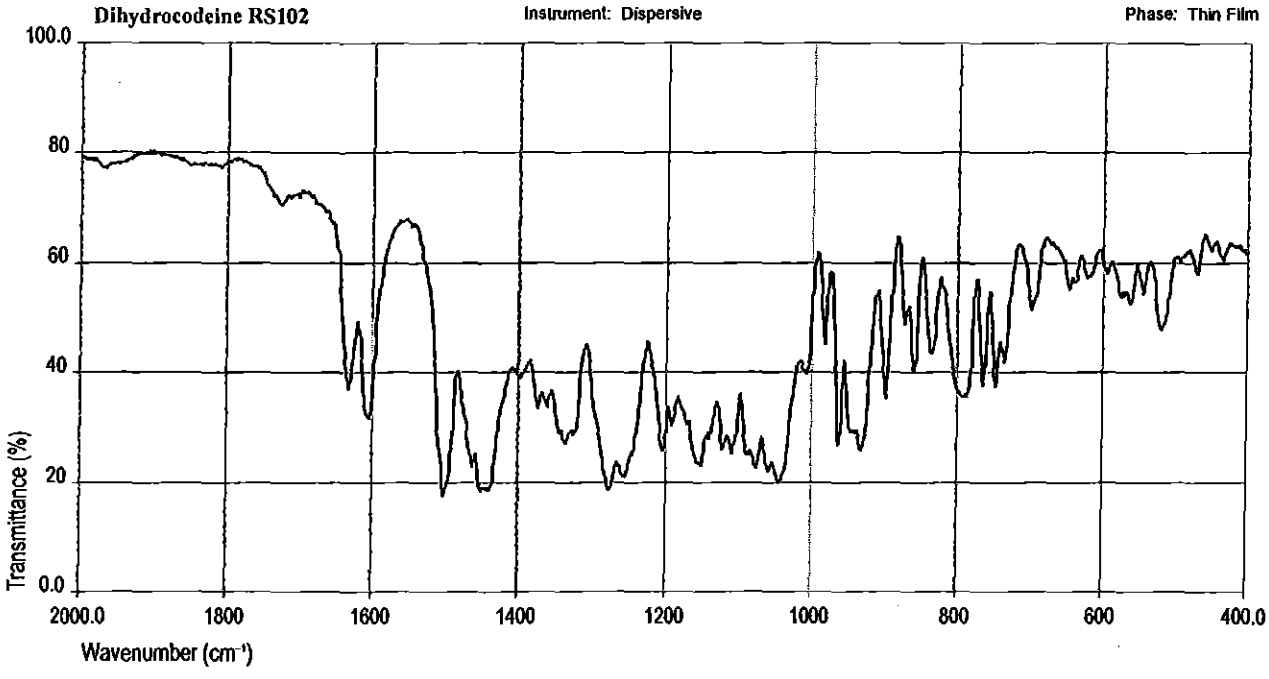


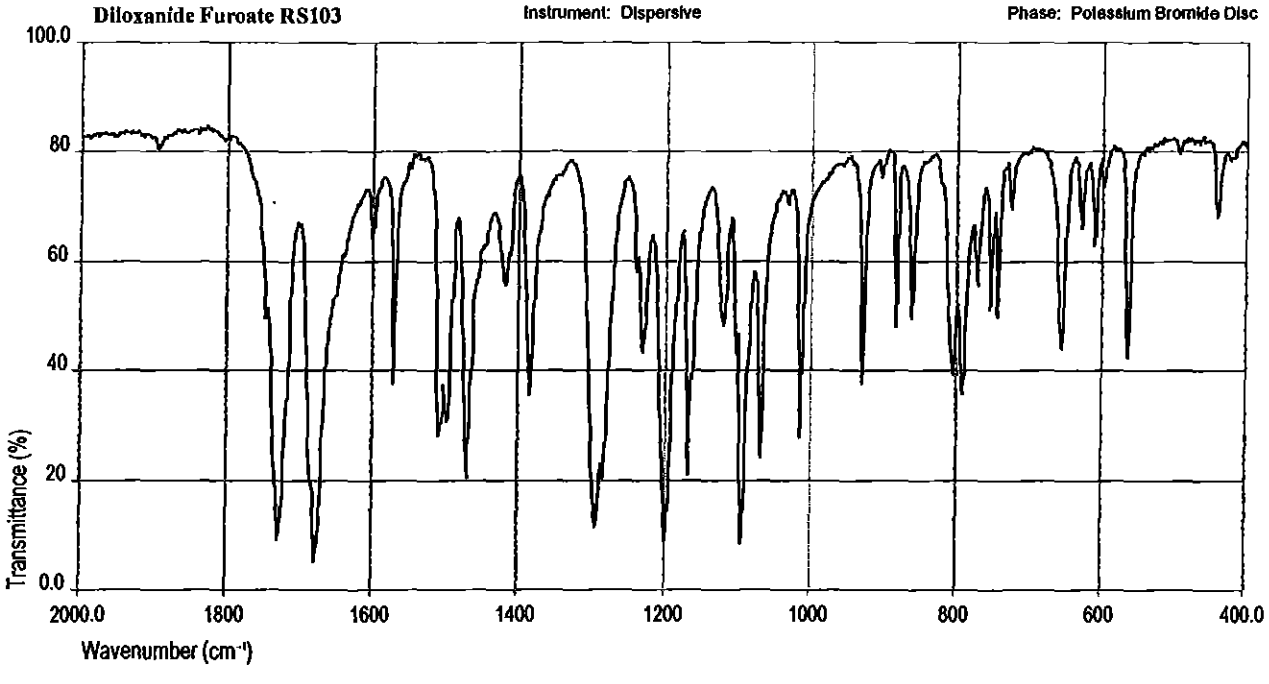
Diflucortolone Valerate RS100 Instrument: Fourier Transform Phase: 5% w/v Solution in Dichloromethane Thickness: 0.1mm

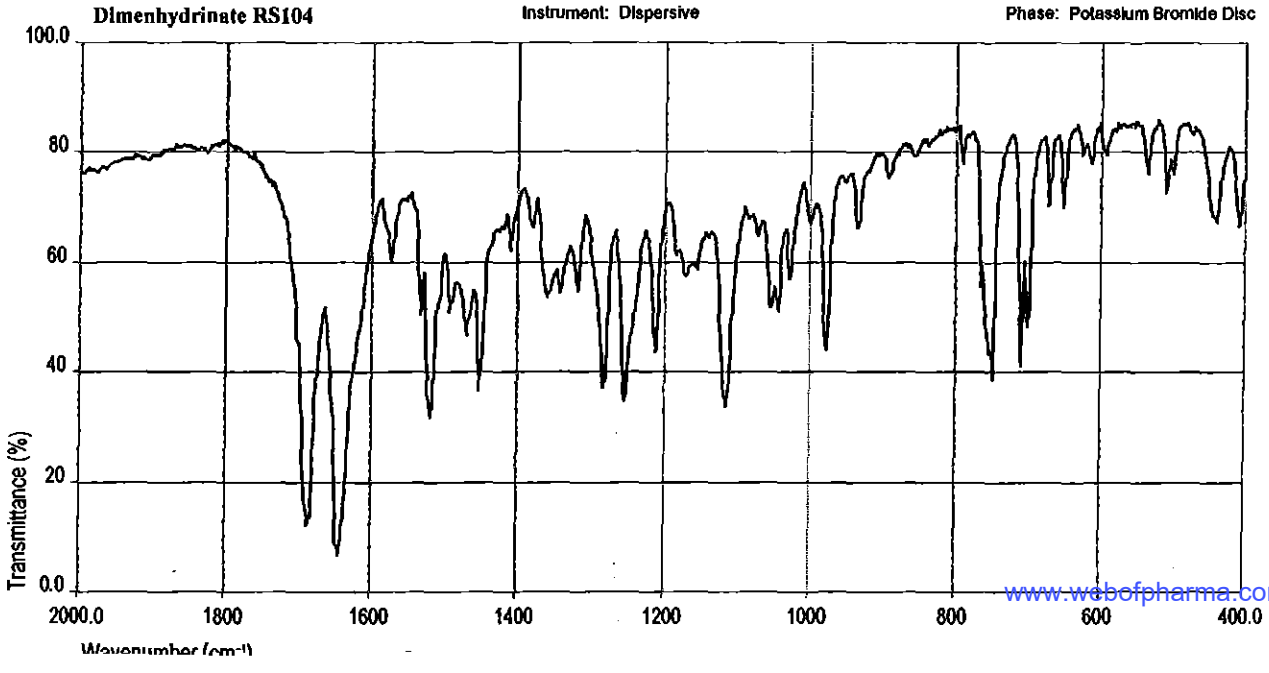


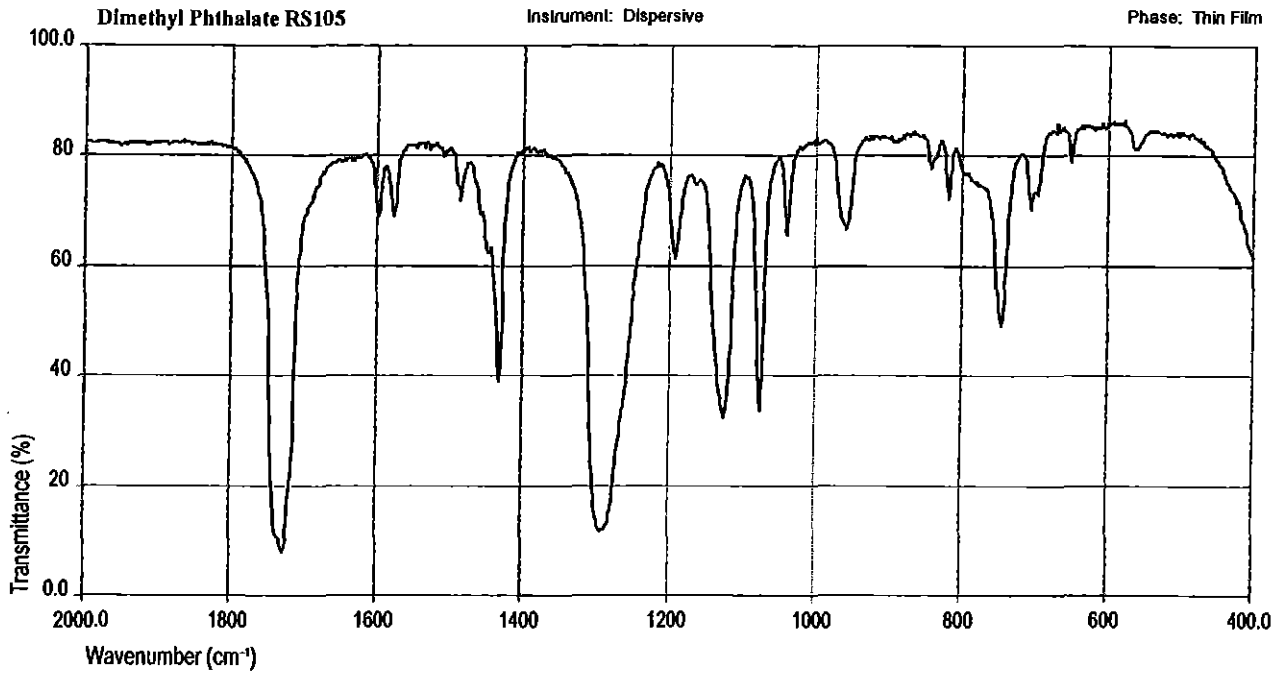
Diflunisal (form B) RS101 Instrument: Dispersive Phase: Potassium Bromide Disc

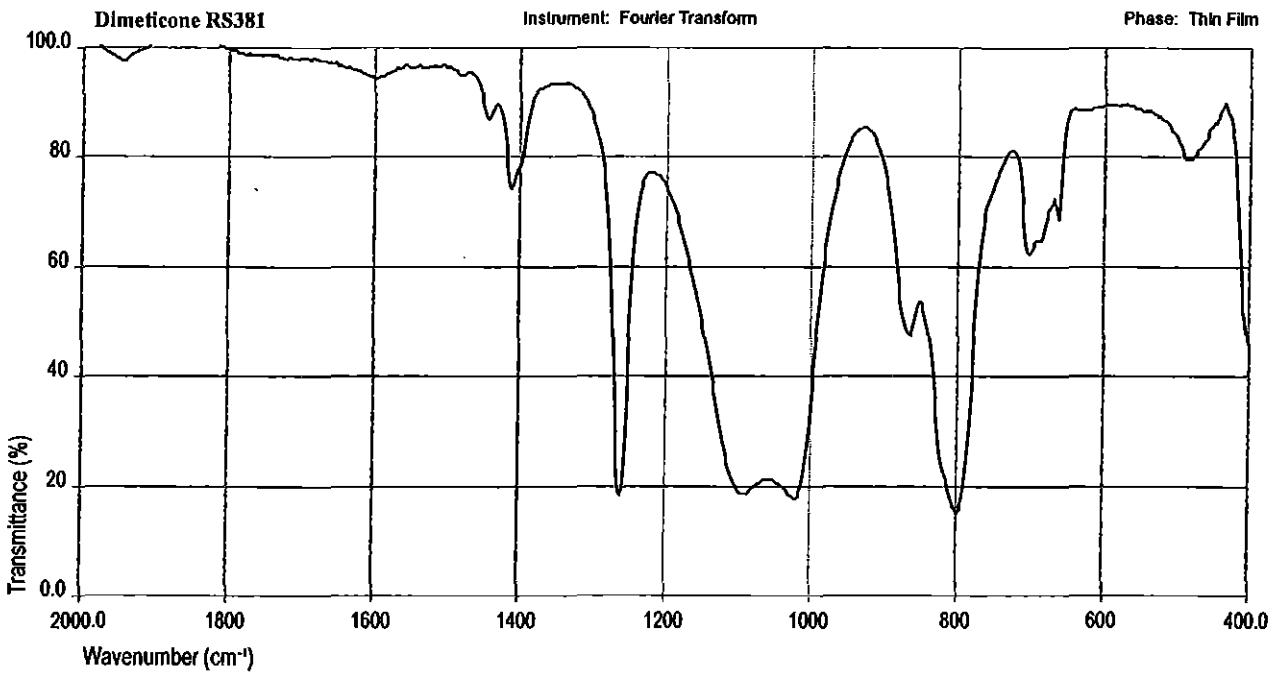


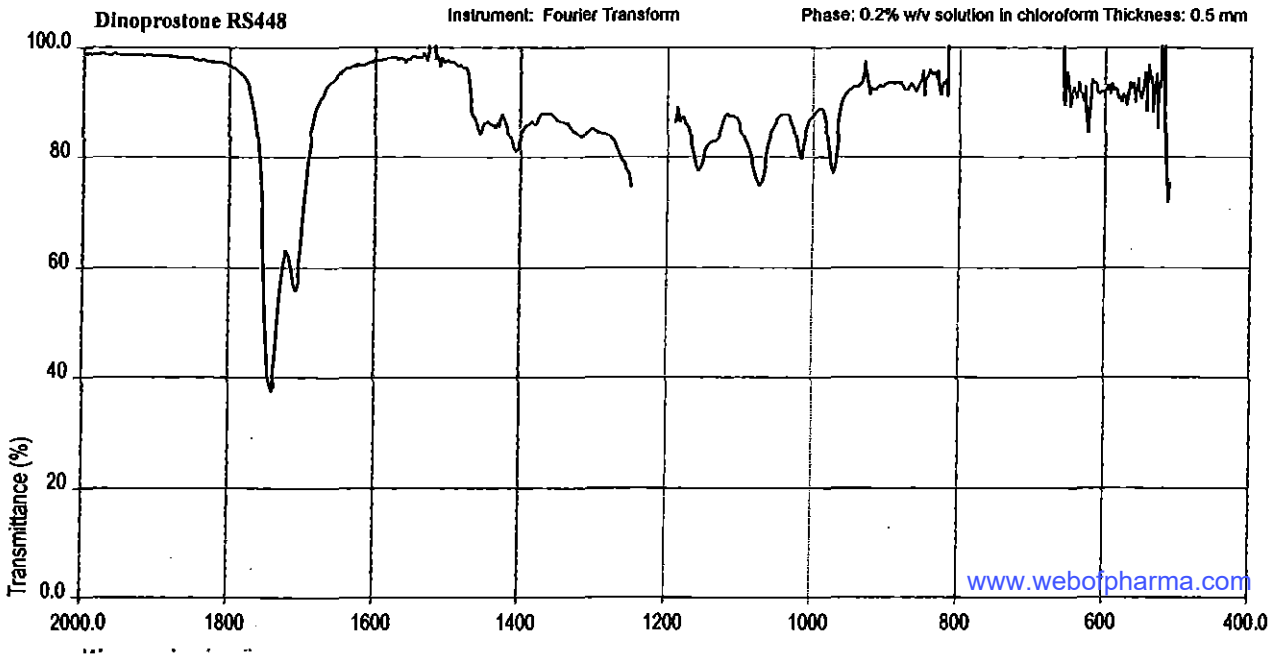


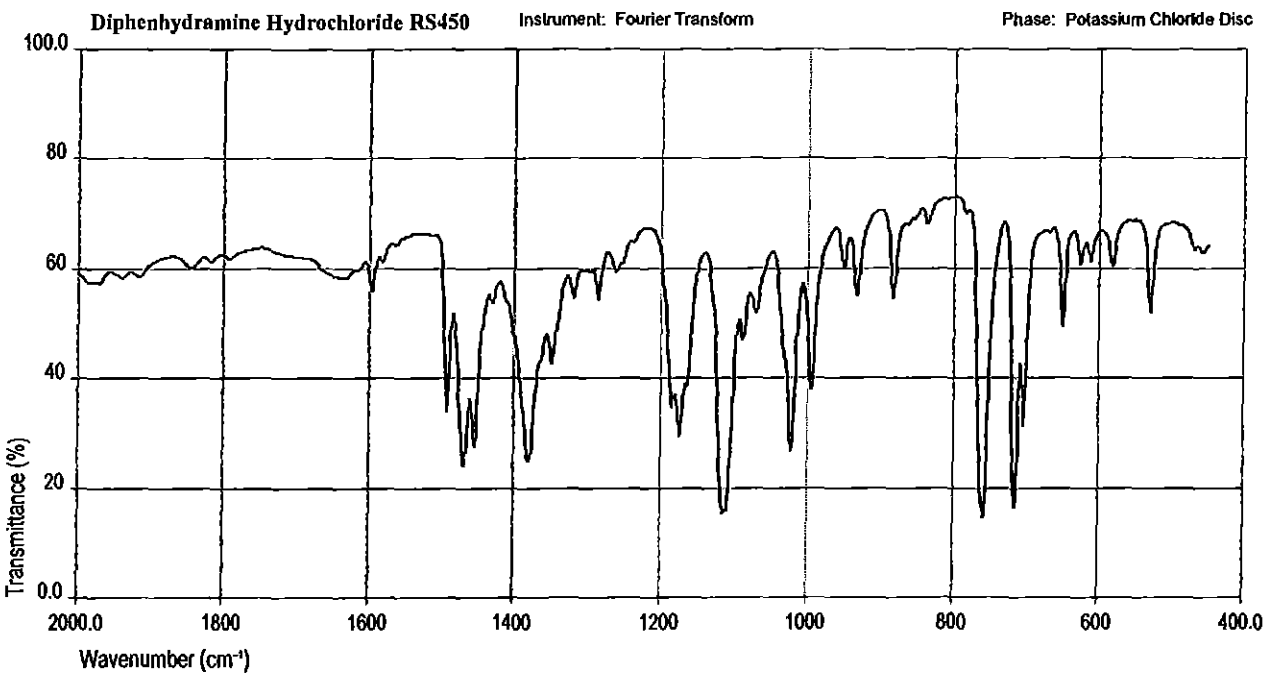


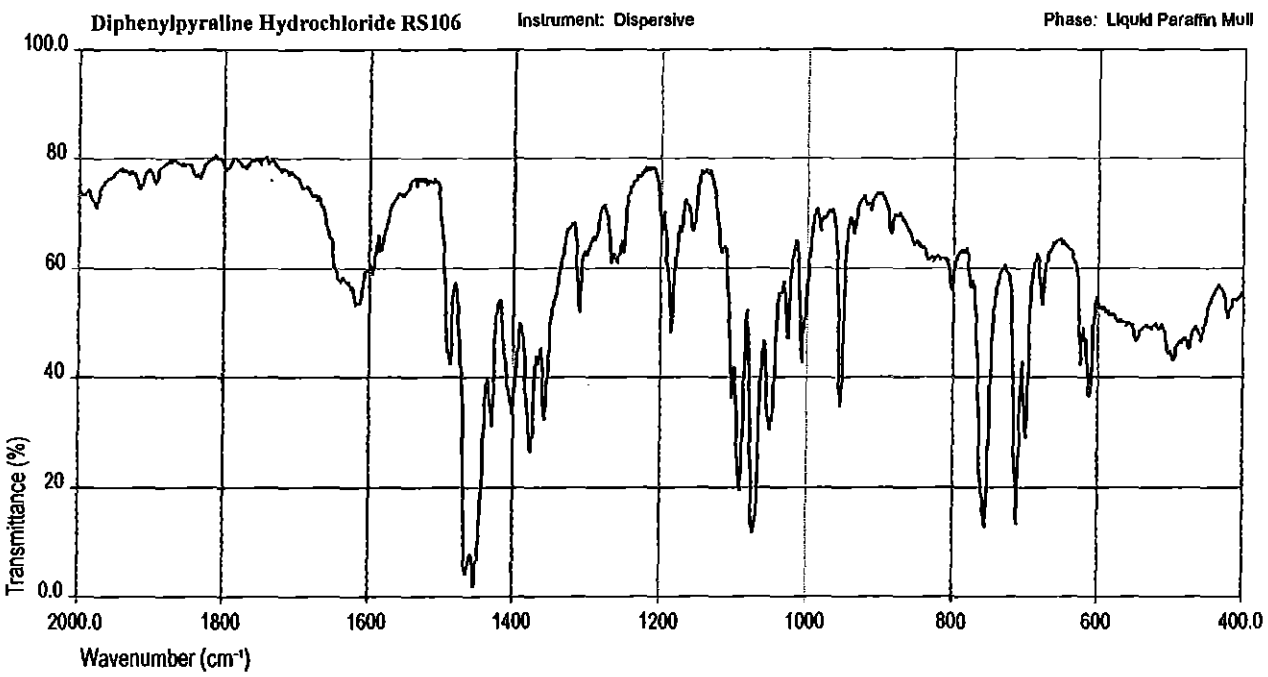


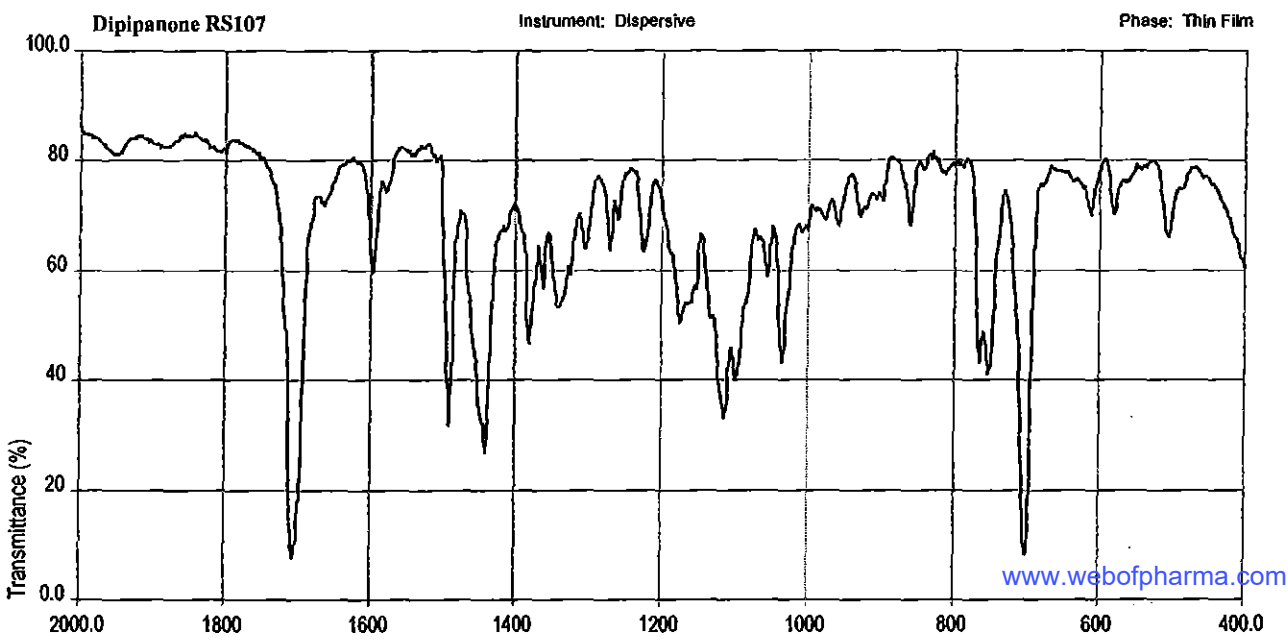








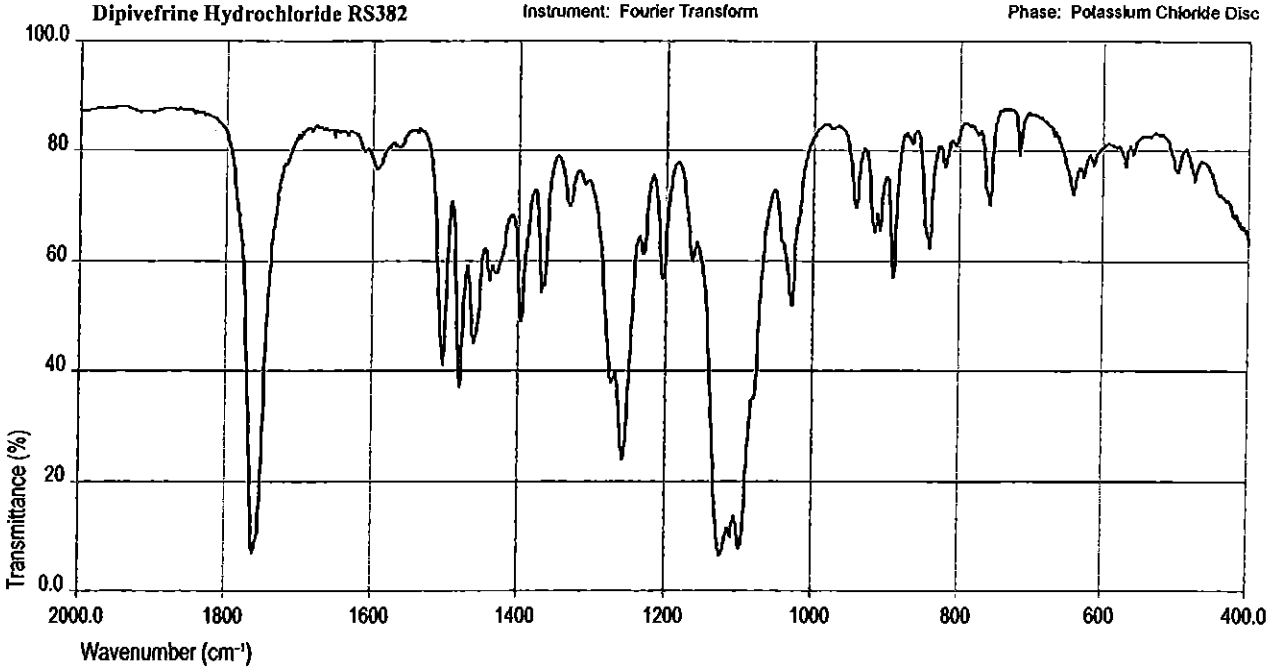




Dipivefrine Hydrochloride RS382

Instrument: Fourier Transform

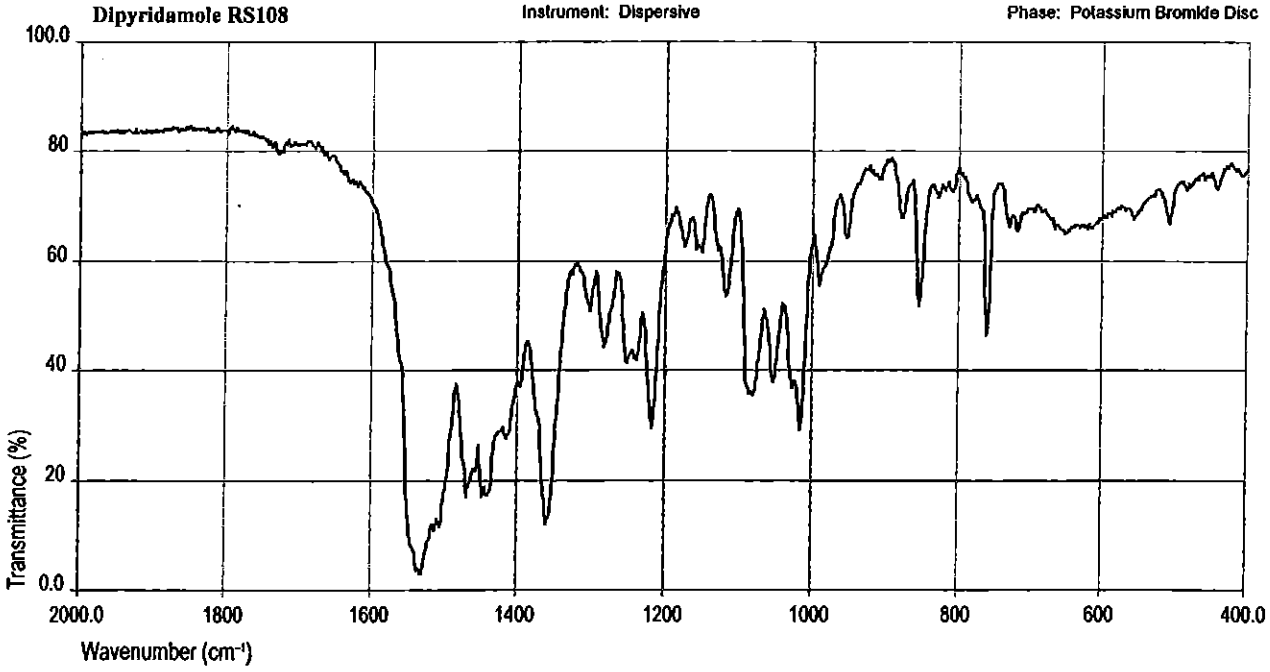
Phase: Potassium Chloride Disc



Dipyridamole RS108

Instrument: Dispersive

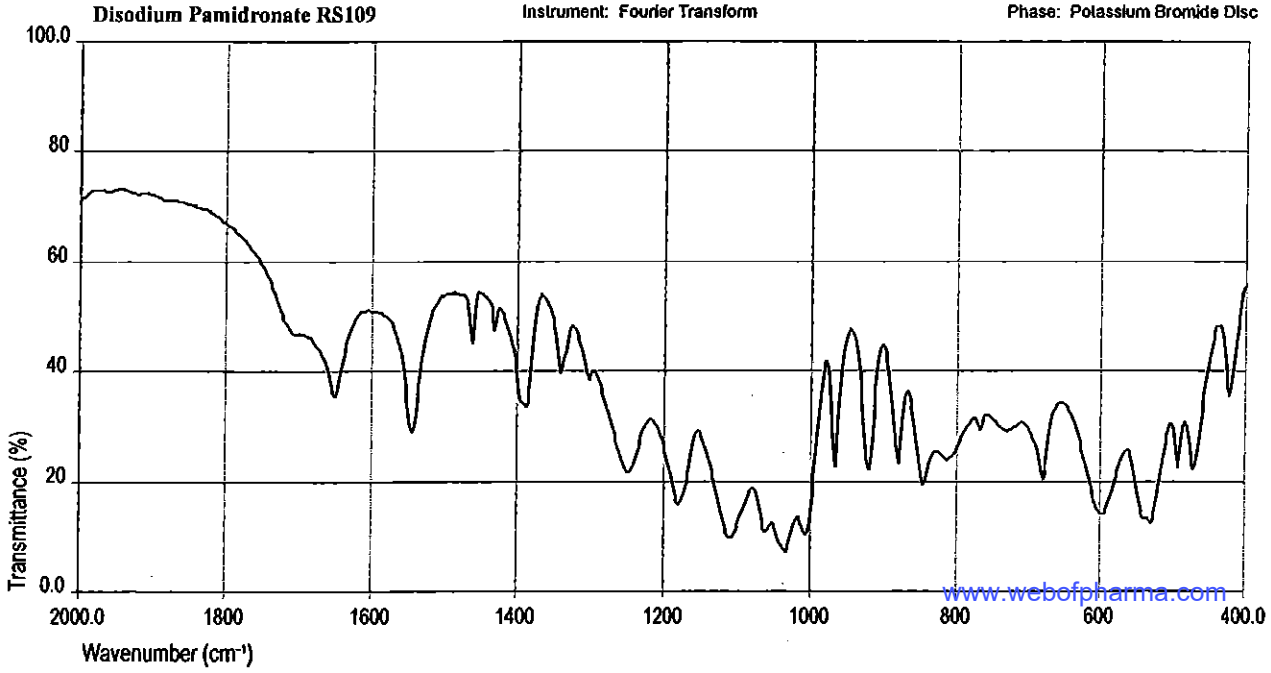
Phase: Potassium Bromide Disc



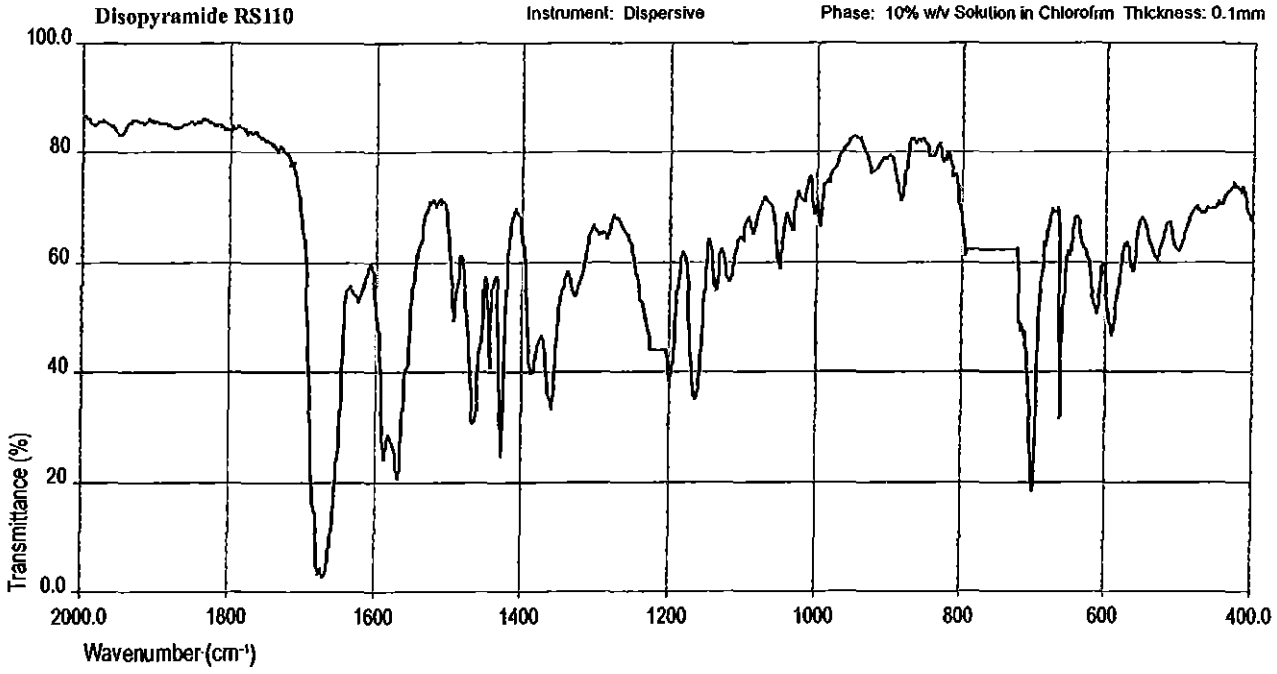
Disodium Pamidronate RS109

Instrument: Fourier Transform

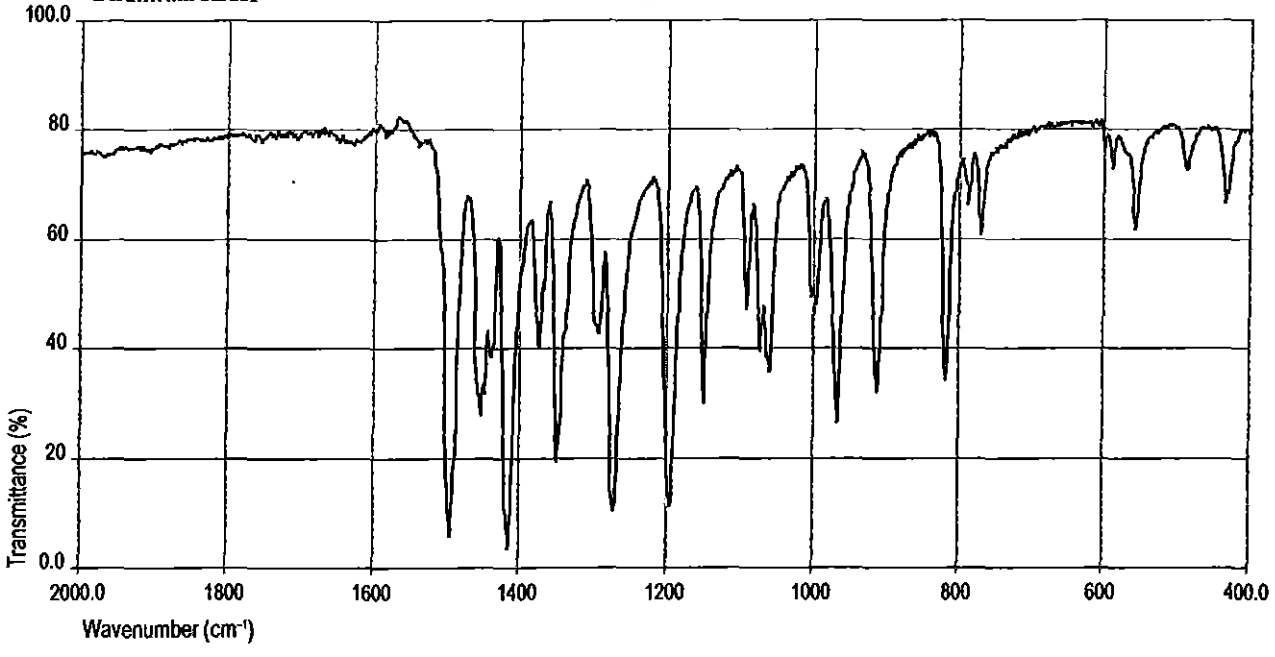
Phase: Potassium Bromide Disc



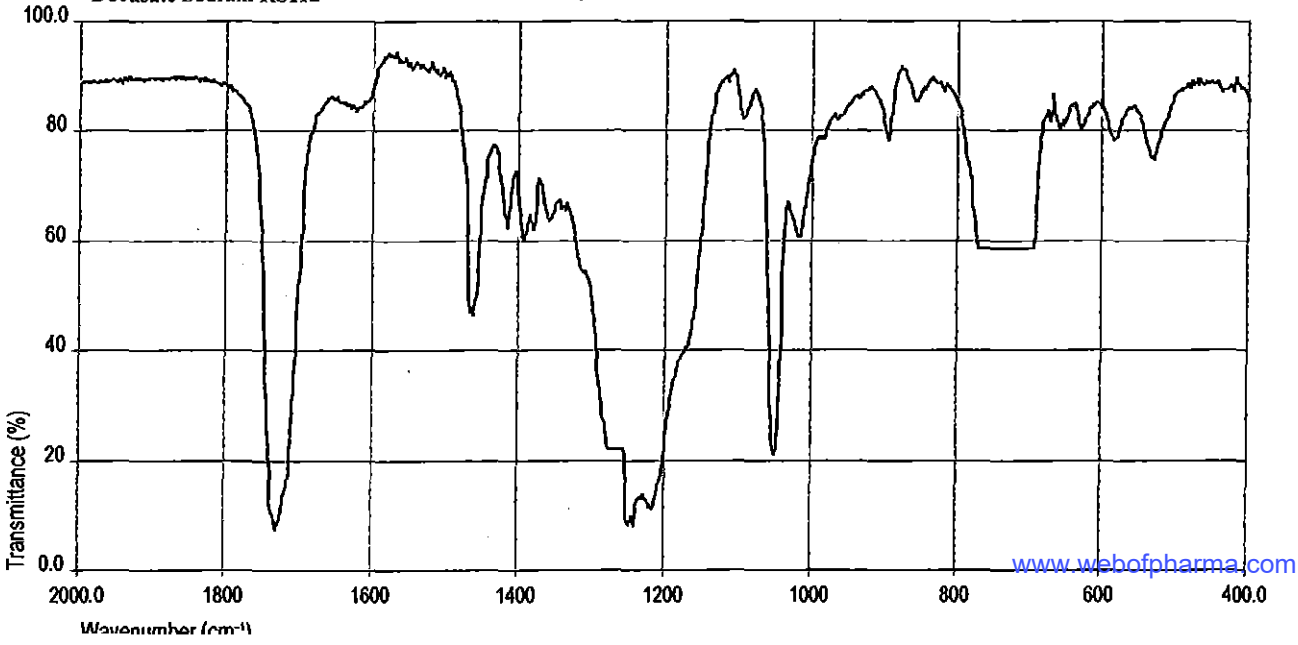
Disopyramide RS110 Instrument: Dispersive Phase: 10% w/v Solution in Chloroform Thickness: 0.1mm



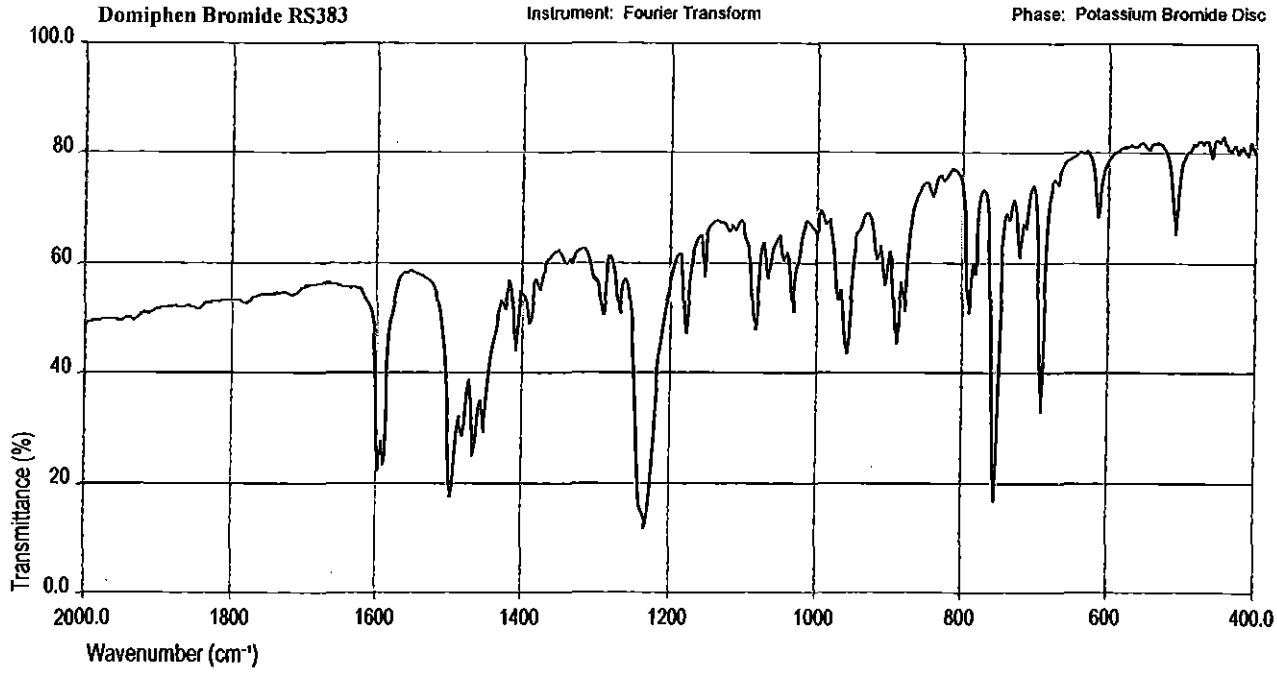
Disulfiram RS111 Instrument: Dispersive Phase: Potassium Bromide Disc



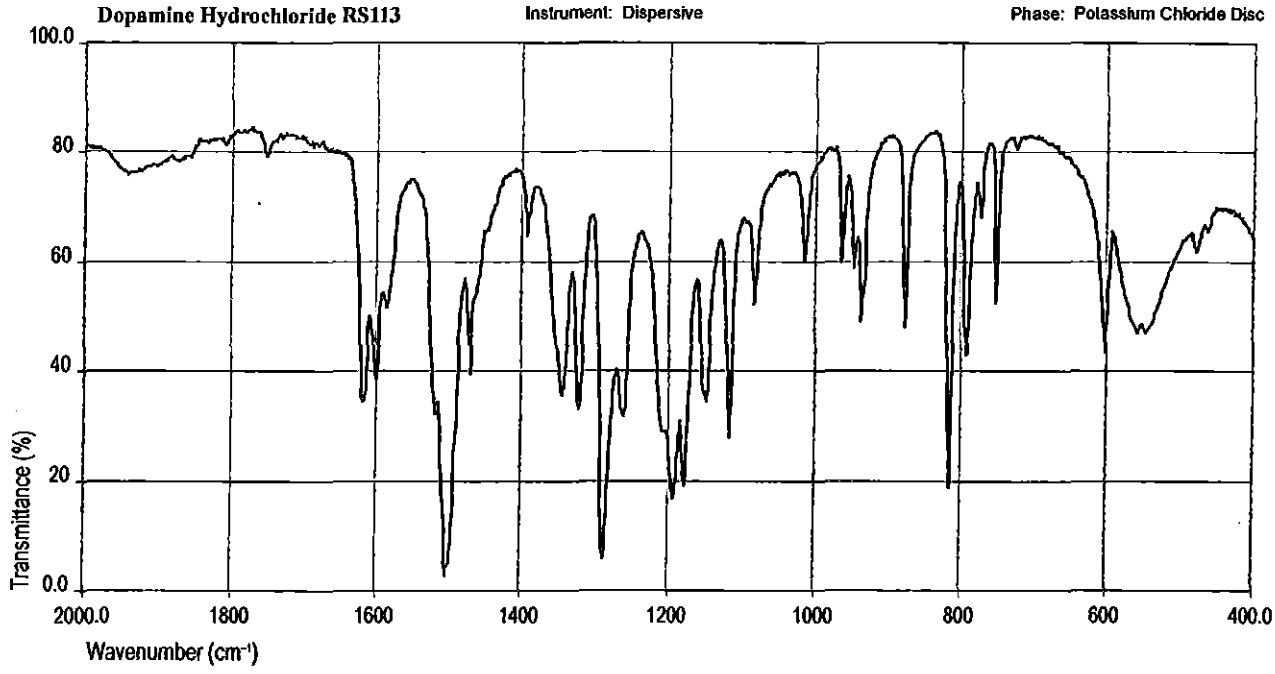
Docusate Sodium RS112 Instrument: Dispersive Phase: 10% w/v Solution in Dichloromethane Thickness: 0.1mm



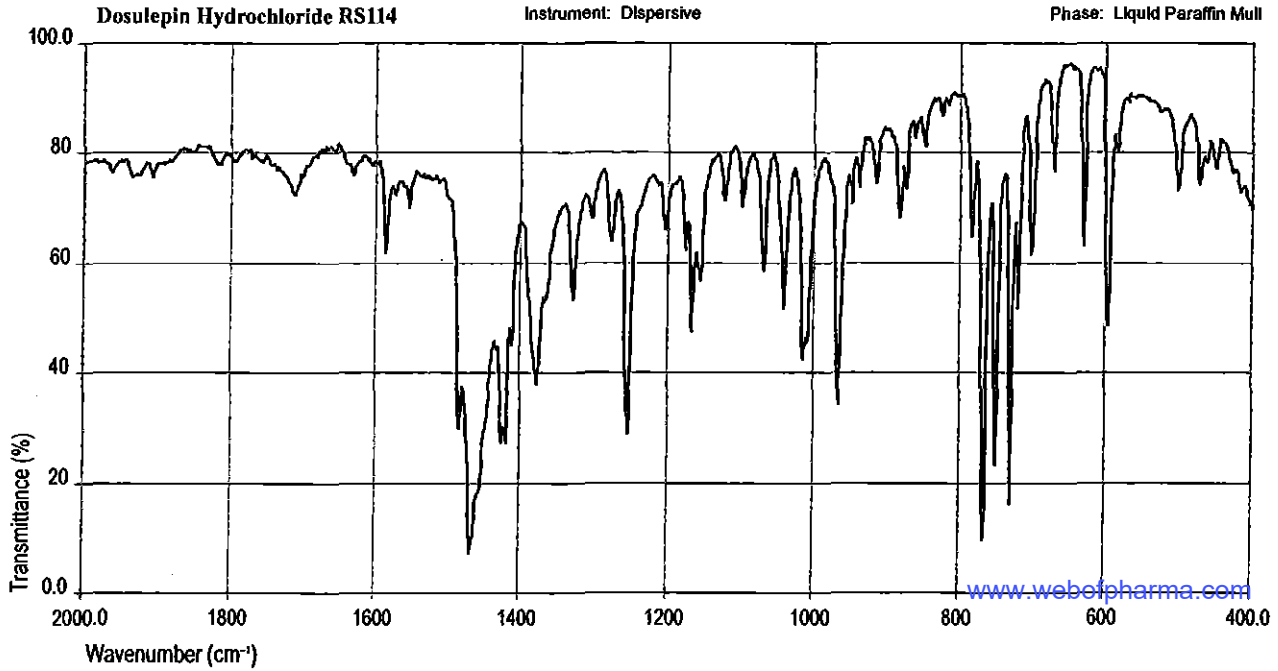
Domiphen Bromide RS383 Instrument: Fourier Transform Phase: Potassium Bromide Disc

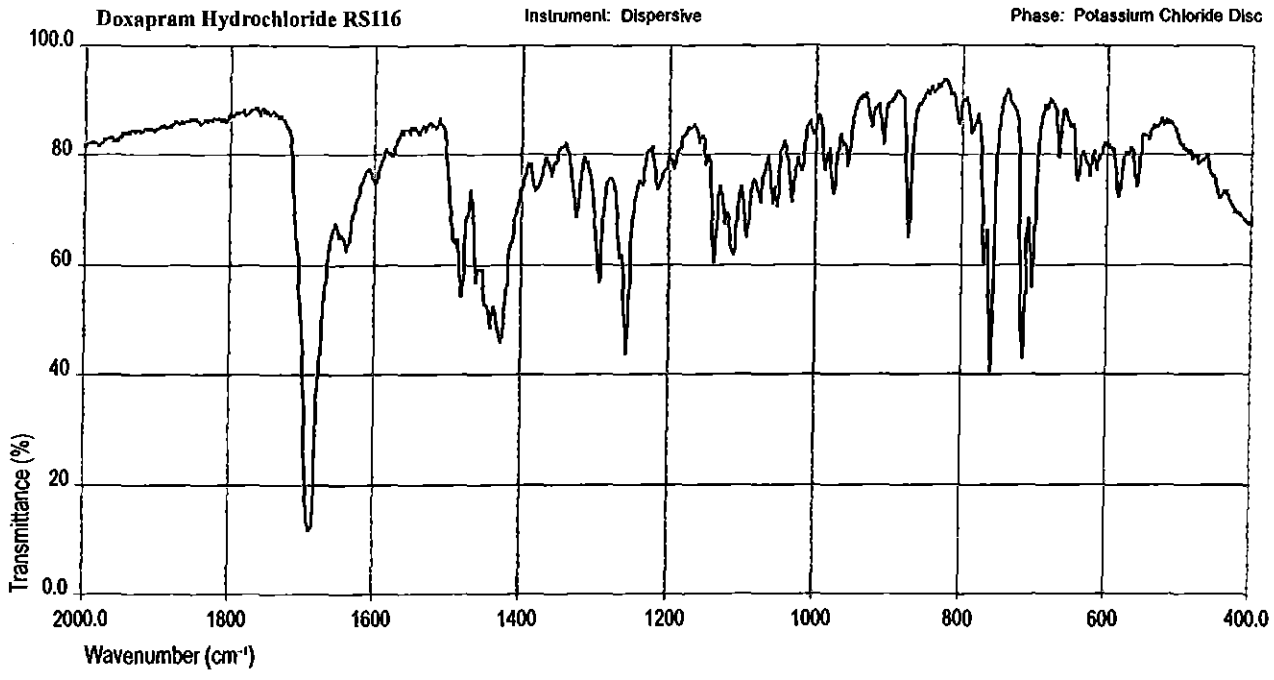


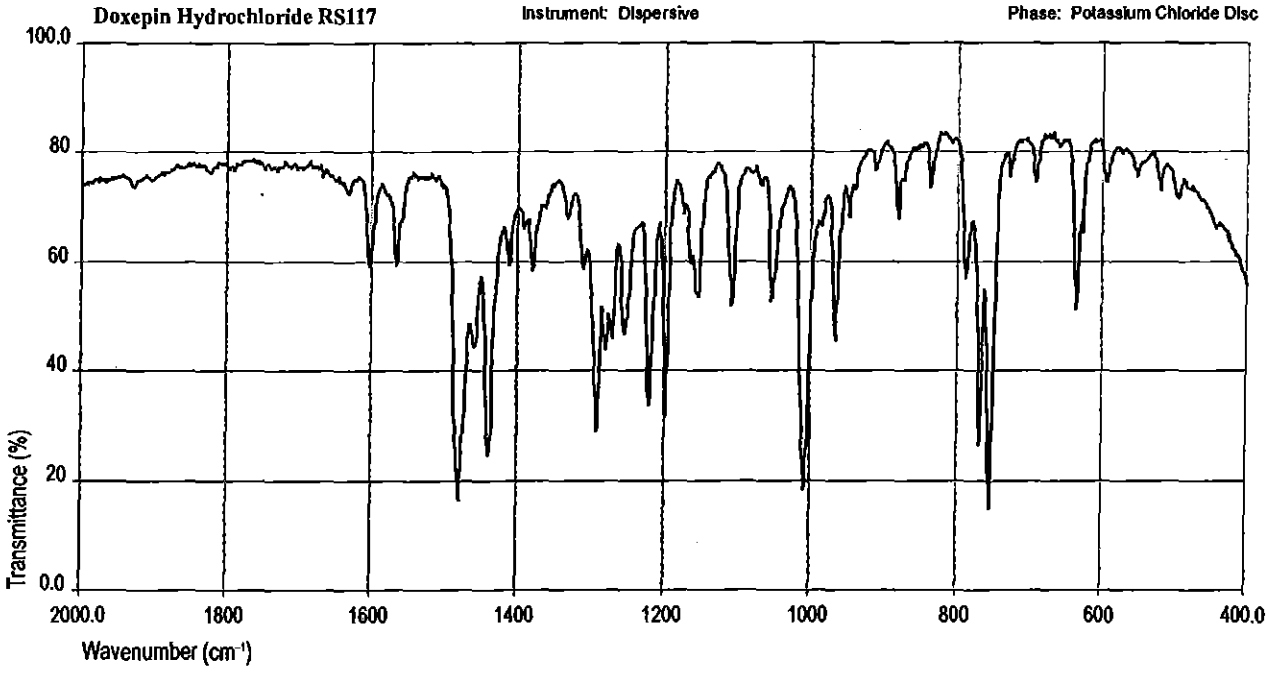
Dopamine Hydrochloride RS113 Instrument: Dispersive Phase: Potassium Chloride Disc

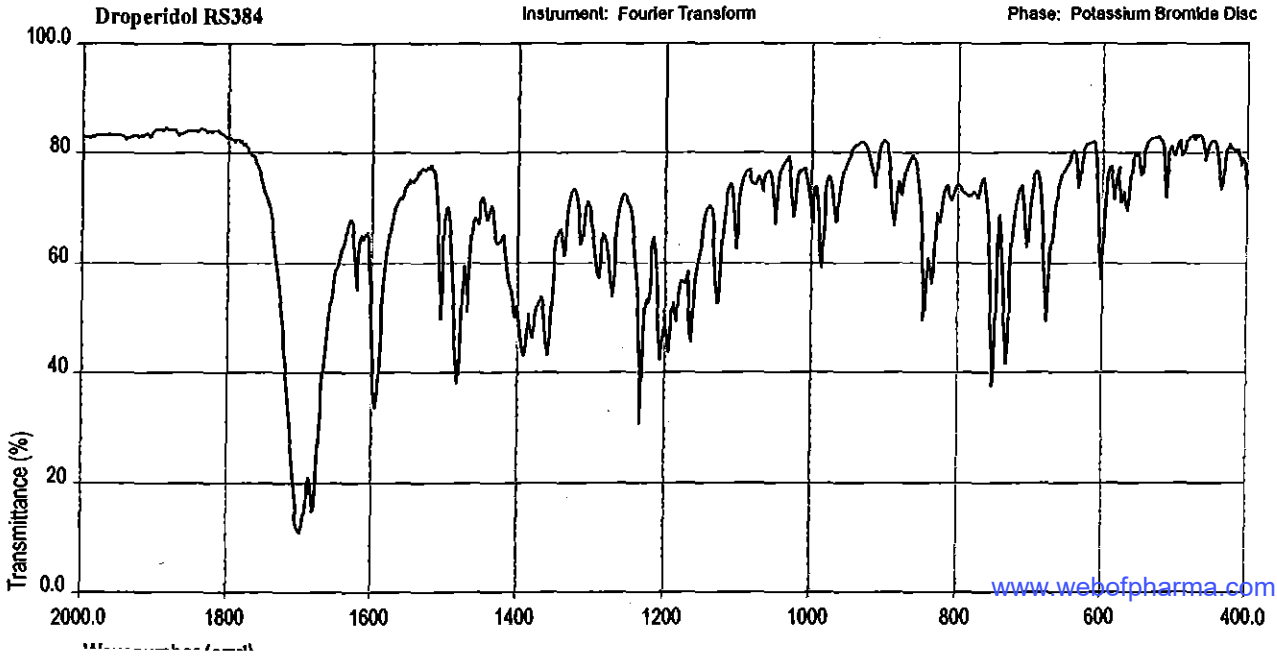


Dosulepin Hydrochloride RS114 Instrument: Dispersive Phase: Liquid Paraffin Mull





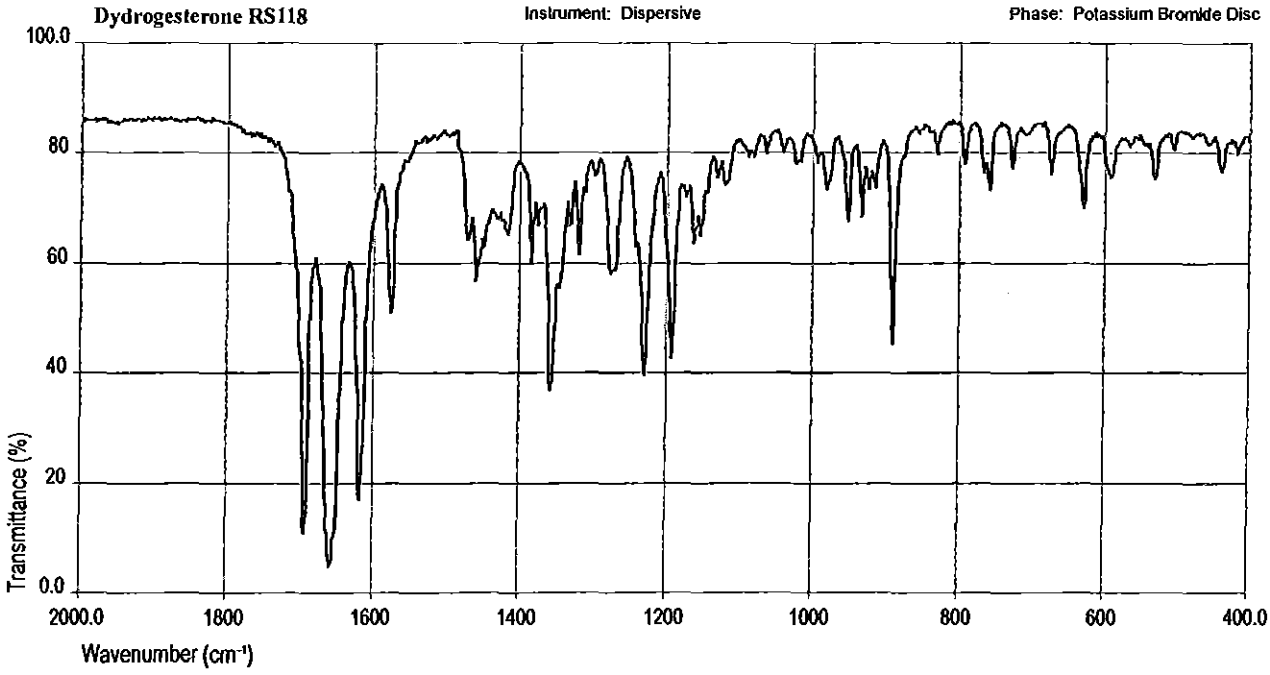




Dydrogesterone RS118

Instrument: Dispersive

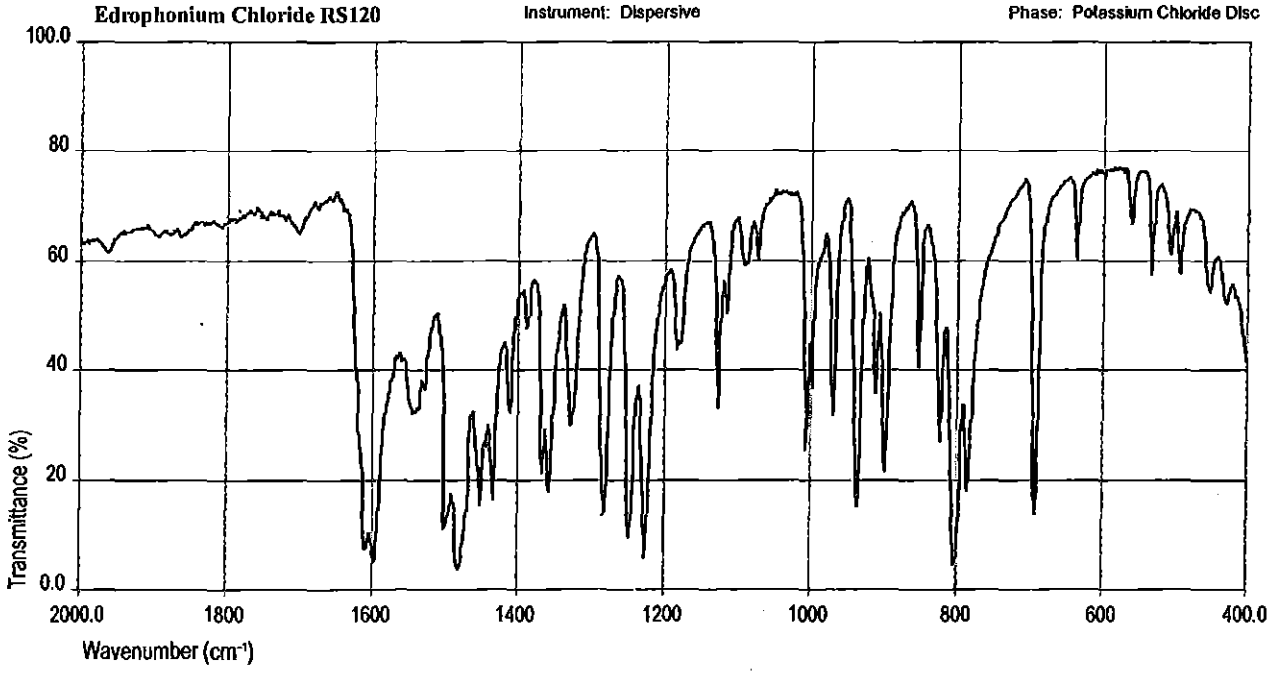
Phase: Potassium Bromide Disc



Edrophonium Chloride RS120

Instrument: Dispersive

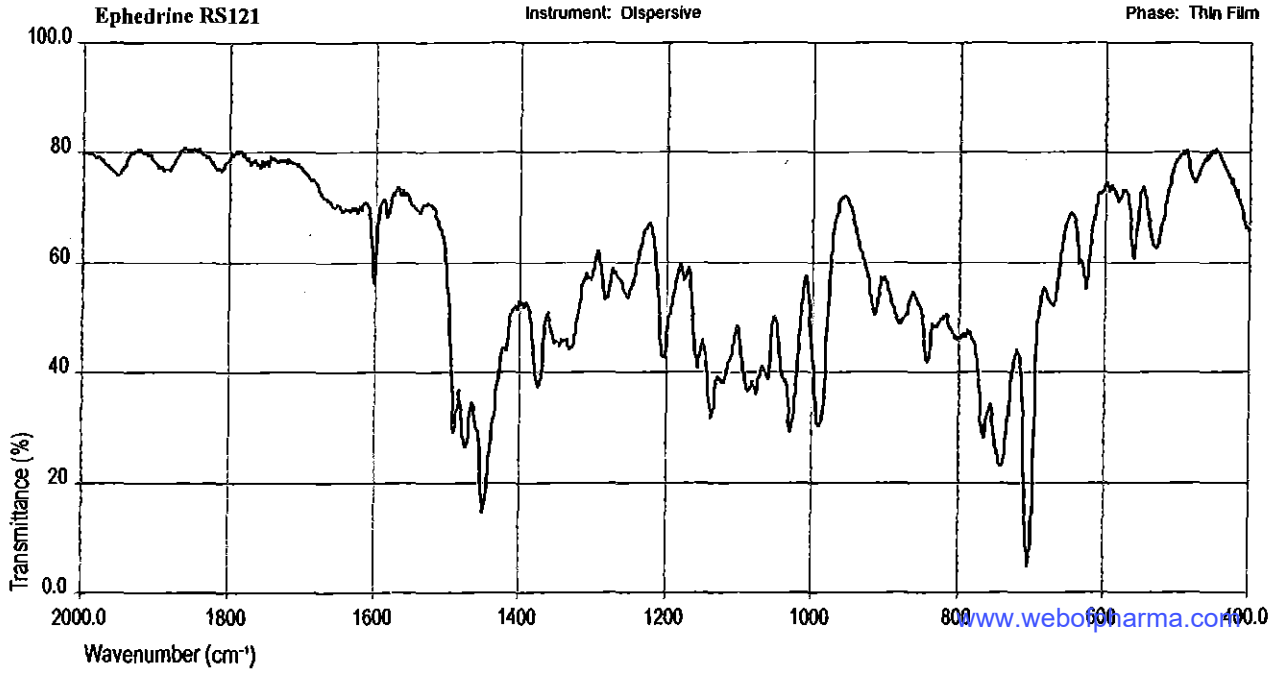
Phase: Potassium Chloride Disc

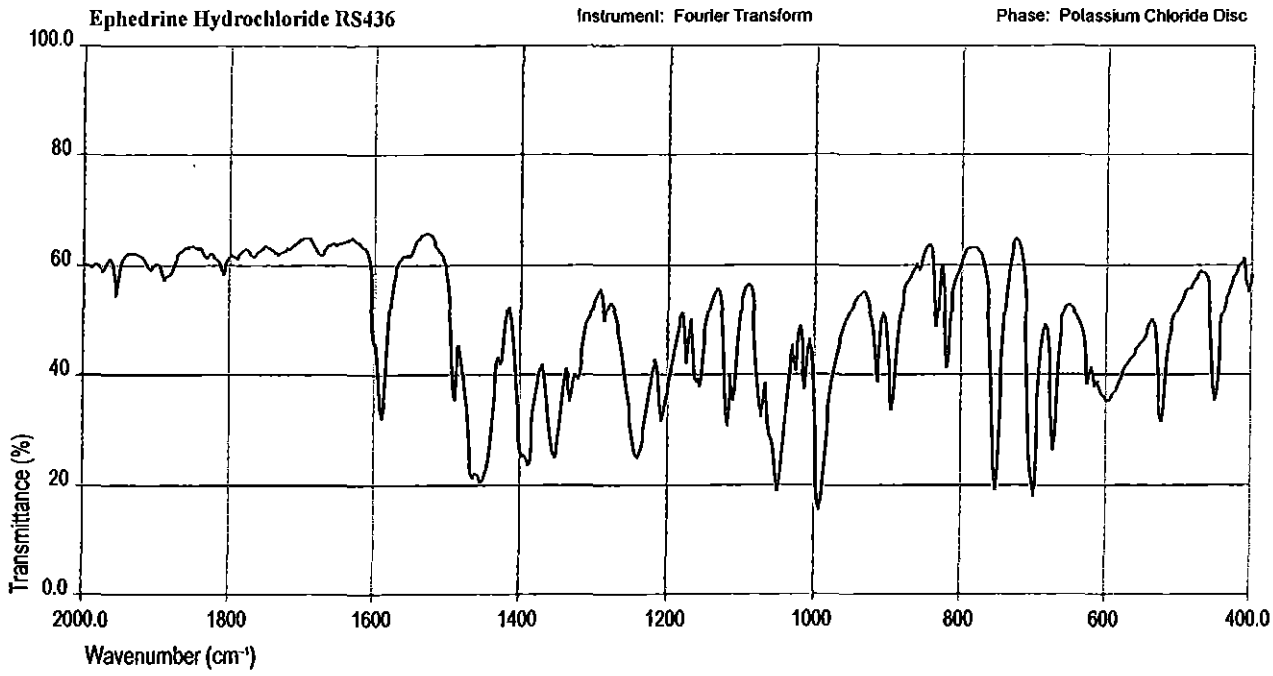


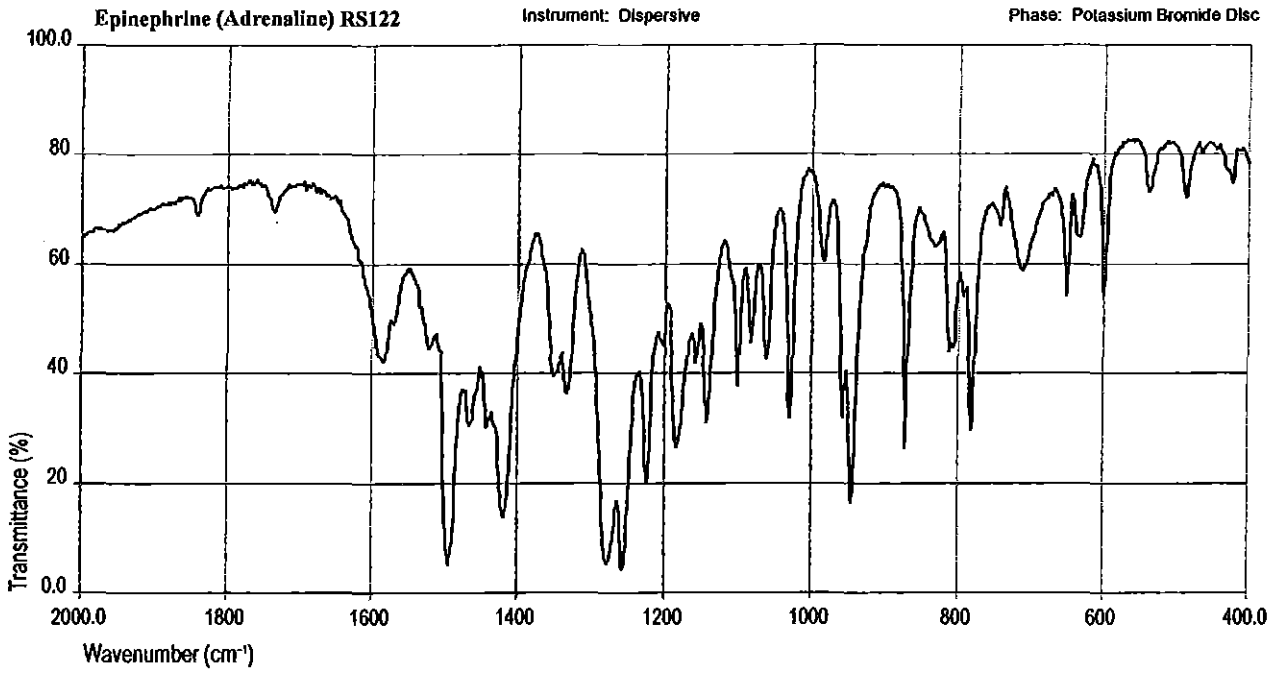
Ephedrine RS121

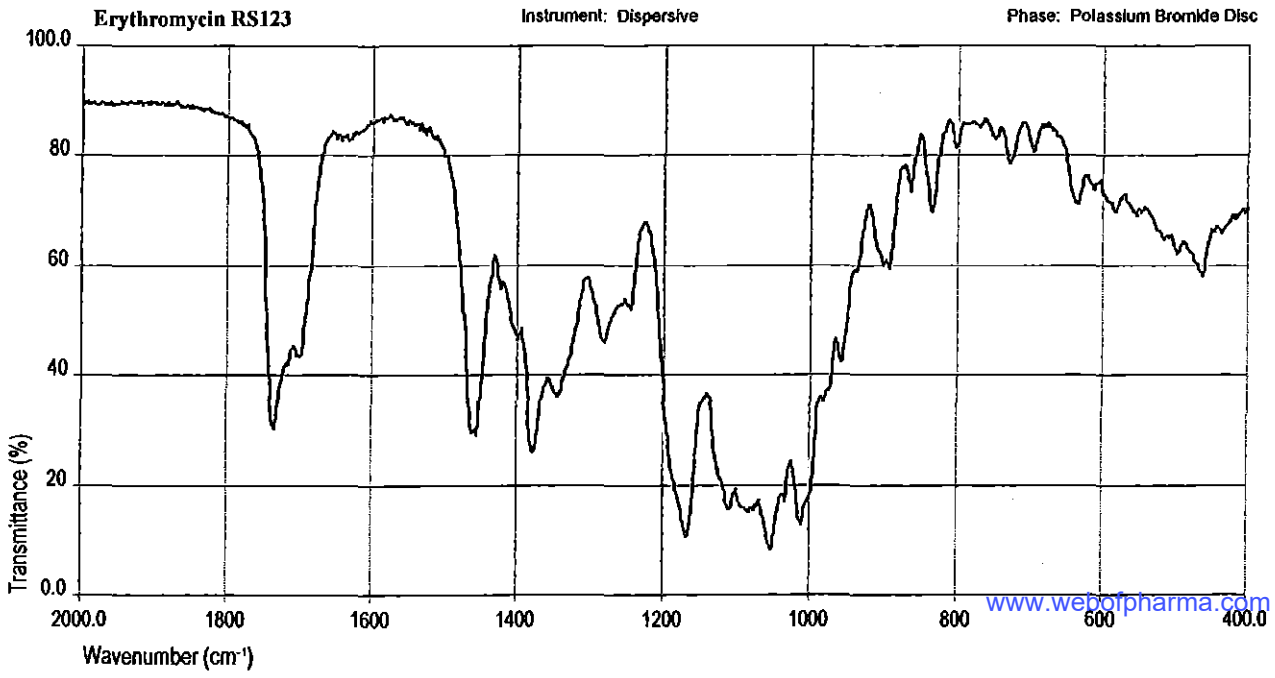
Instrument: Dispersive

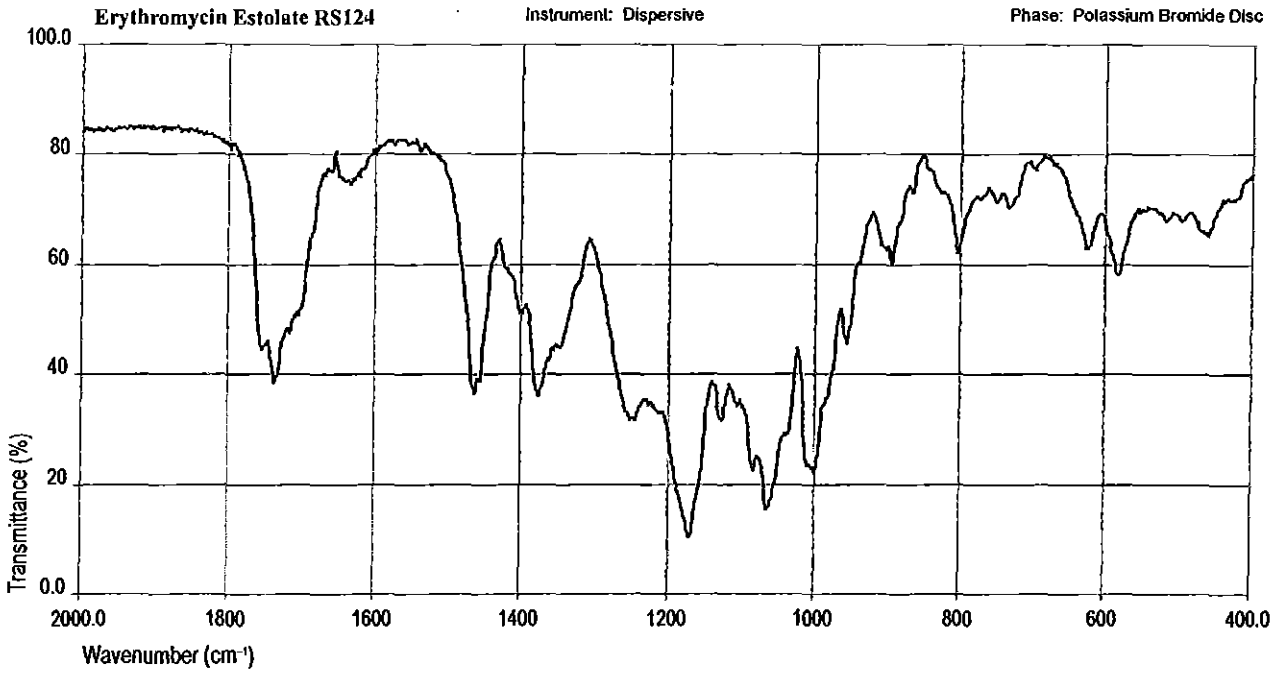
Phase: Thin Film

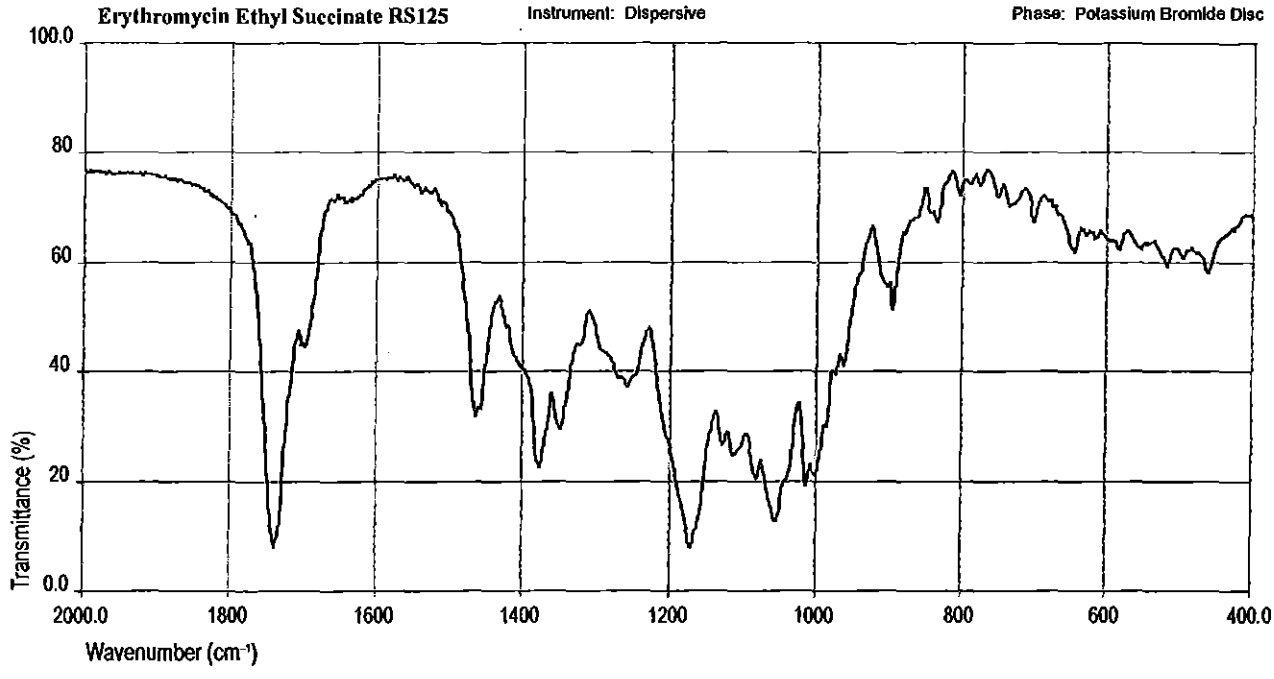


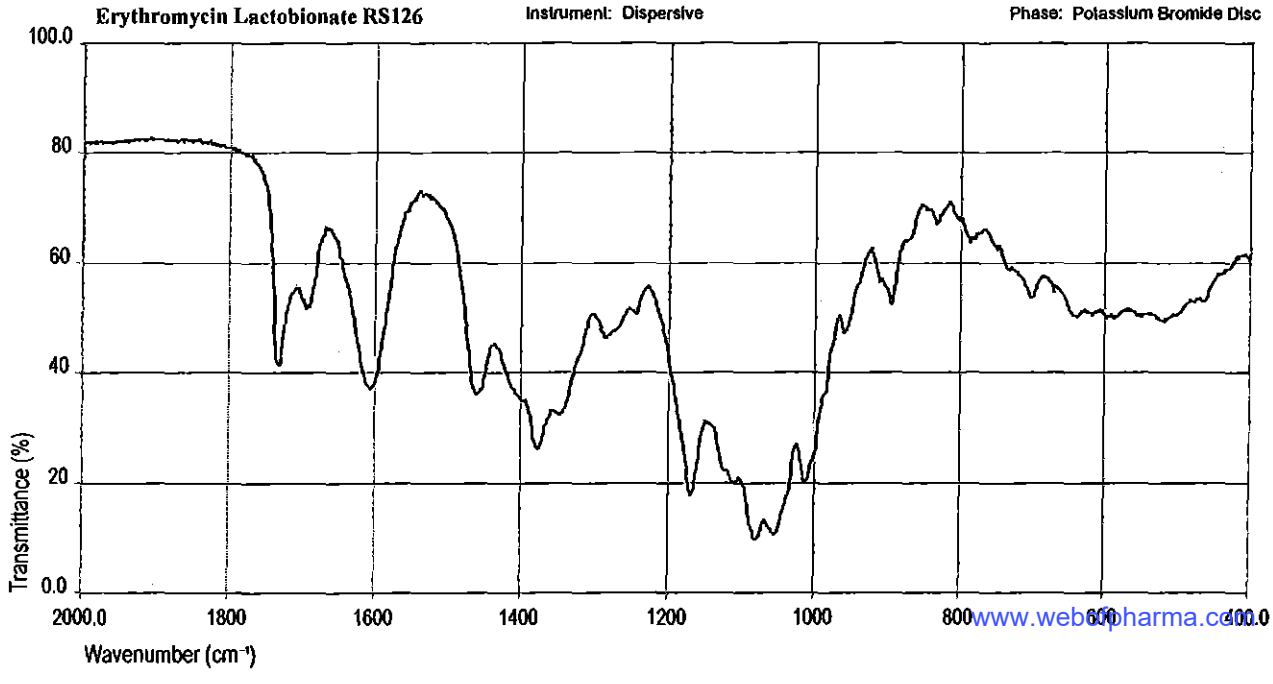


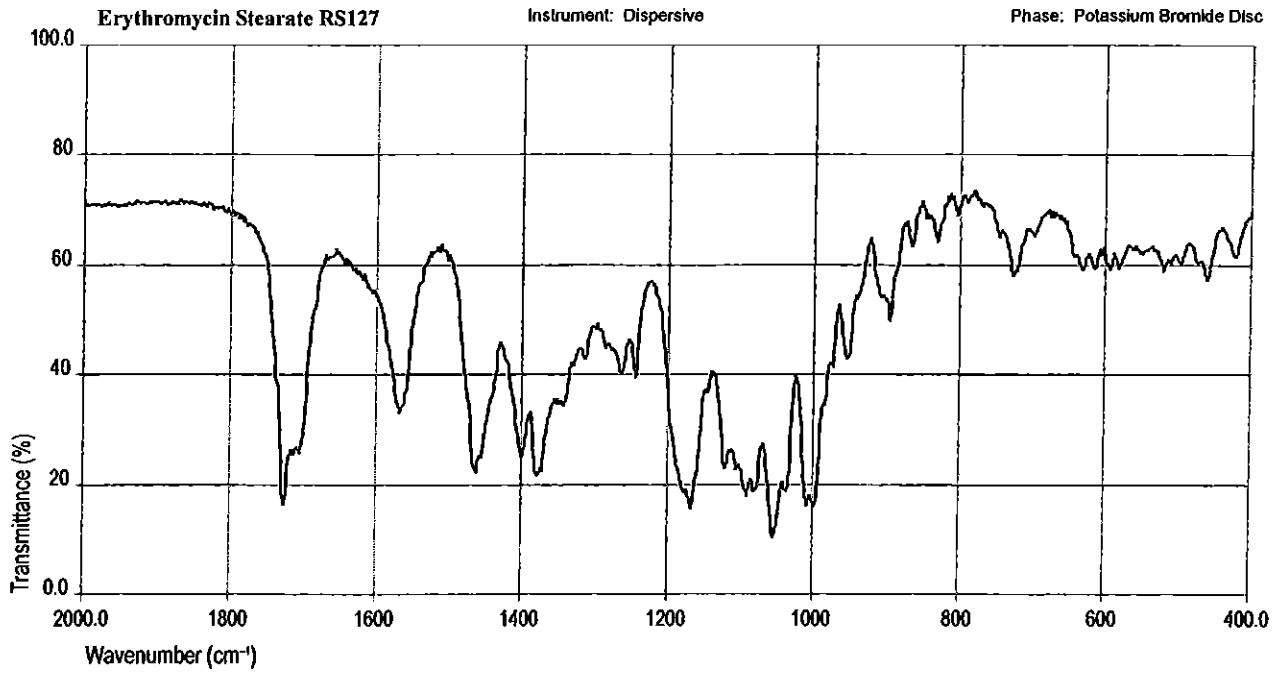


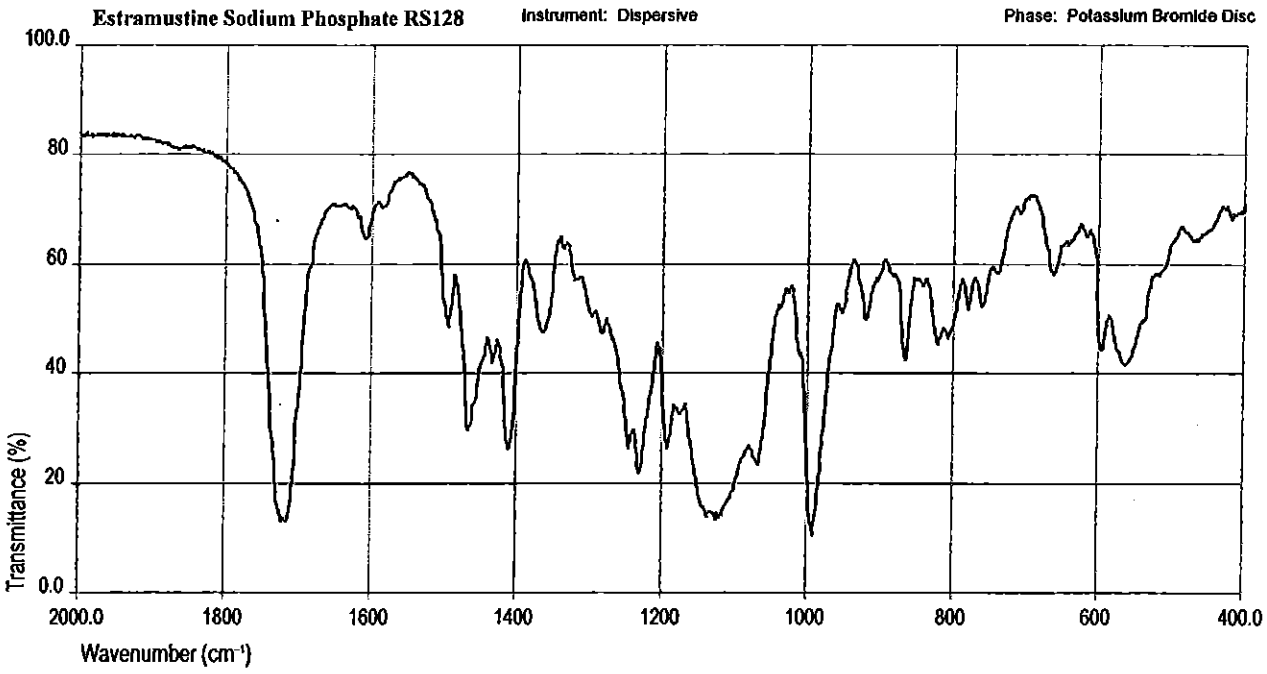


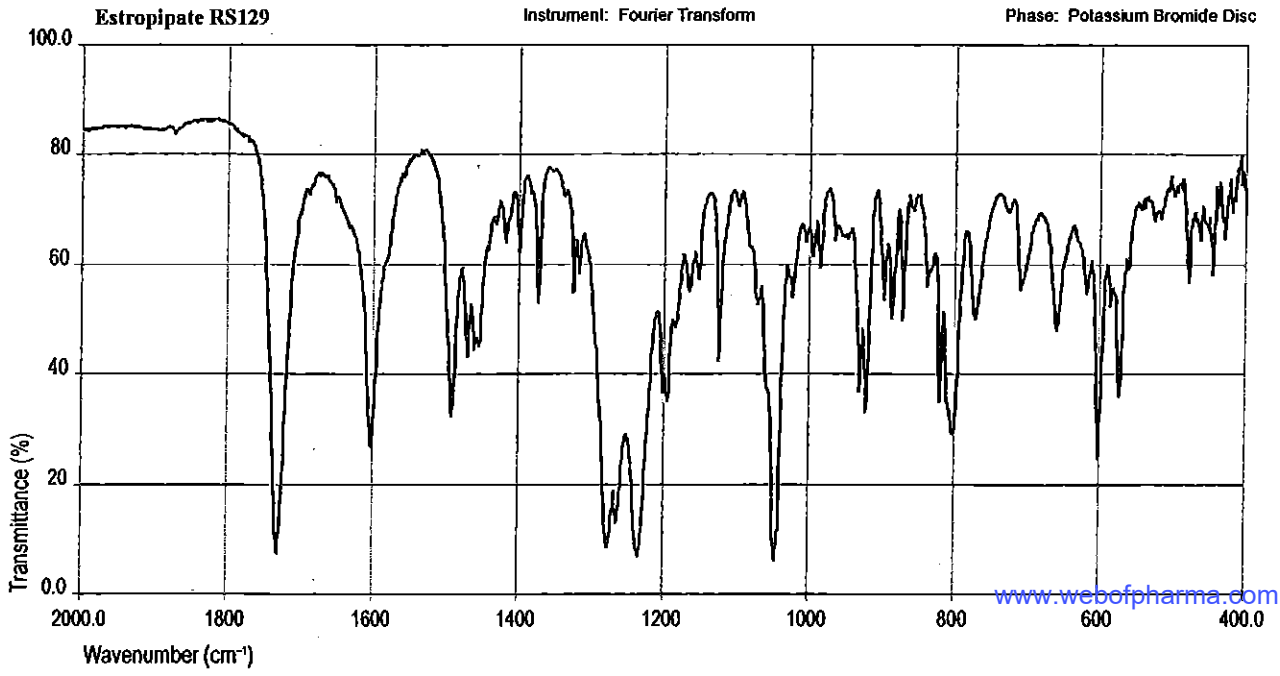


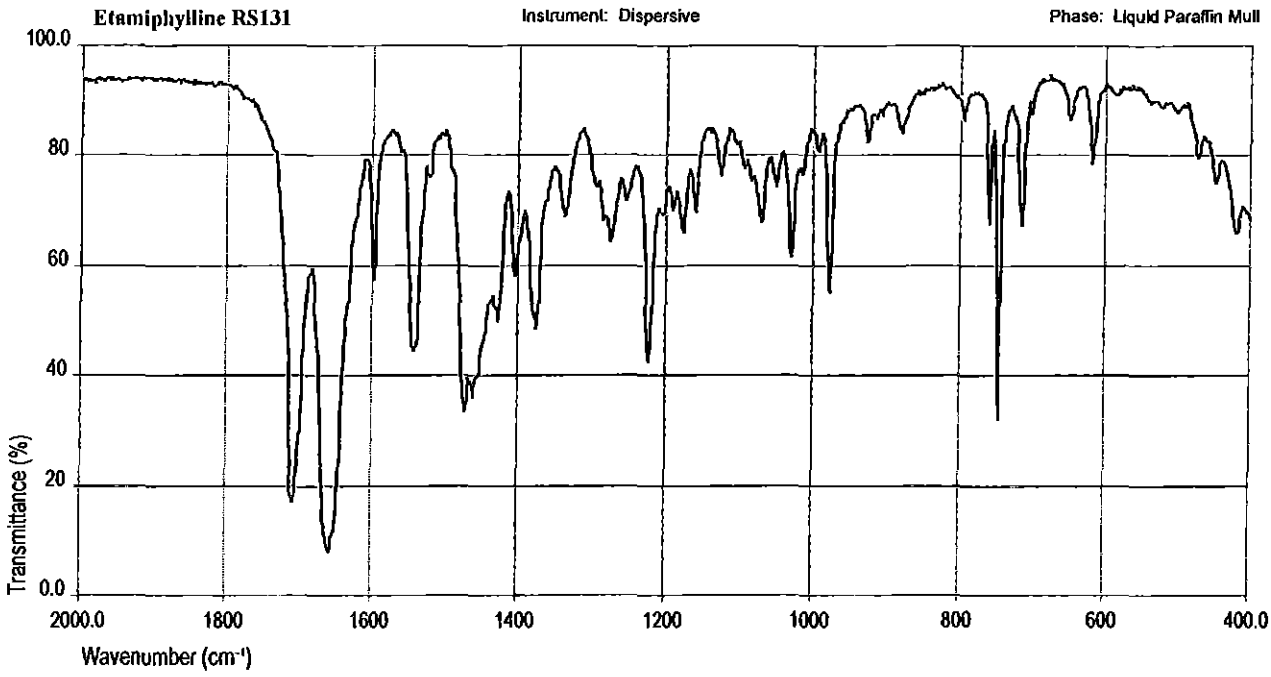


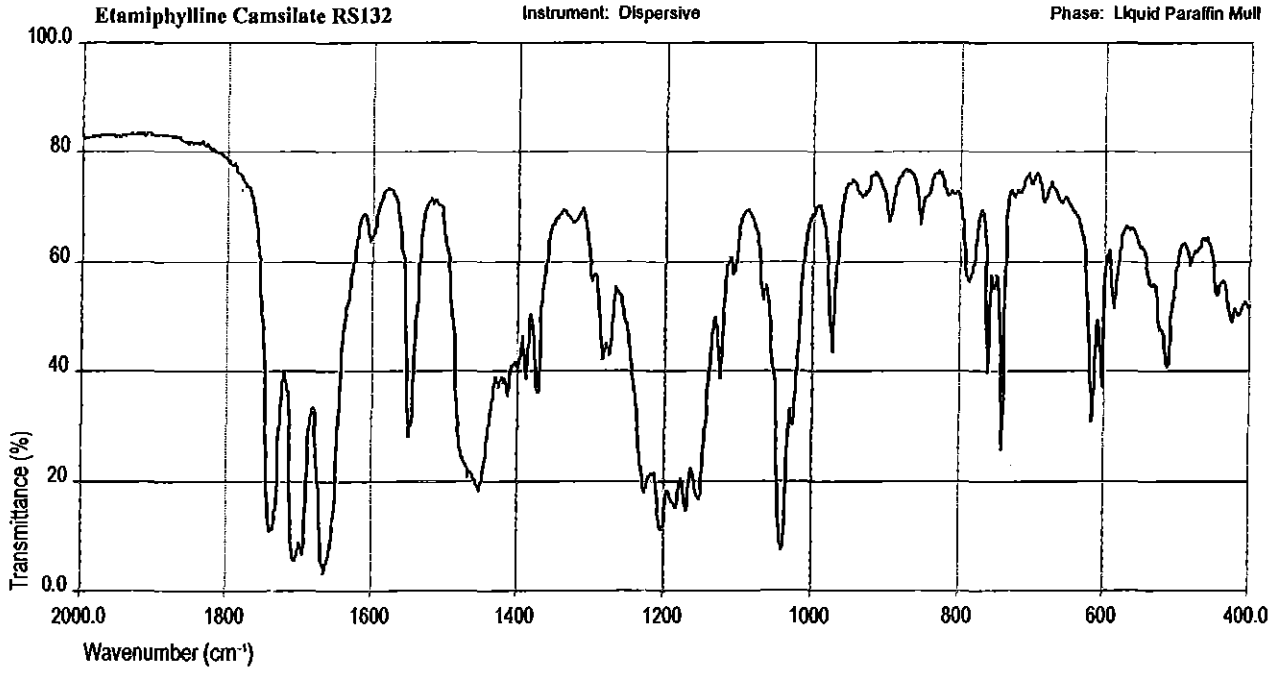


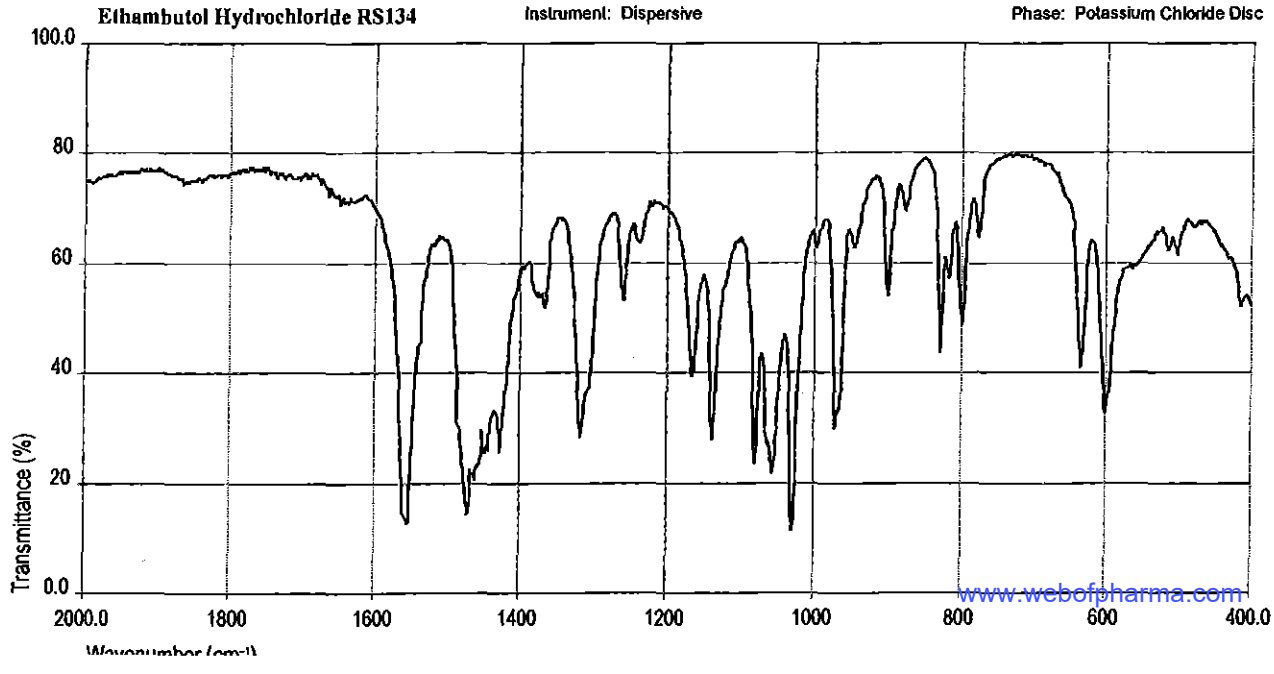




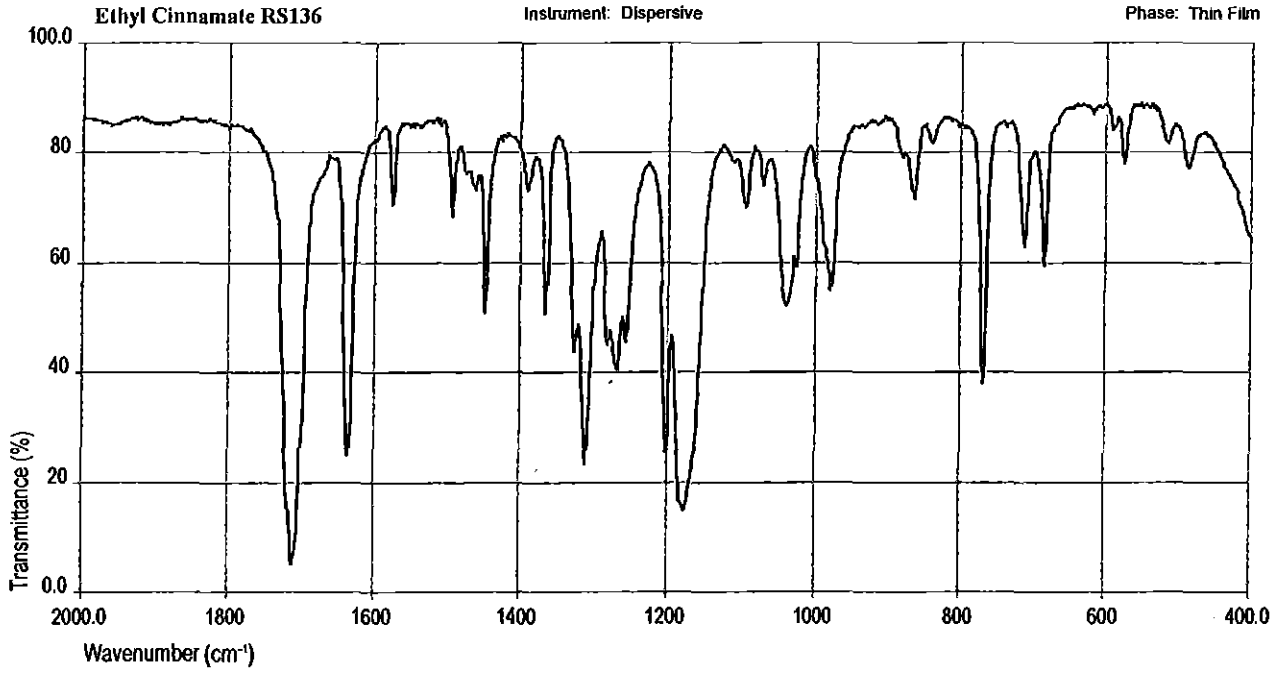




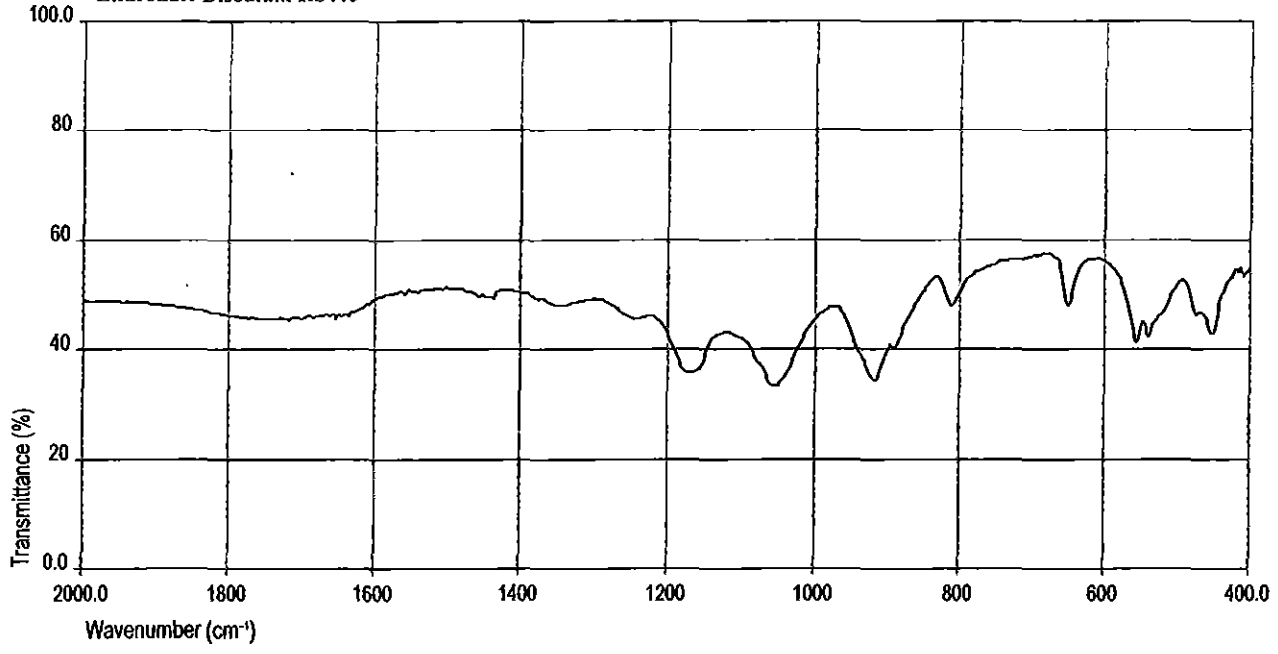




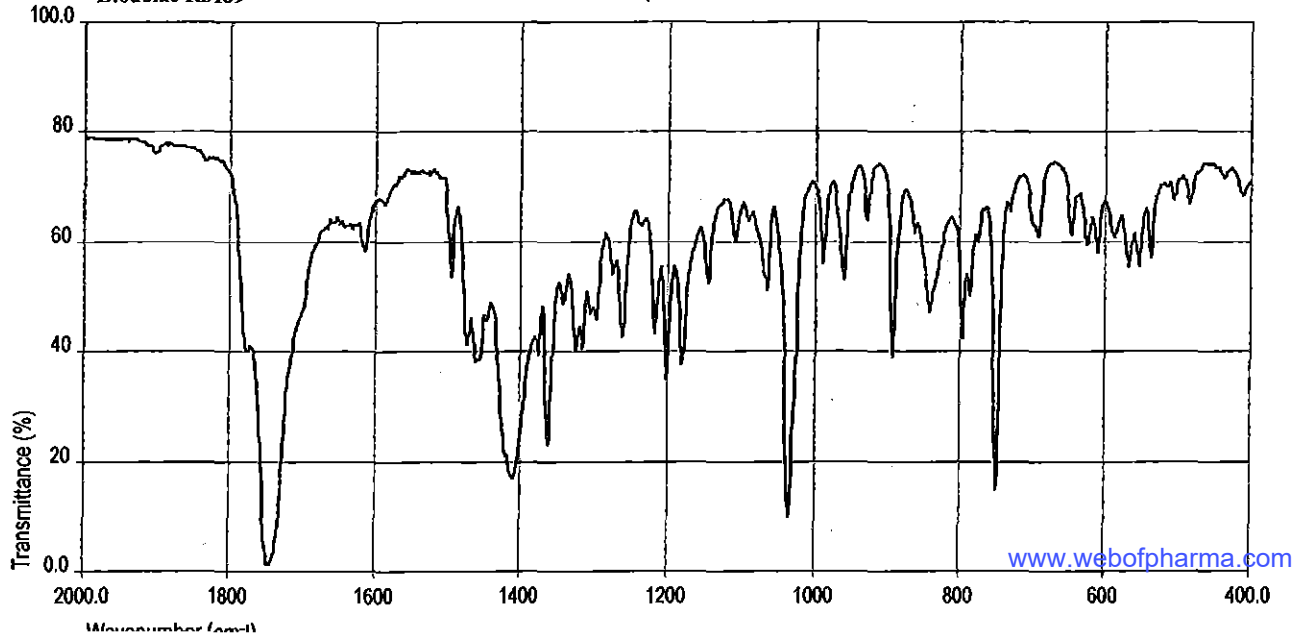
Ethyl Cinnamate RS136 Instrument: Dispersive Phase: Thin Film

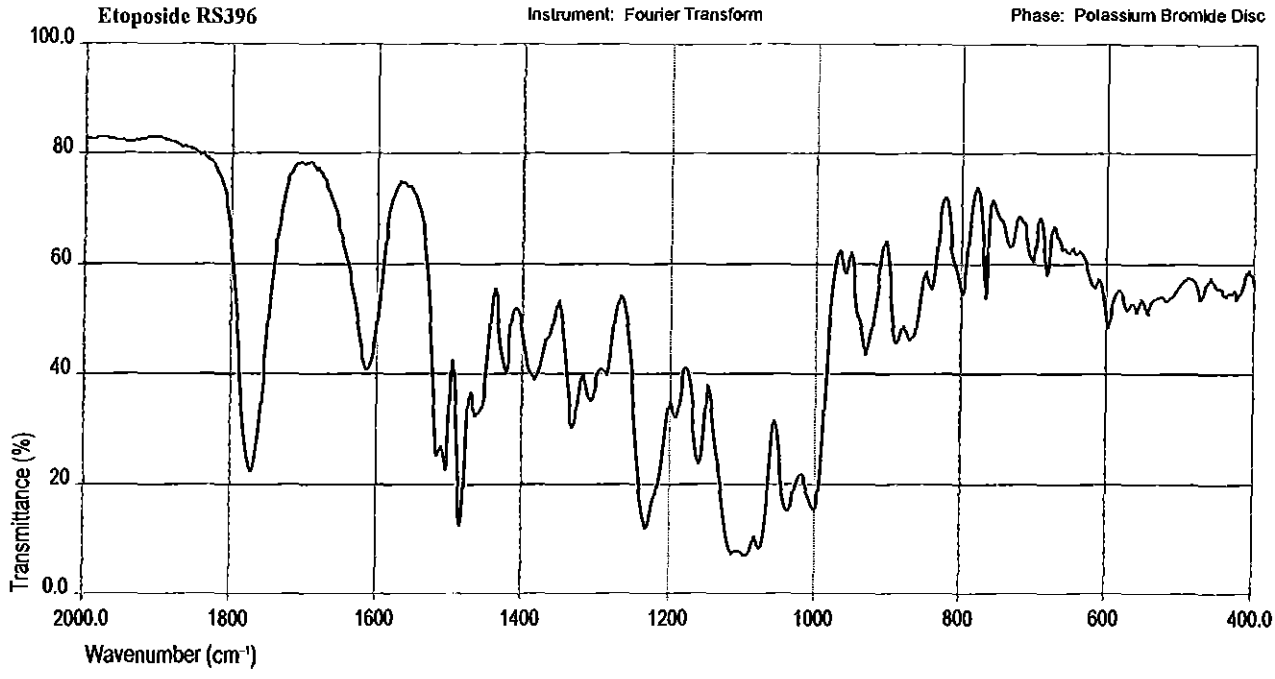


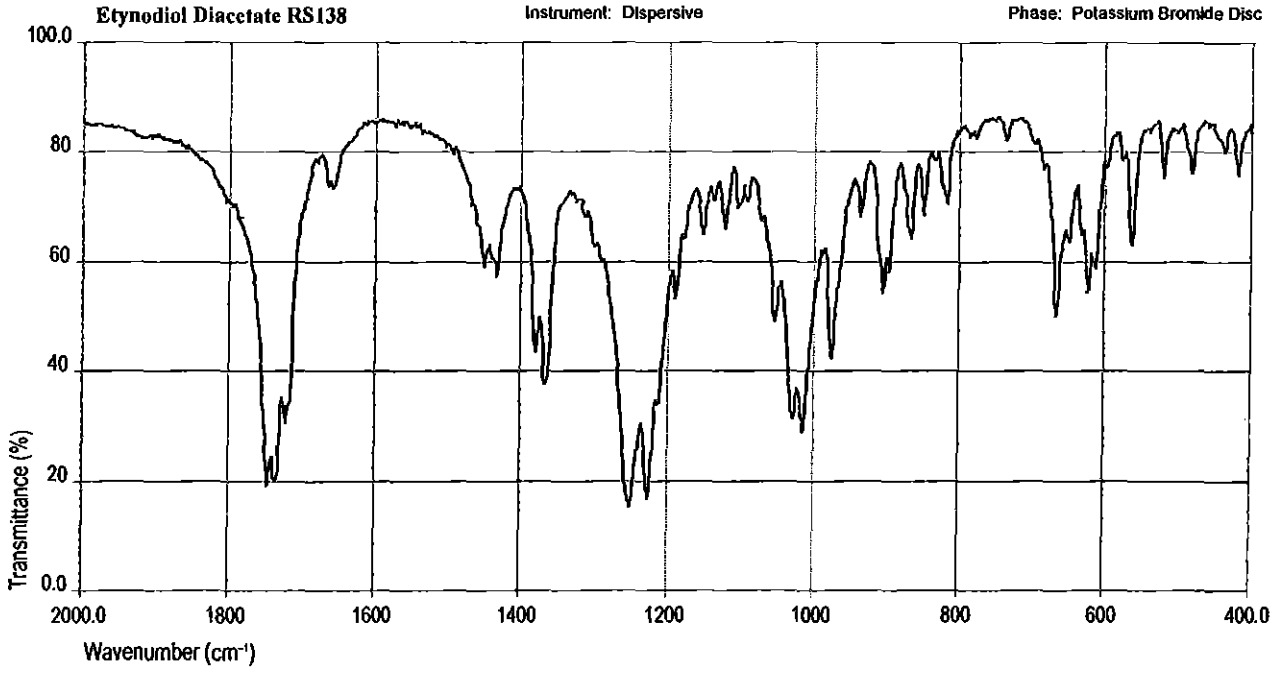
Etidronate Disodium RS440 Instrument: Fourier Transform Phase: Potassium Bromide Disc

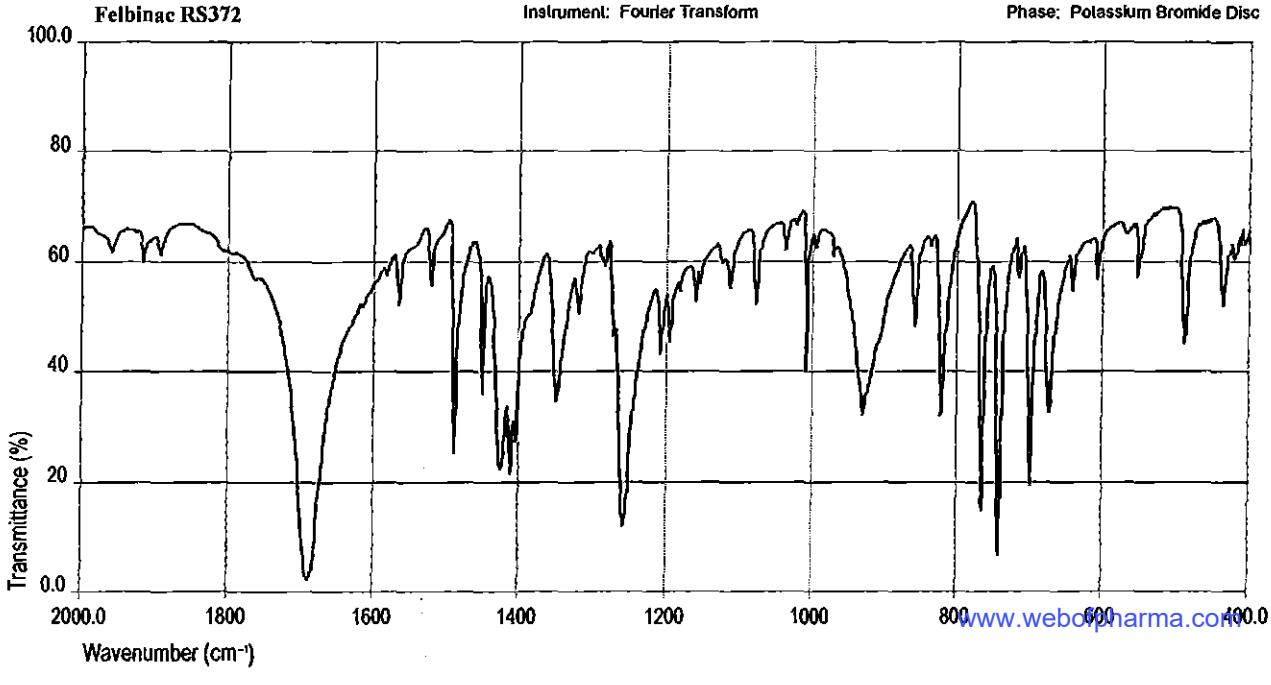


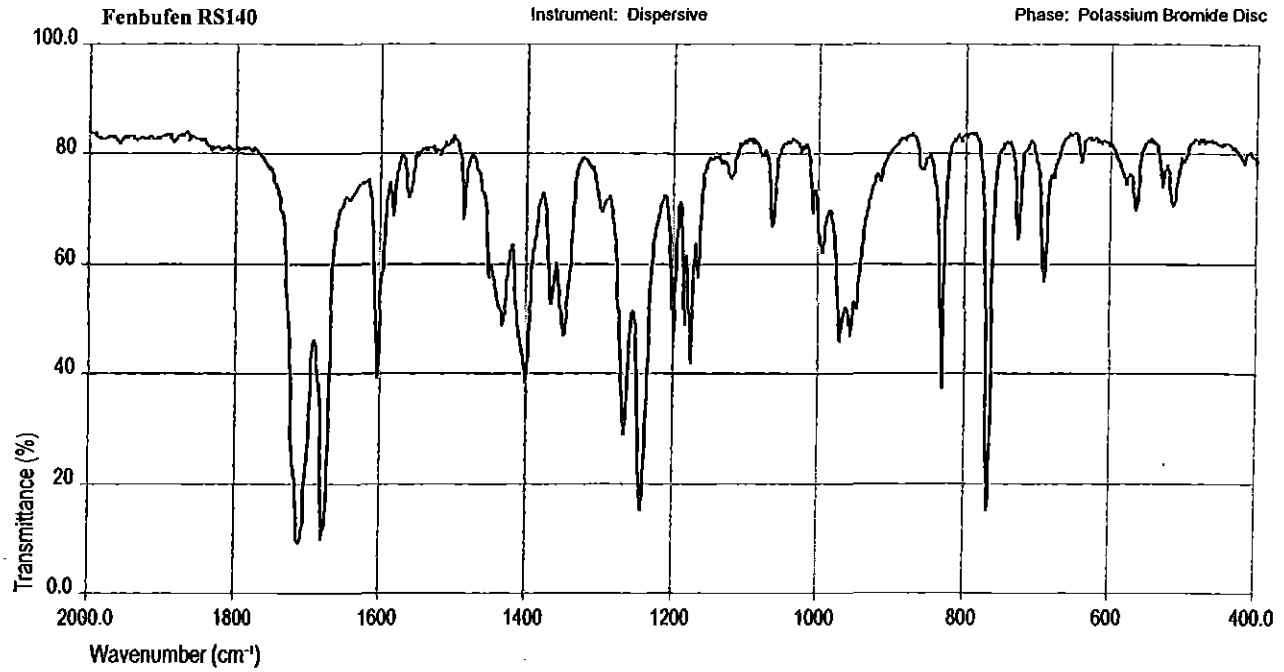
Etodolac RS139 Instrument: Dispersive Phase: Potassium Bromide Disc

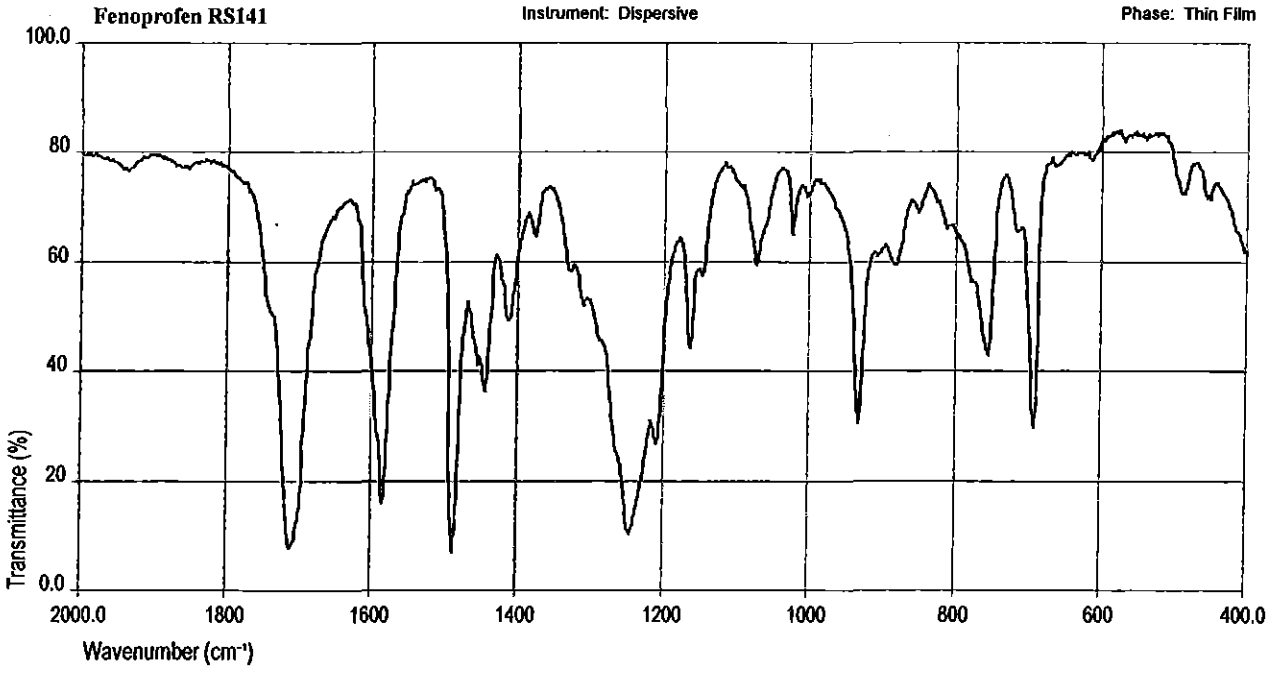


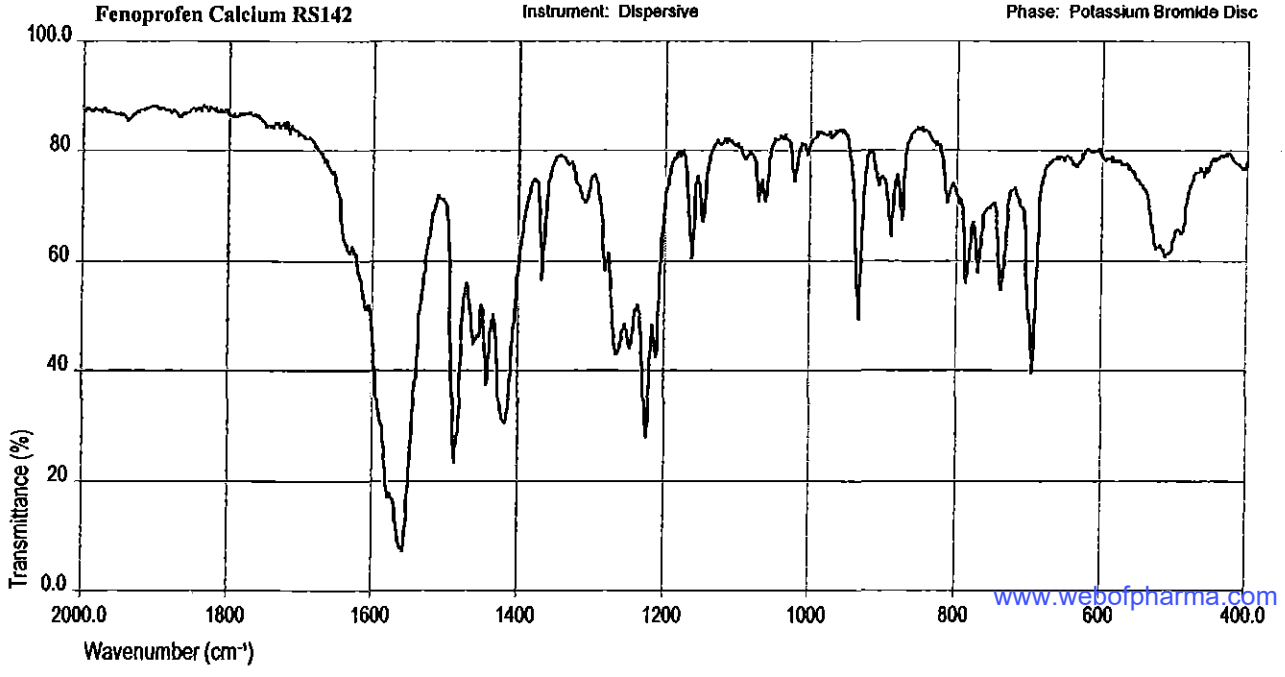


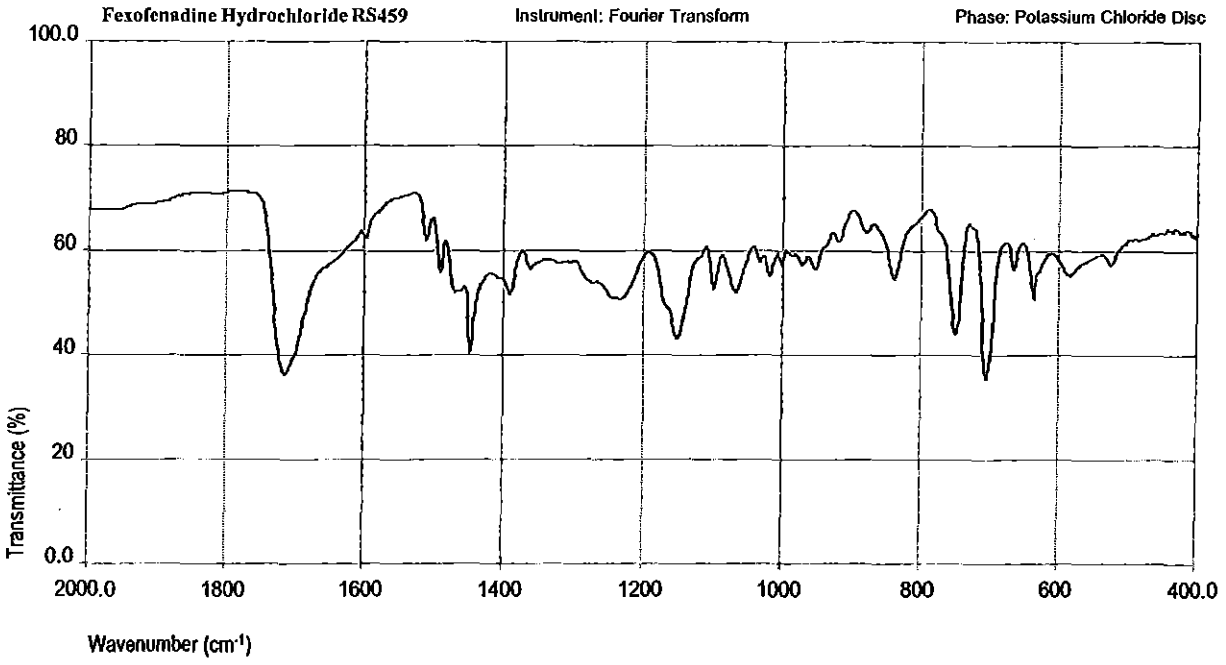


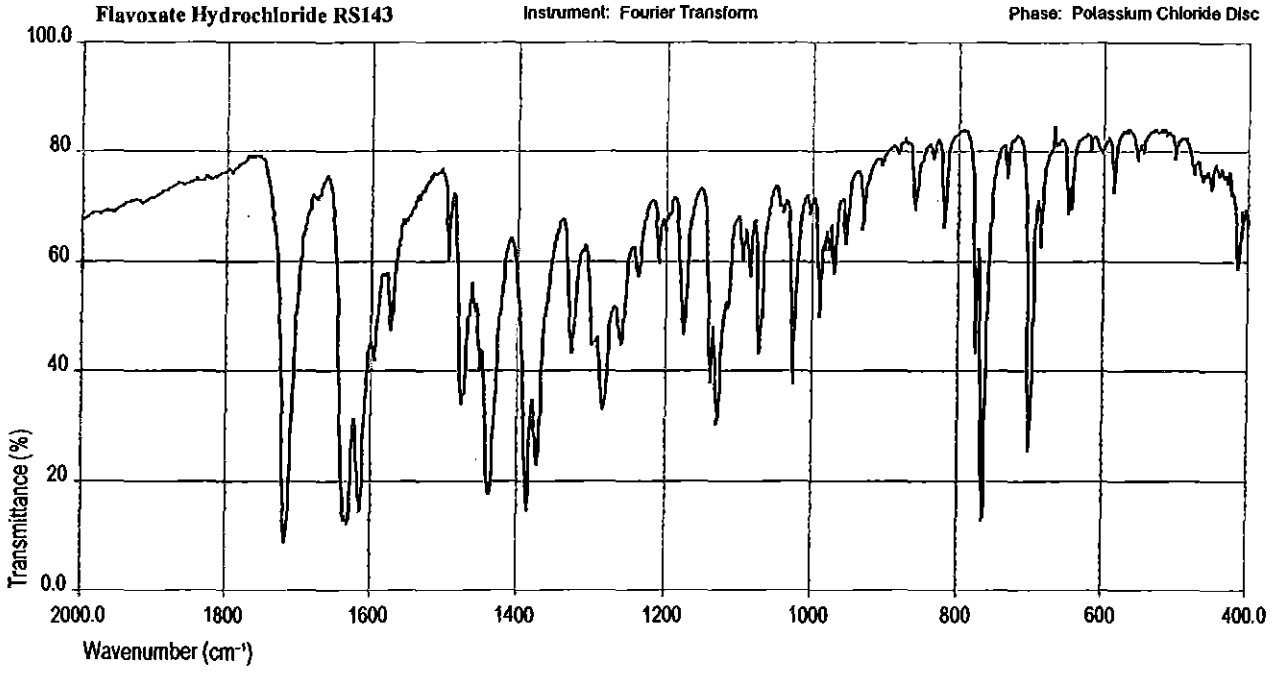


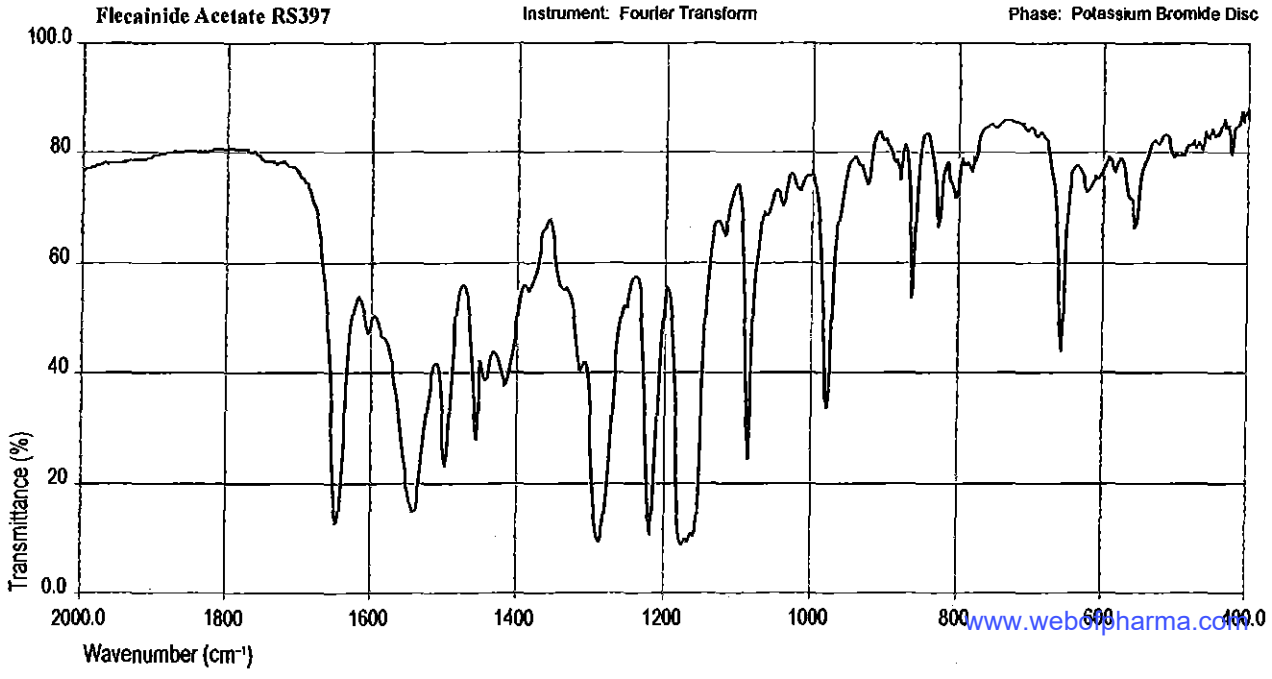




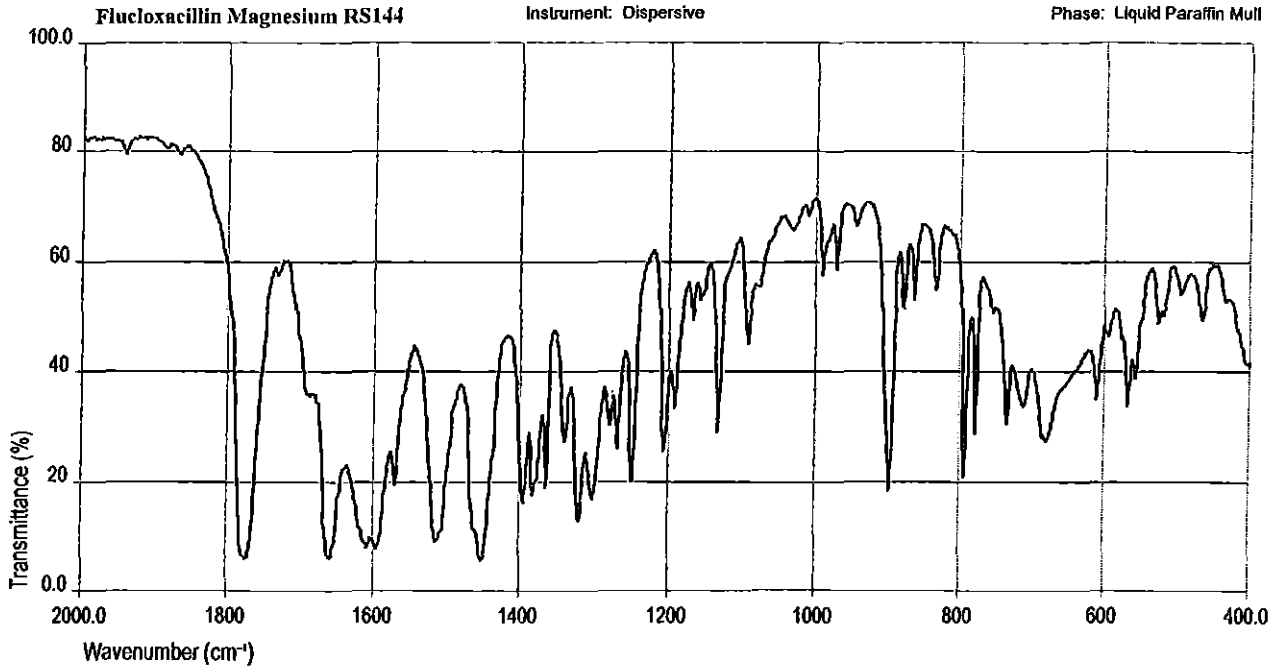




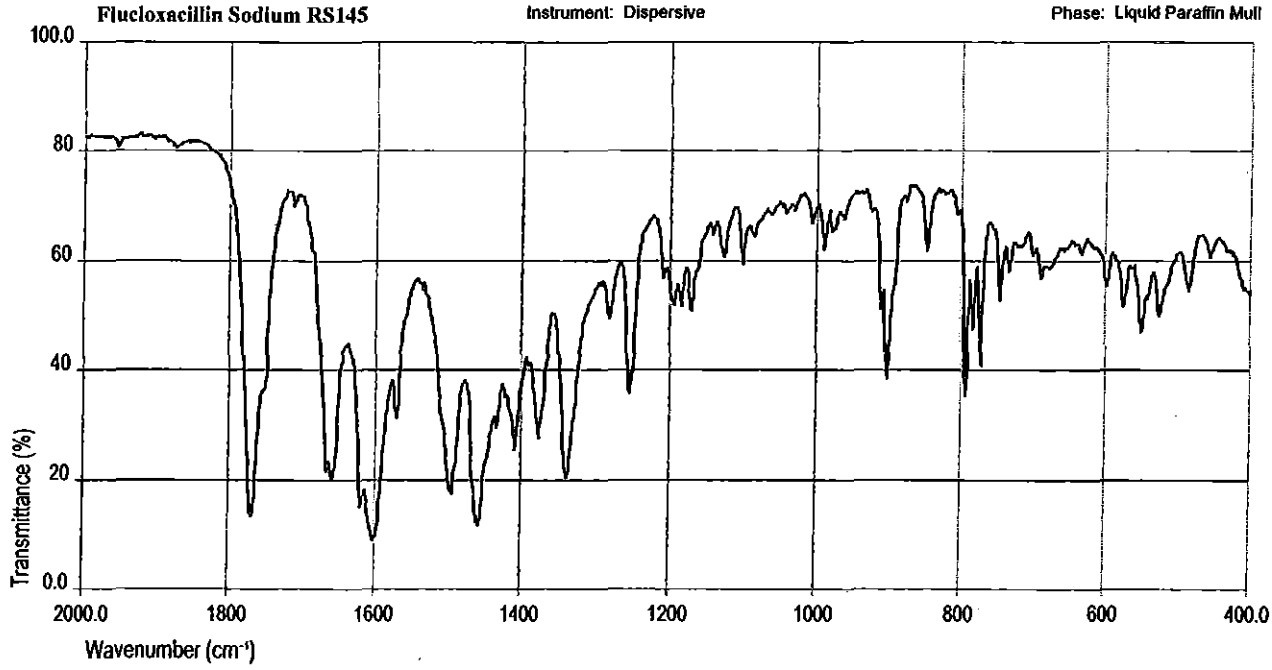




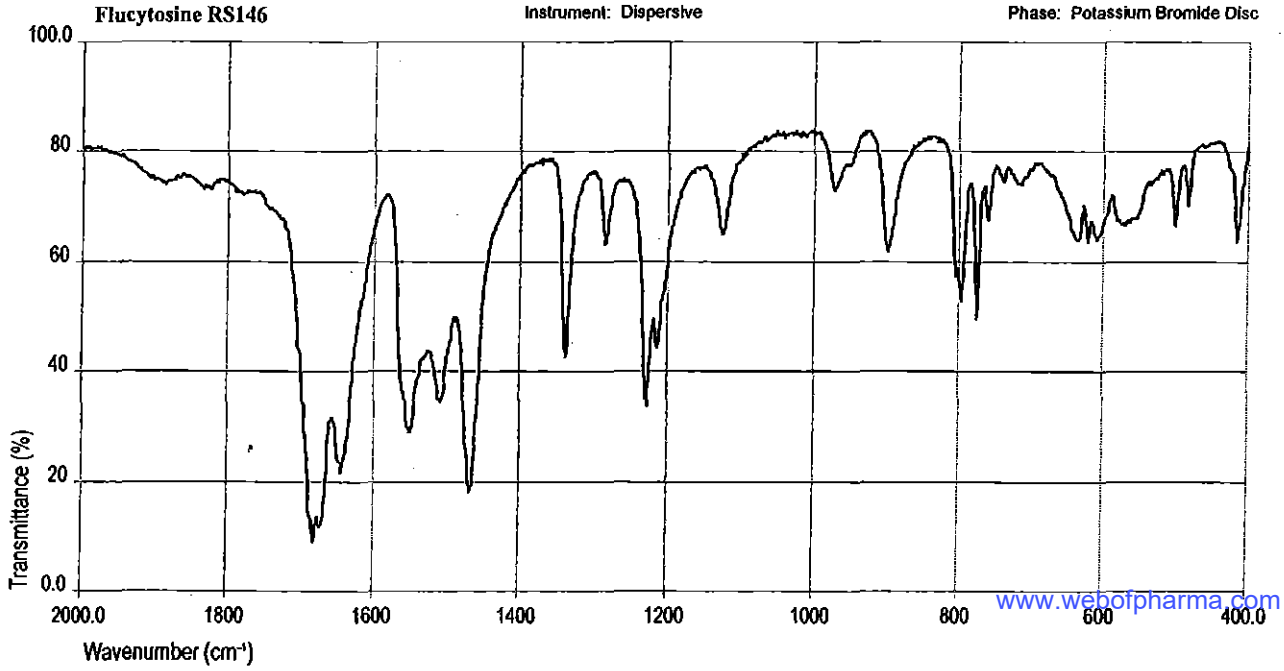
Flucloxacillin Magnesium RS144 Instrument: Dispersive Phase: Liquid Paraffin Mull



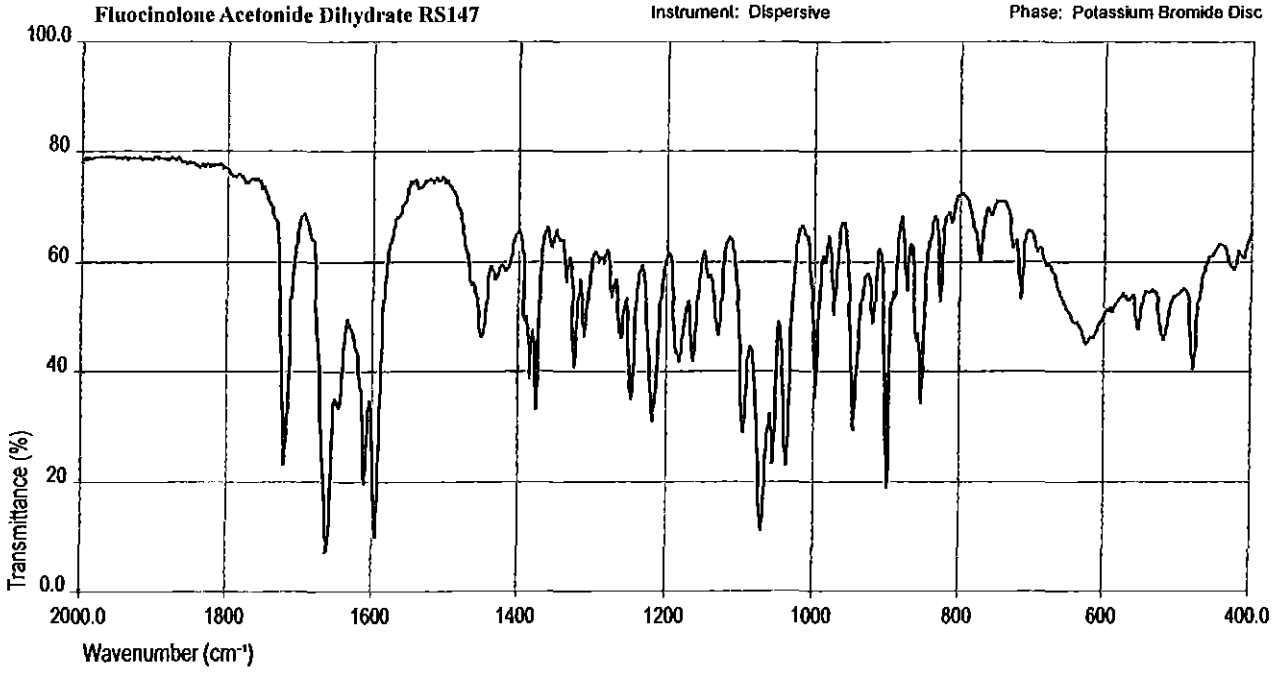
Flucloxacillin Sodium RS145 Instrument: Dispersive Phase: Liquid Paraffin Mull



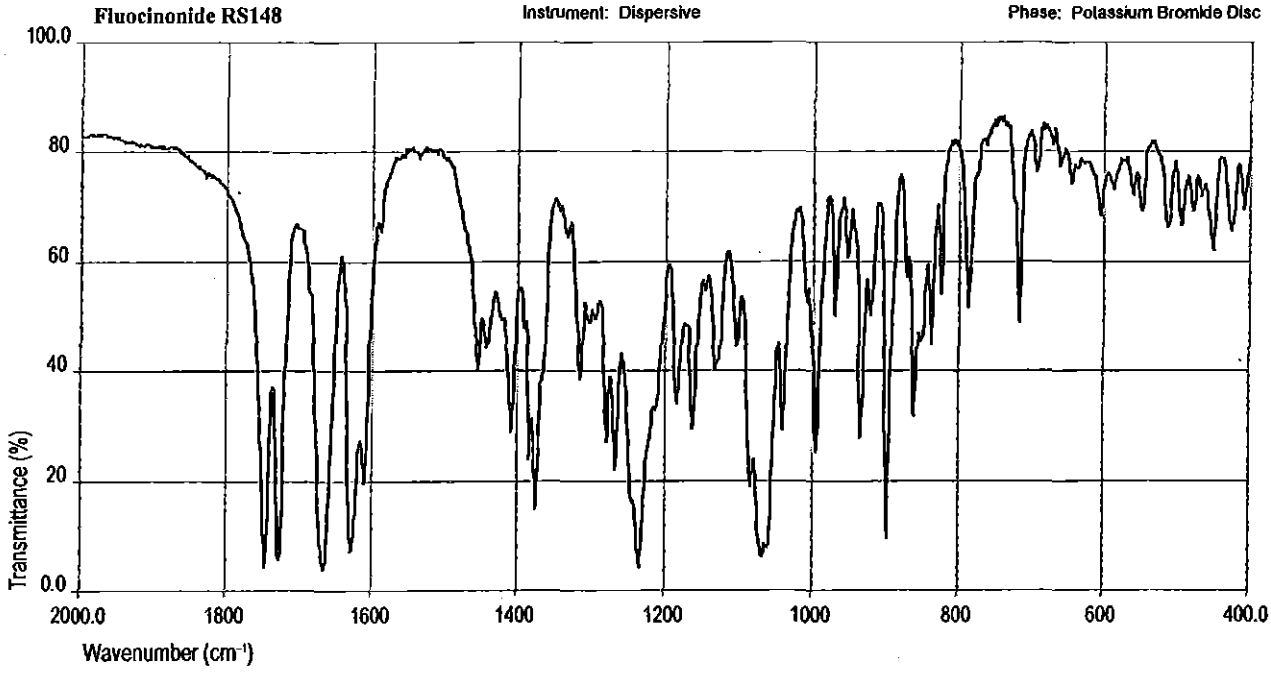
Flucytosine RS146 Instrument: Dispersive Phase: Potassium Bromide Disc



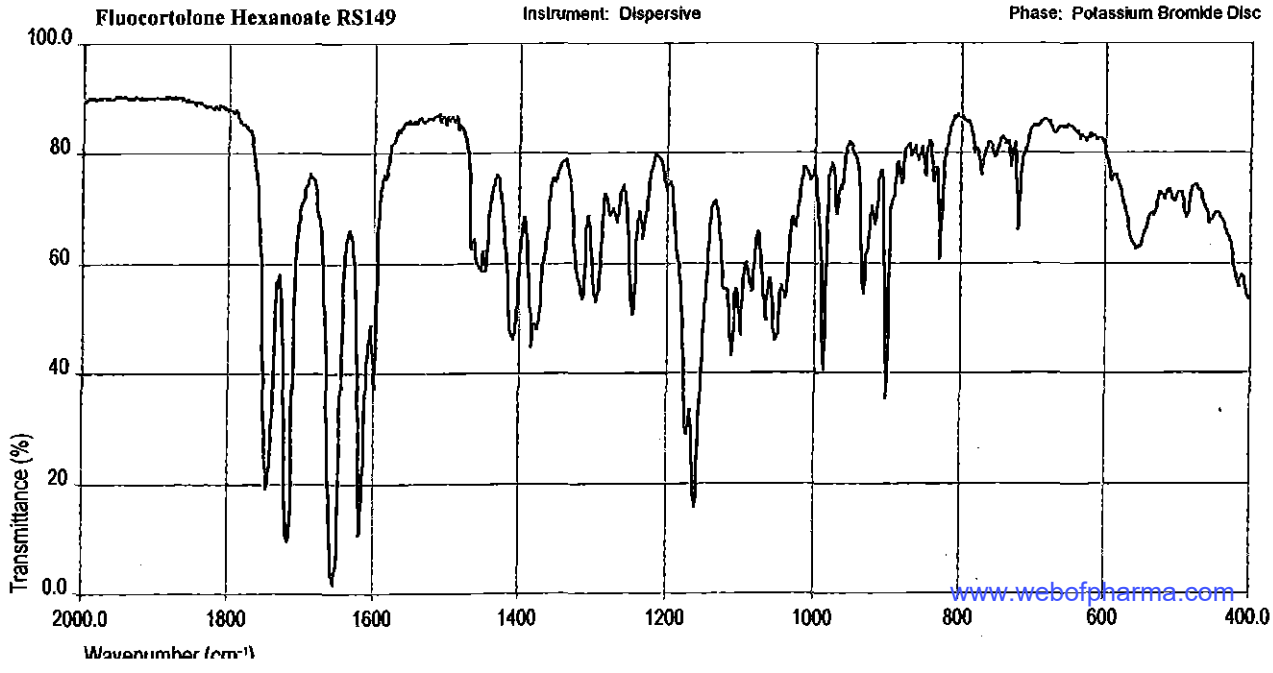
Fluocinolone Acetonide Dihydrate RS147 Instrument: Dispersive Phase: Potassium Bromide Disc



Fluocinonide RS148 Instrument: Dispersive Phase: Potassium Bromide Disc



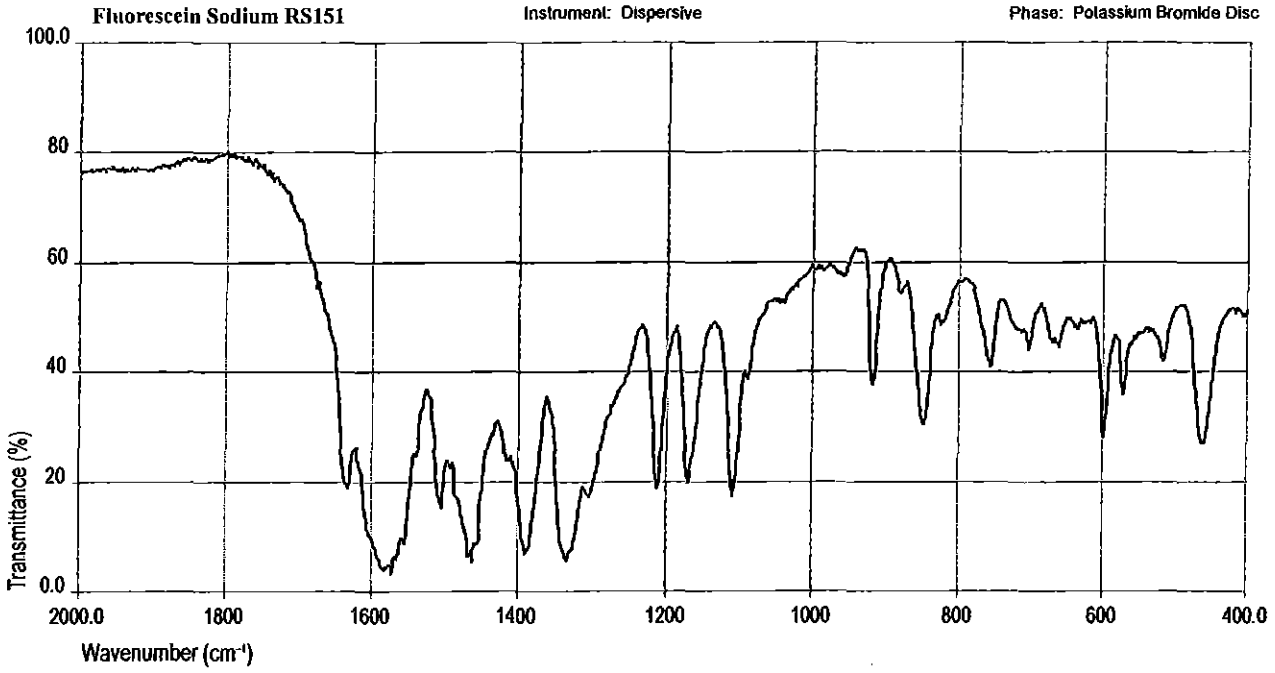
Fluocortolone Hexanoate RS149 Instrument: Dispersive Phase: Potassium Bromide Disc



Fluorescein Sodium RS151

Instrument: Dispersive

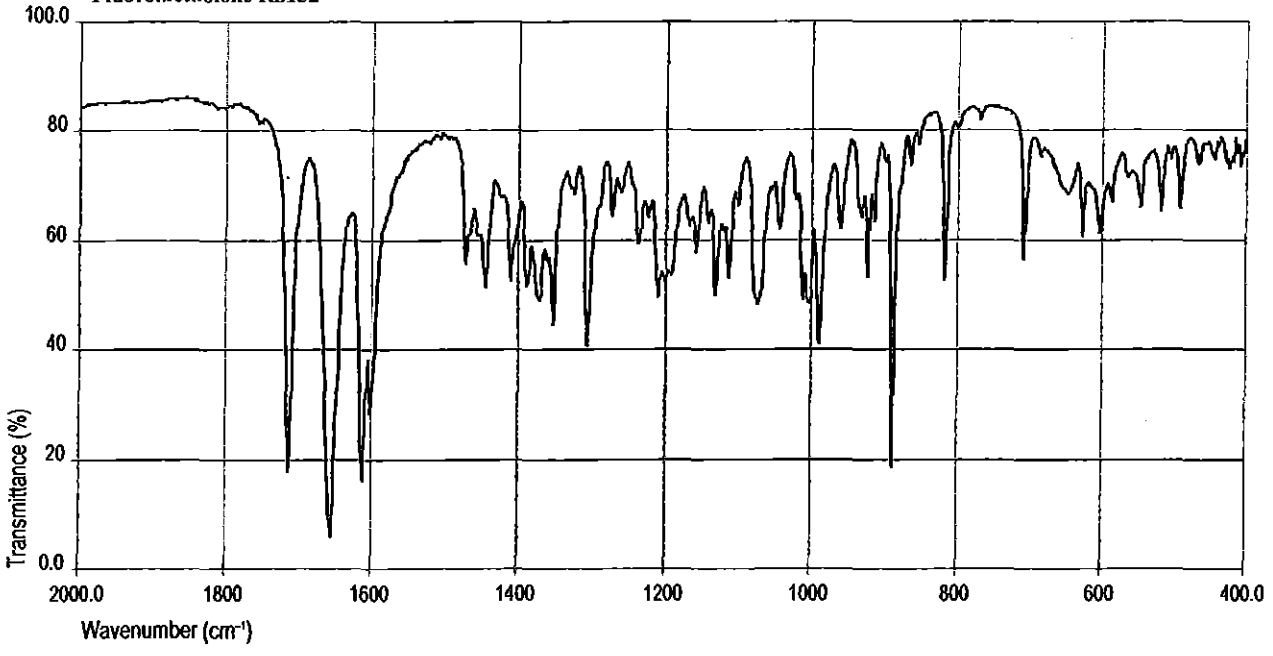
Phase: Potassium Bromide Disc



Fluorometholone RS152

Instrument: Fourier Transform

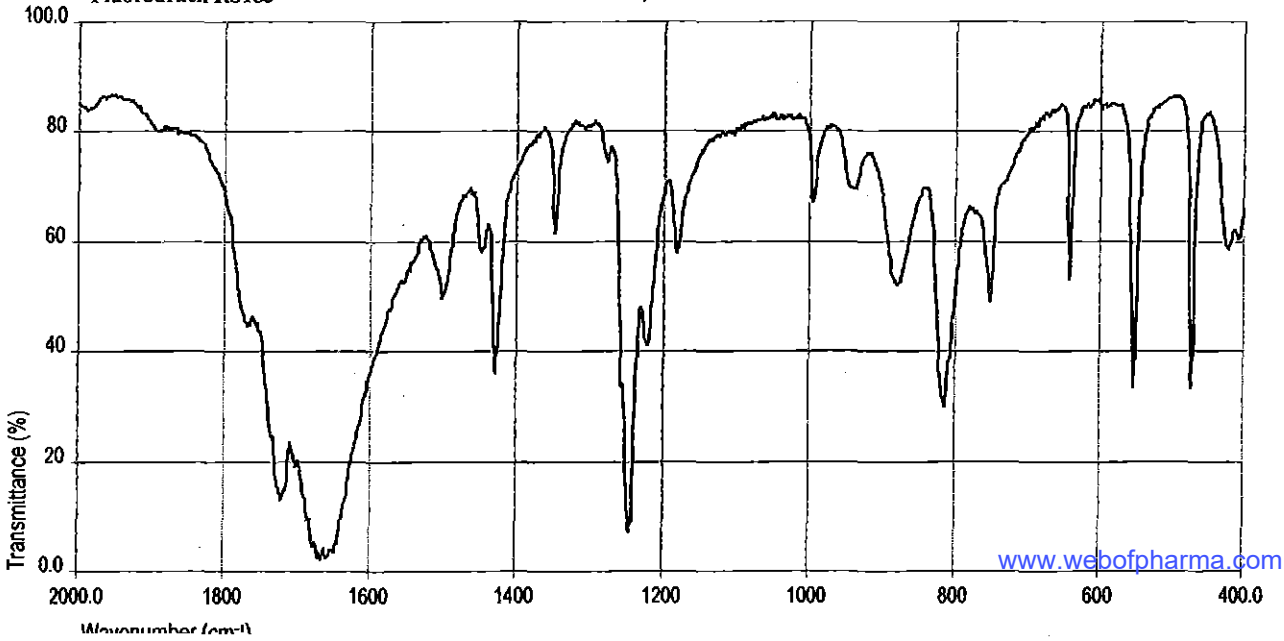
Phase: Potassium Bromide Disc

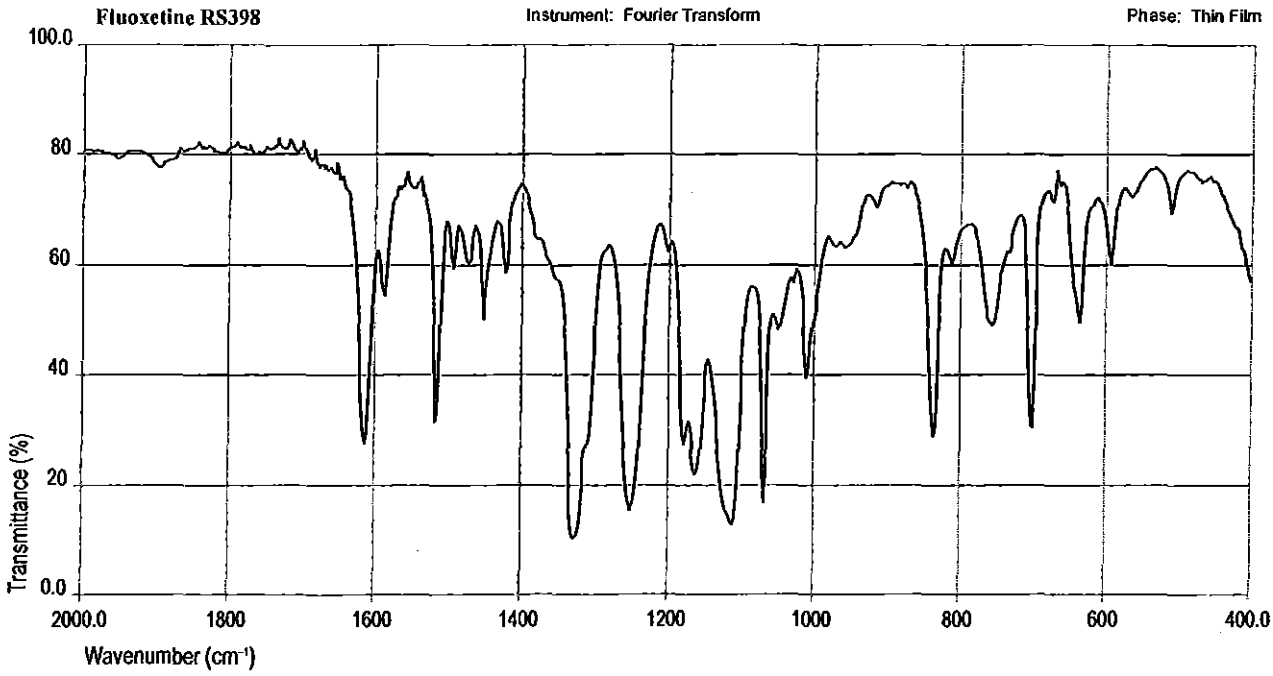


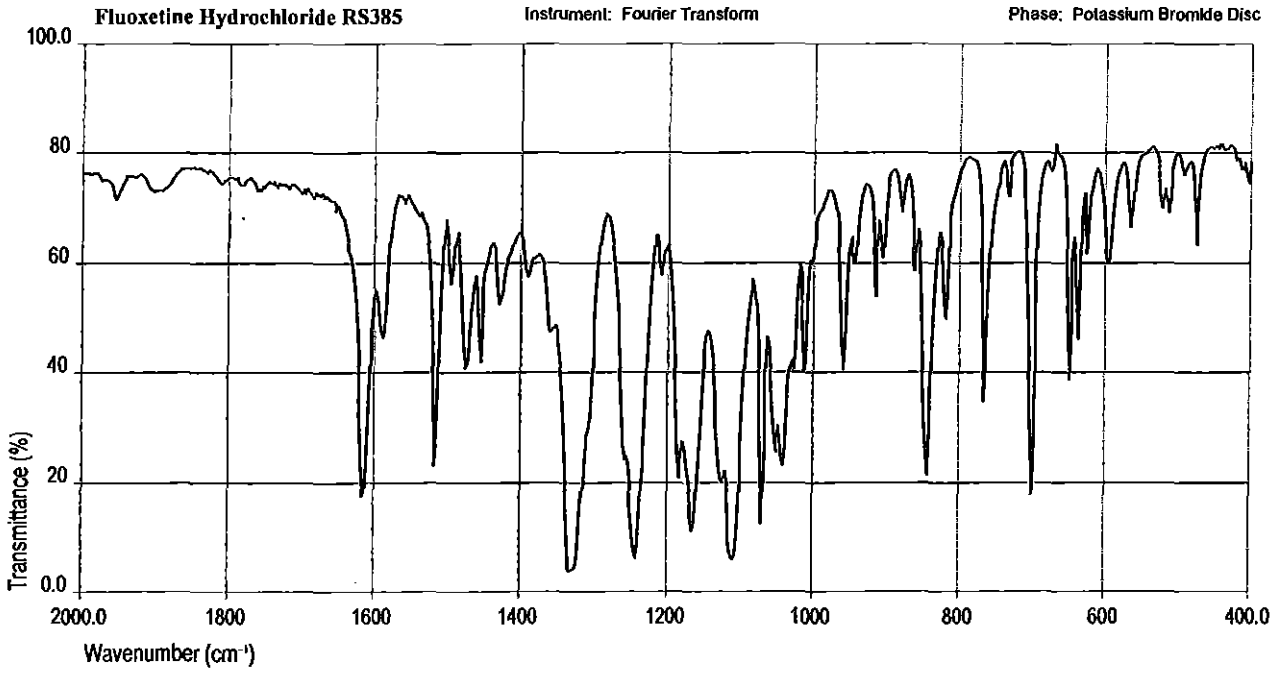
Fluorouracil RS153

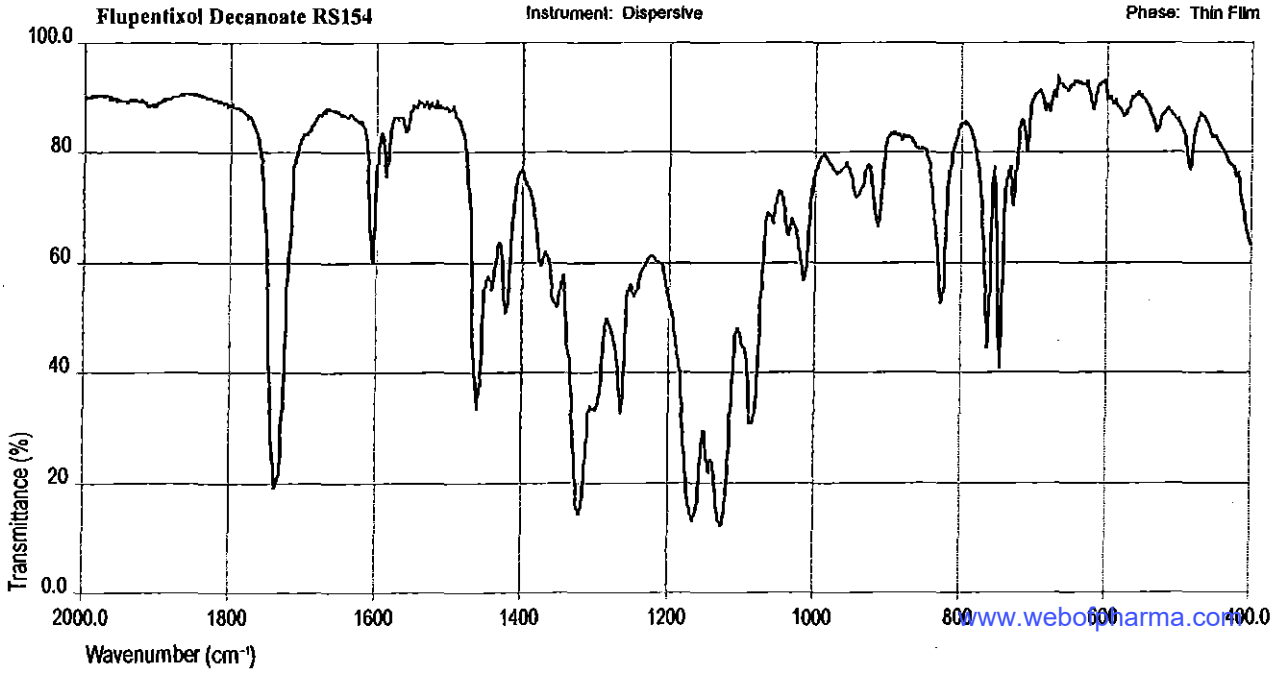
Instrument: Dispersive

Phase: Potassium Bromide Disc

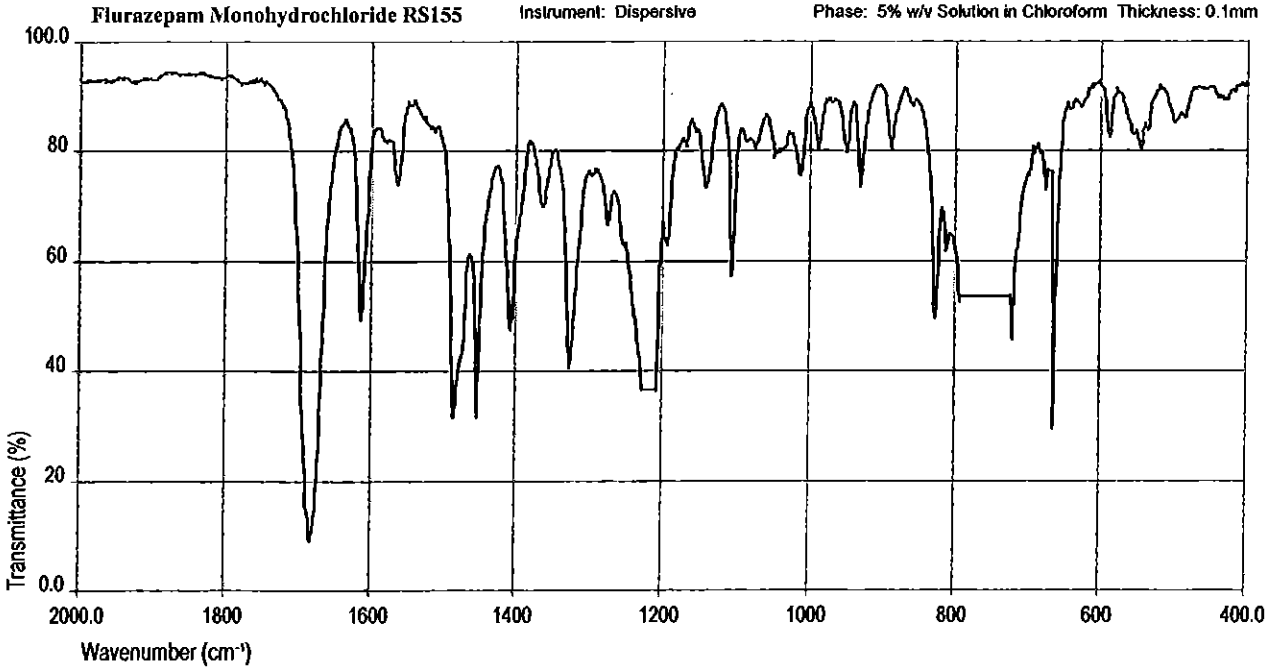




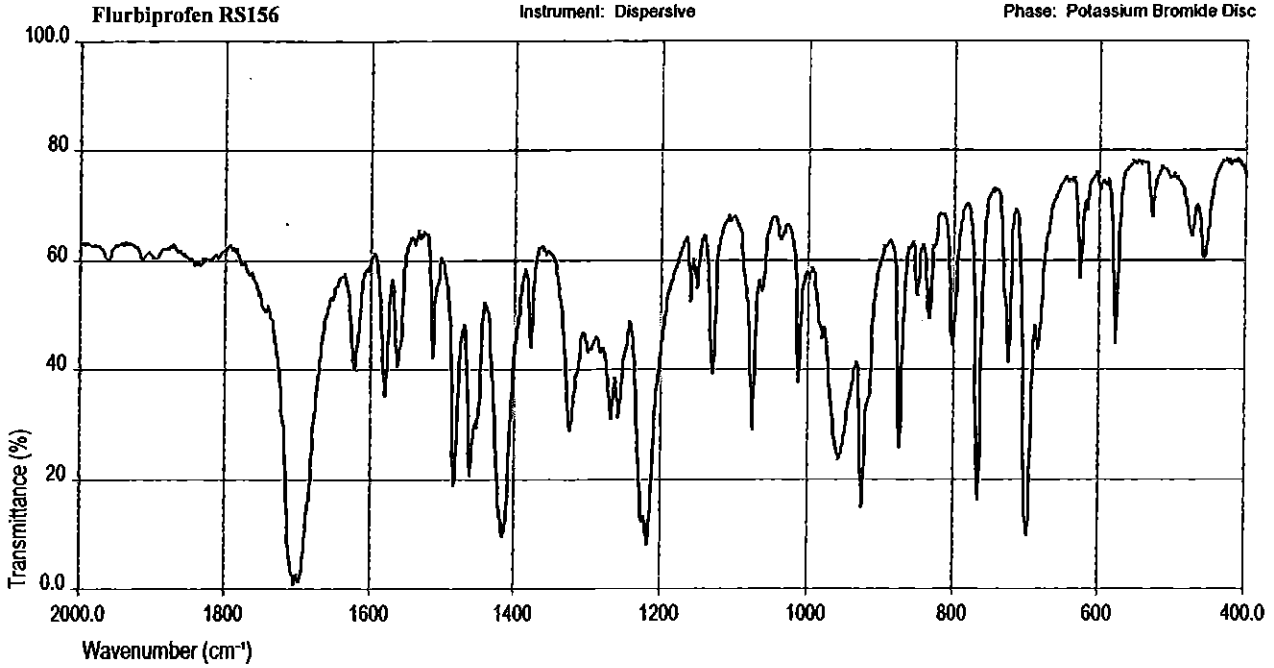




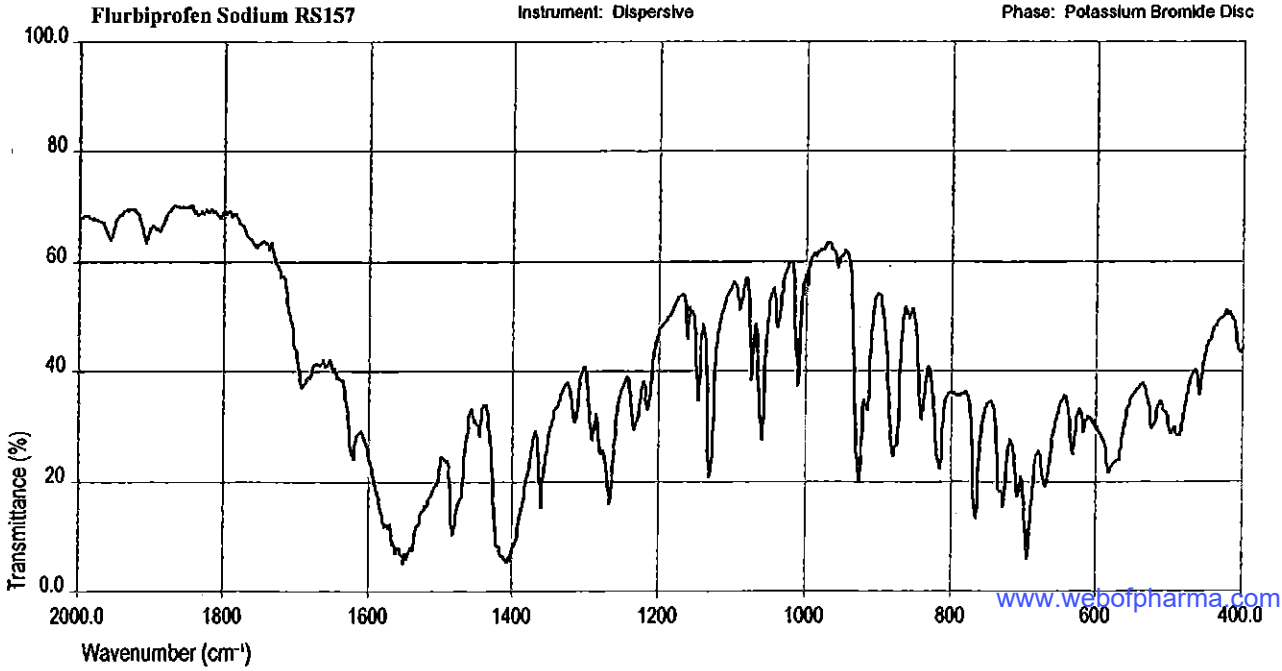
Flurazepam Monohydrochloride RS155 Instrument: Dispersive Phase: 5% w/v Solution in Chloroform Thickness: 0.1mm



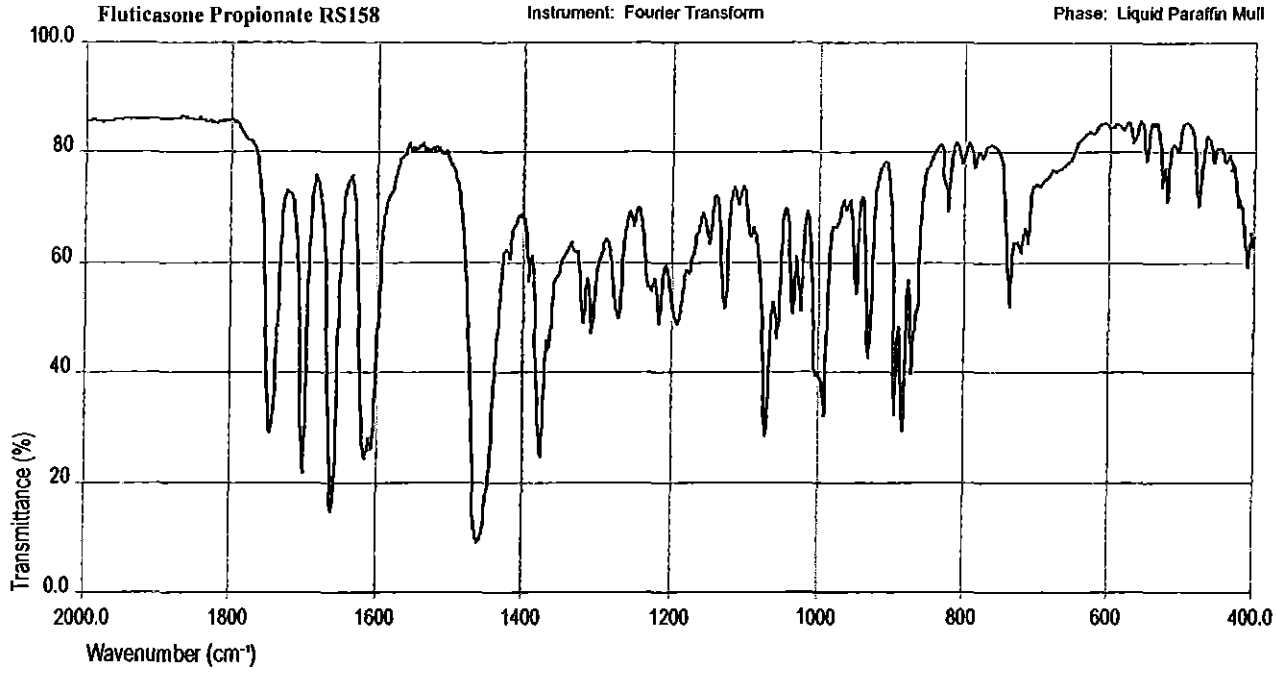
Flurbiprofen RS156 Instrument: Dispersive Phase: Potassium Bromide Disc



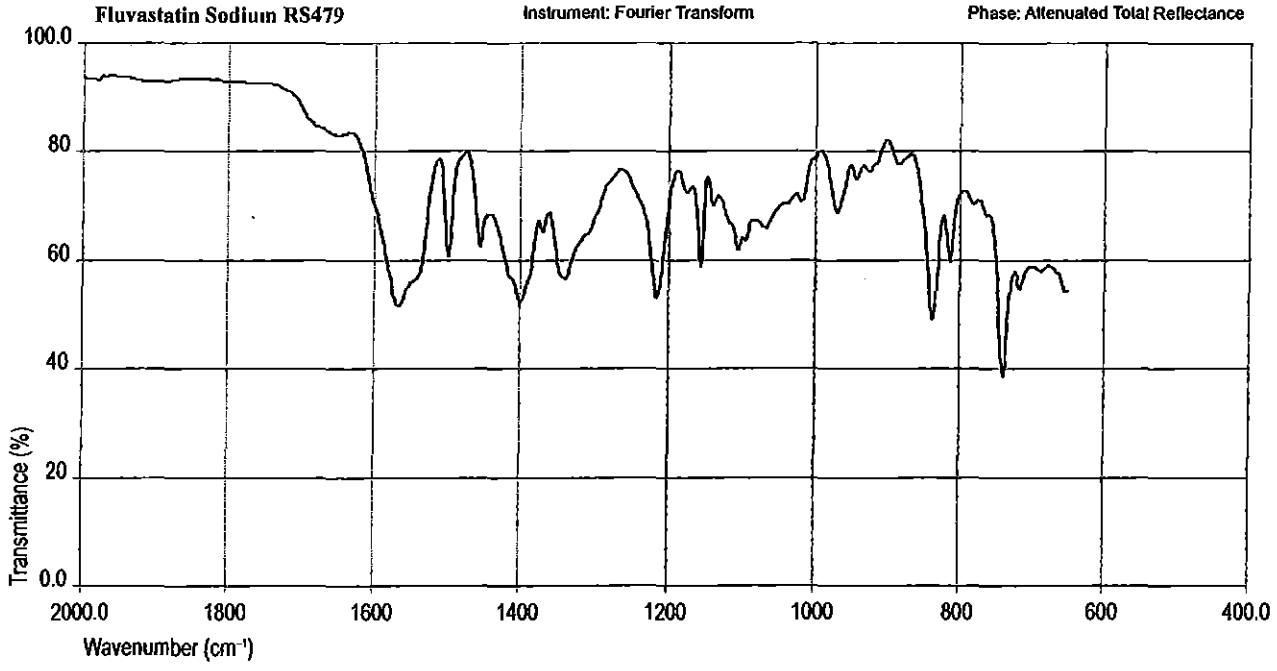
Flurbiprofen Sodium RS157 Instrument: Dispersive Phase: Potassium Bromide Disc



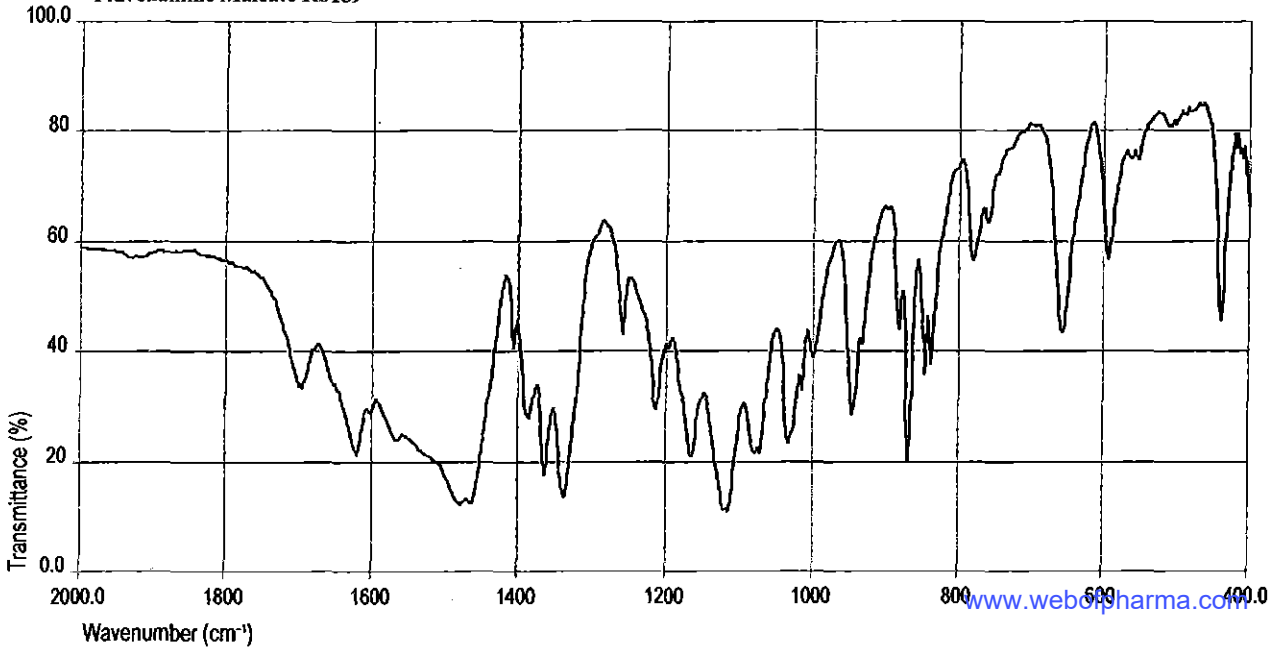
Fluticasone Propionate RS158 Instrument: Fourier Transform Phase: Liquid Paraffin Mull

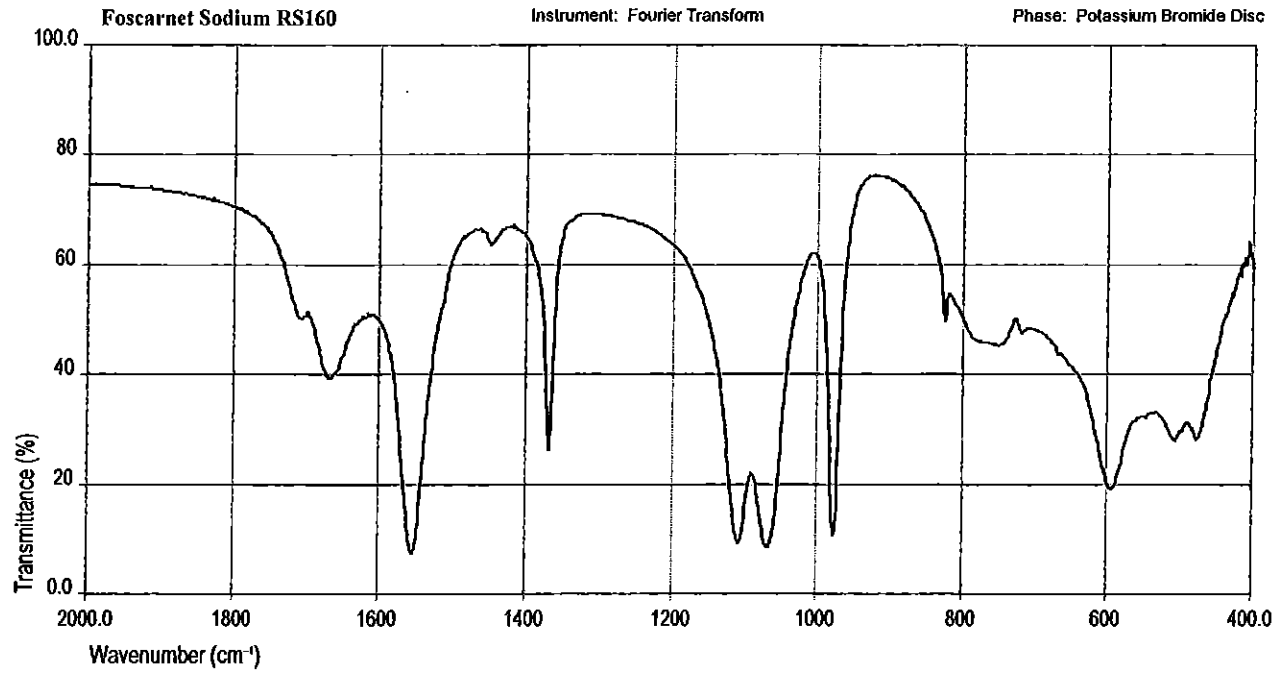


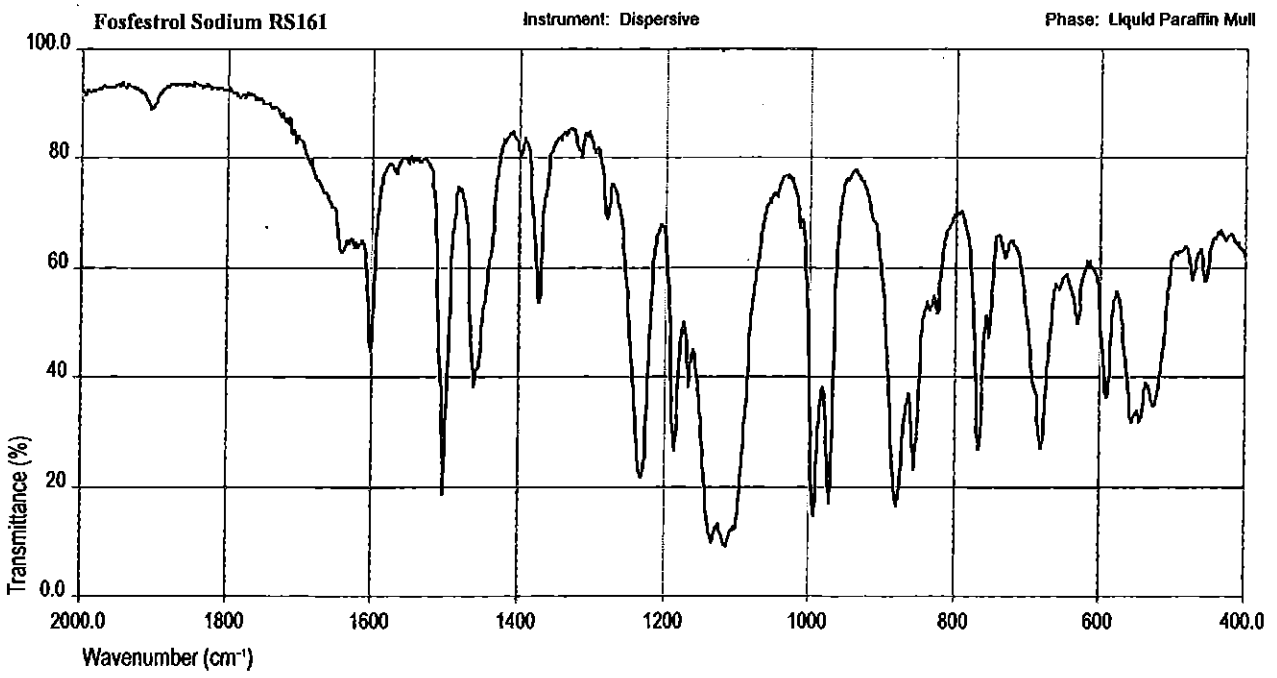
Fluvastatin Sodium RS479 Instrument: Fourier Transform Phase: Attenuated Total Reflectance

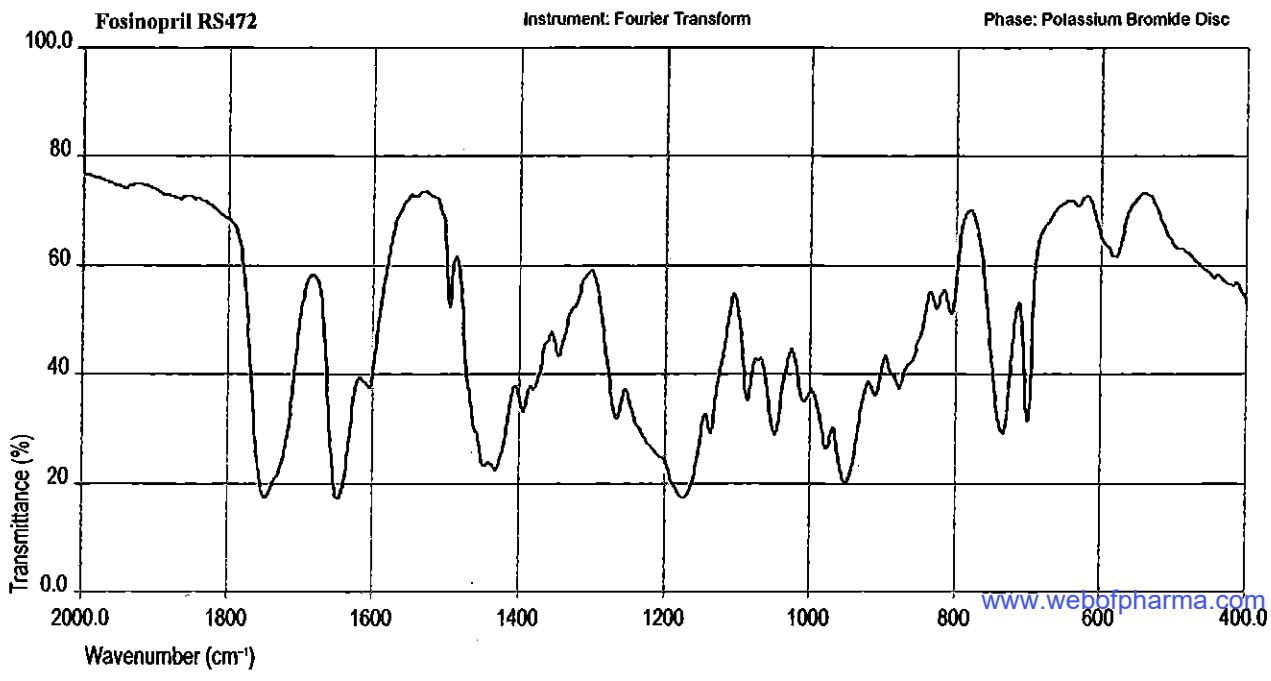


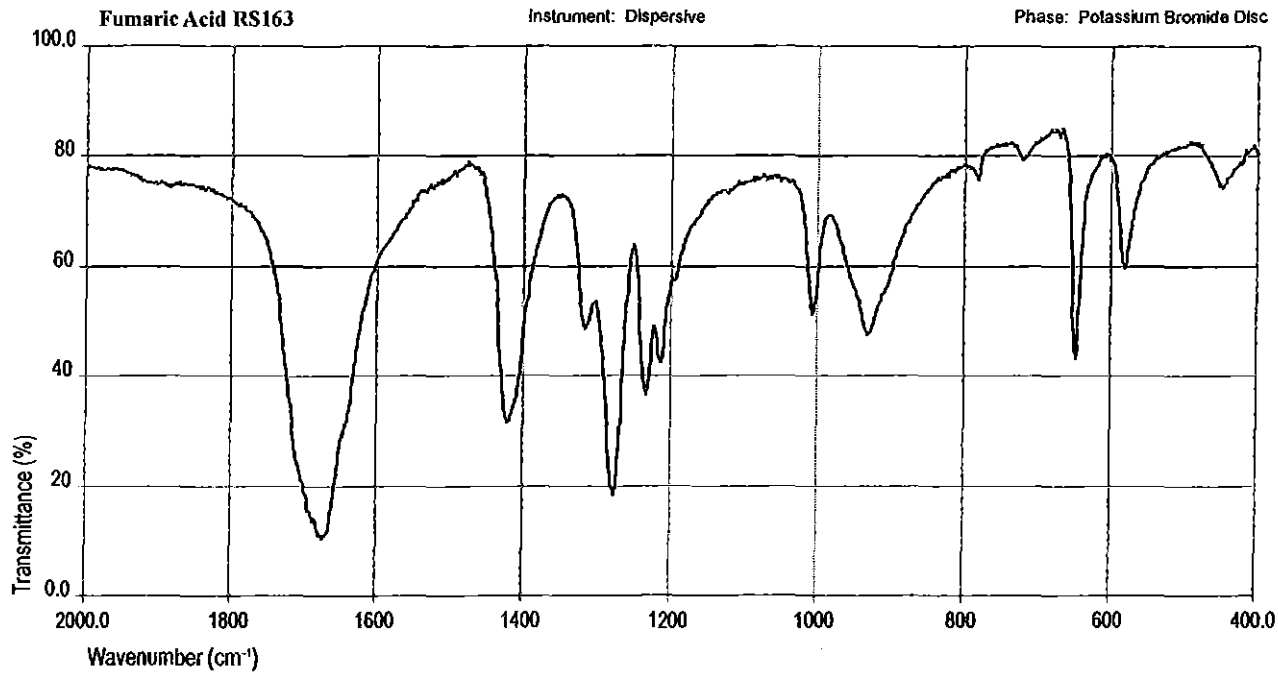
Fluvoxamine Maleate RS159 Instrument: Fourier Transform Phase: Potassium Bromide Disc

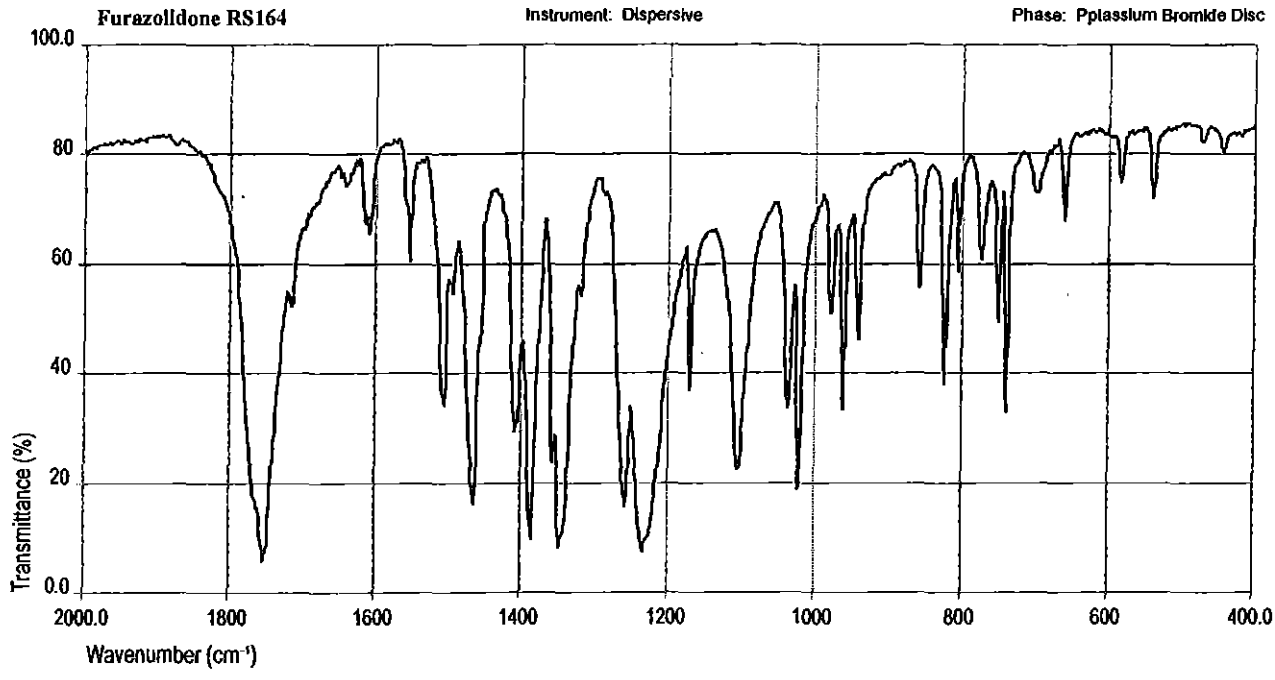


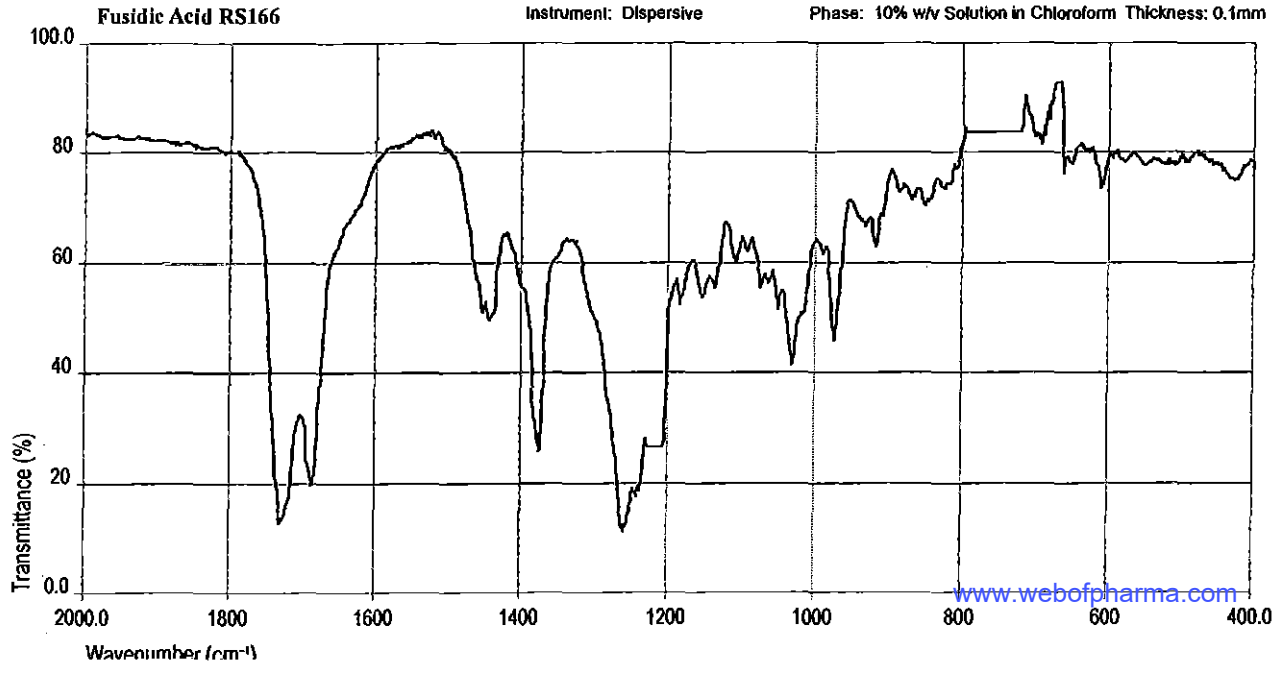


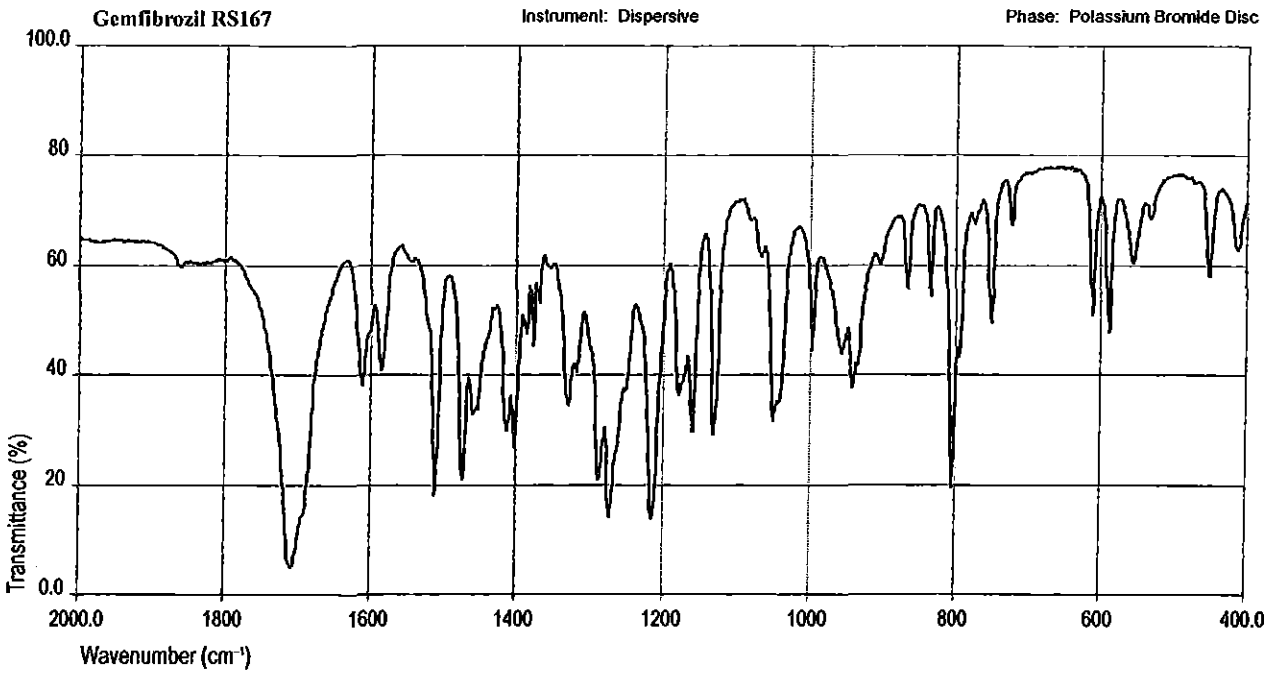


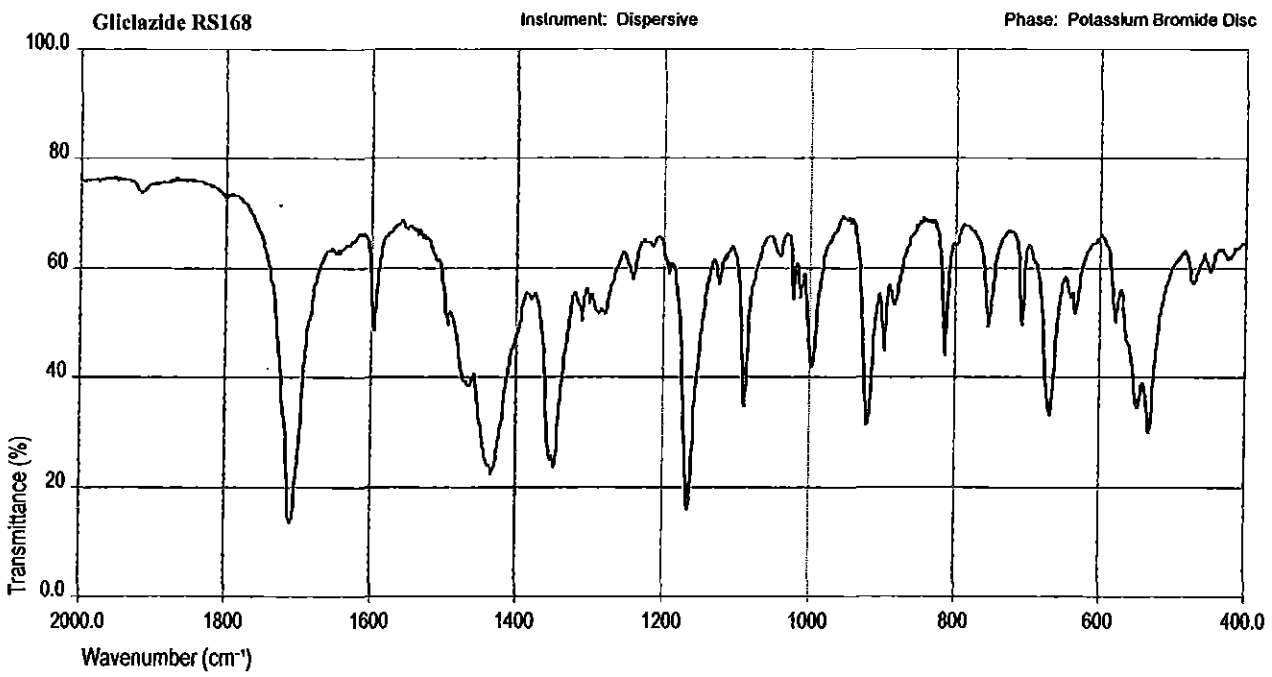


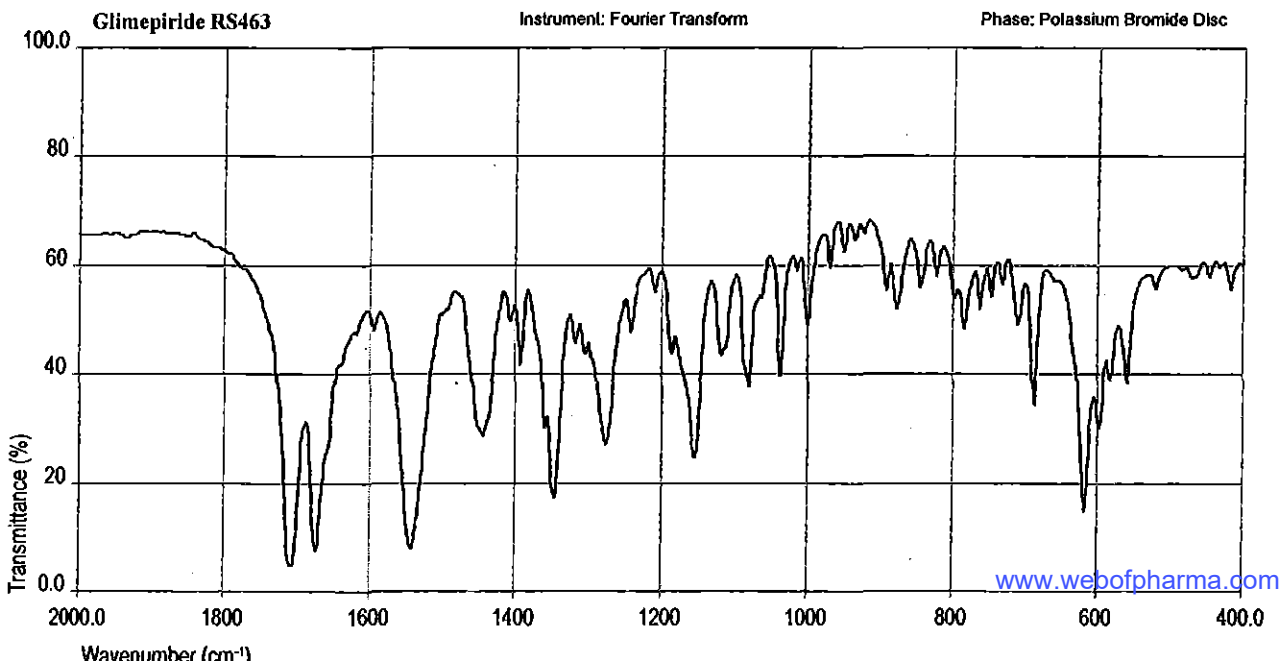








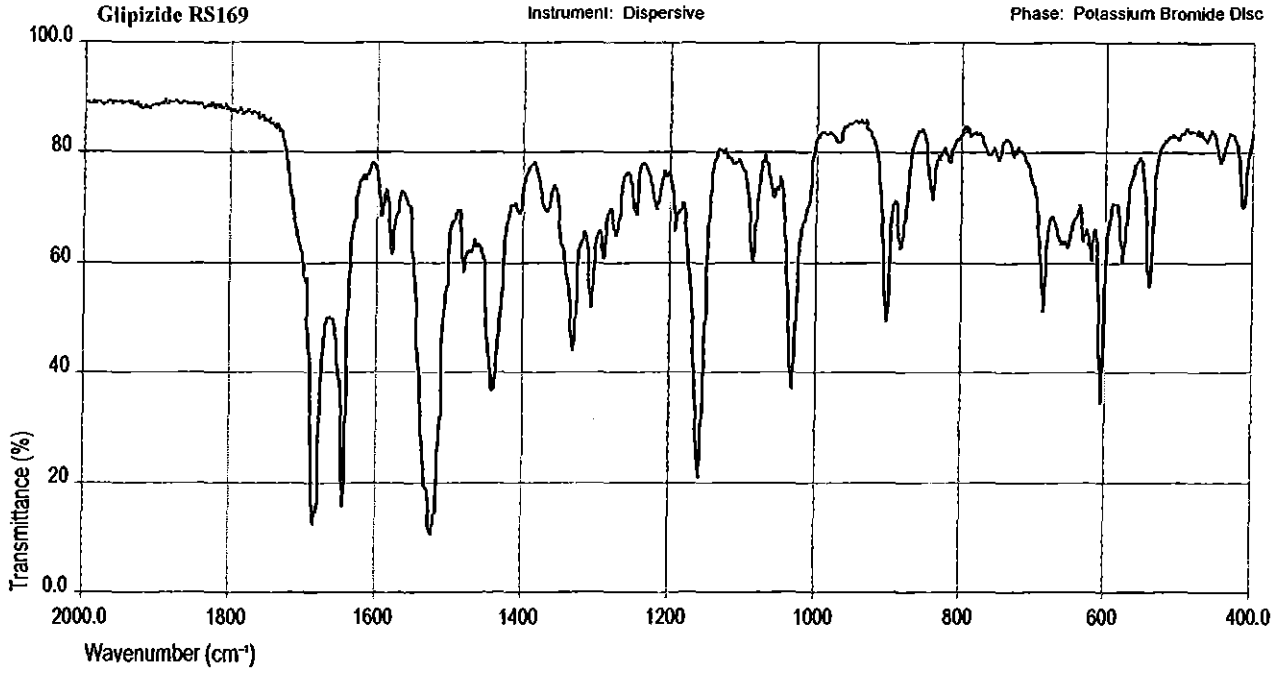




Glipizide RS169

Instrument: Dispersive

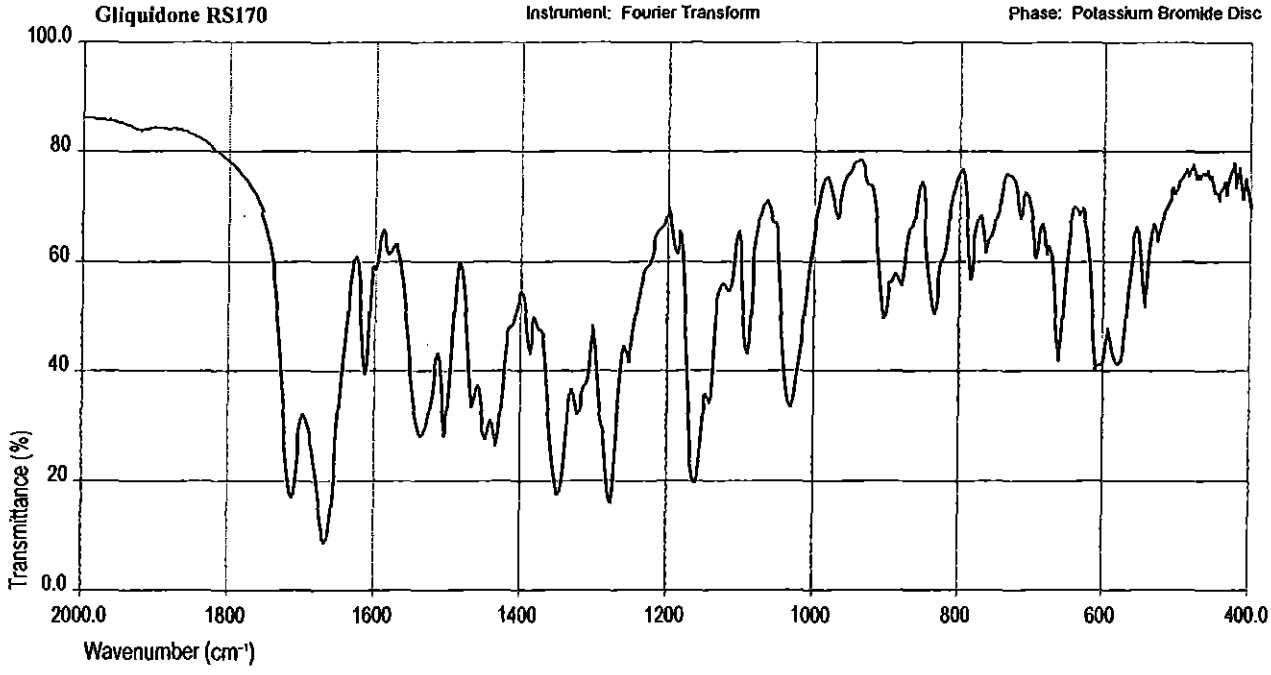
Phase: Potassium Bromide Disc



Gliquidone RS170

Instrument: Fourier Transform

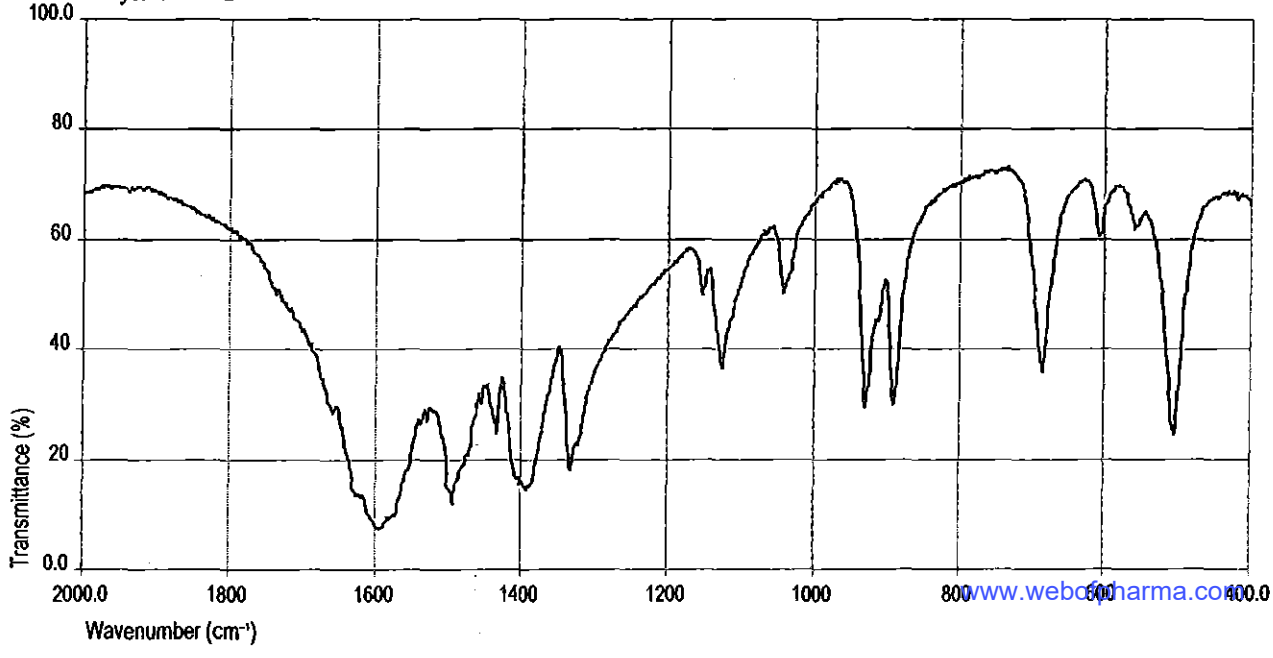
Phase: Potassium Bromide Disc

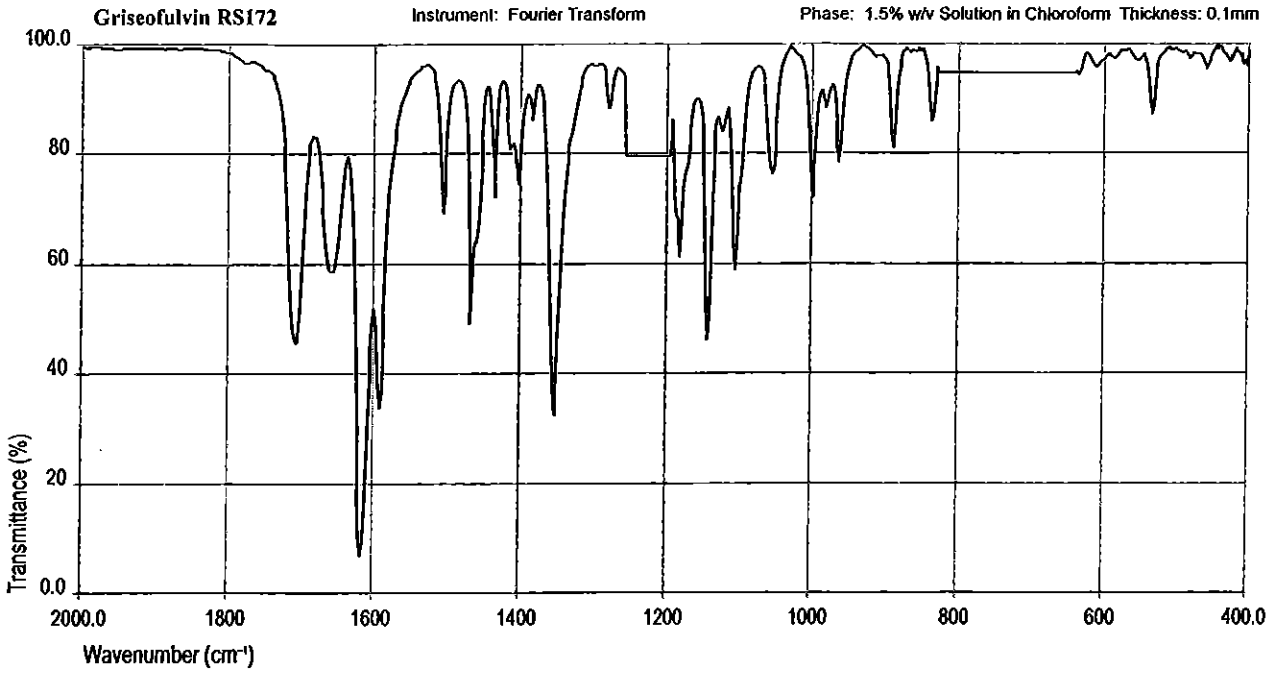


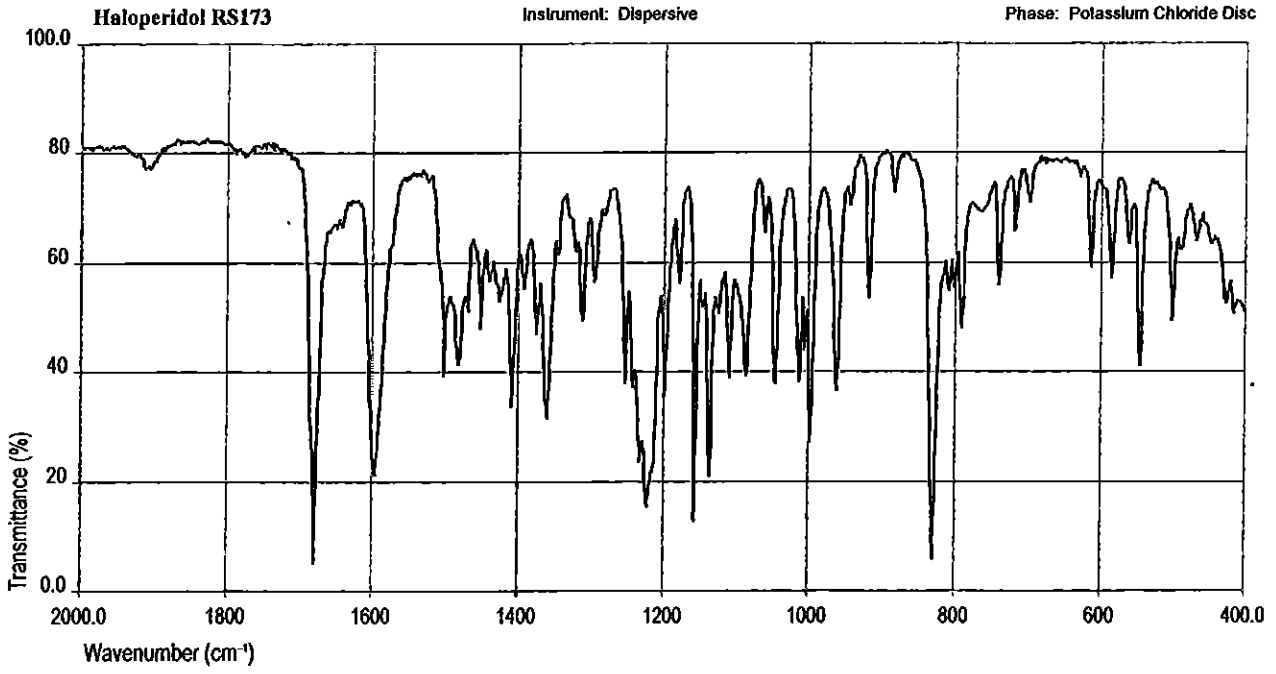
Glycine RS171

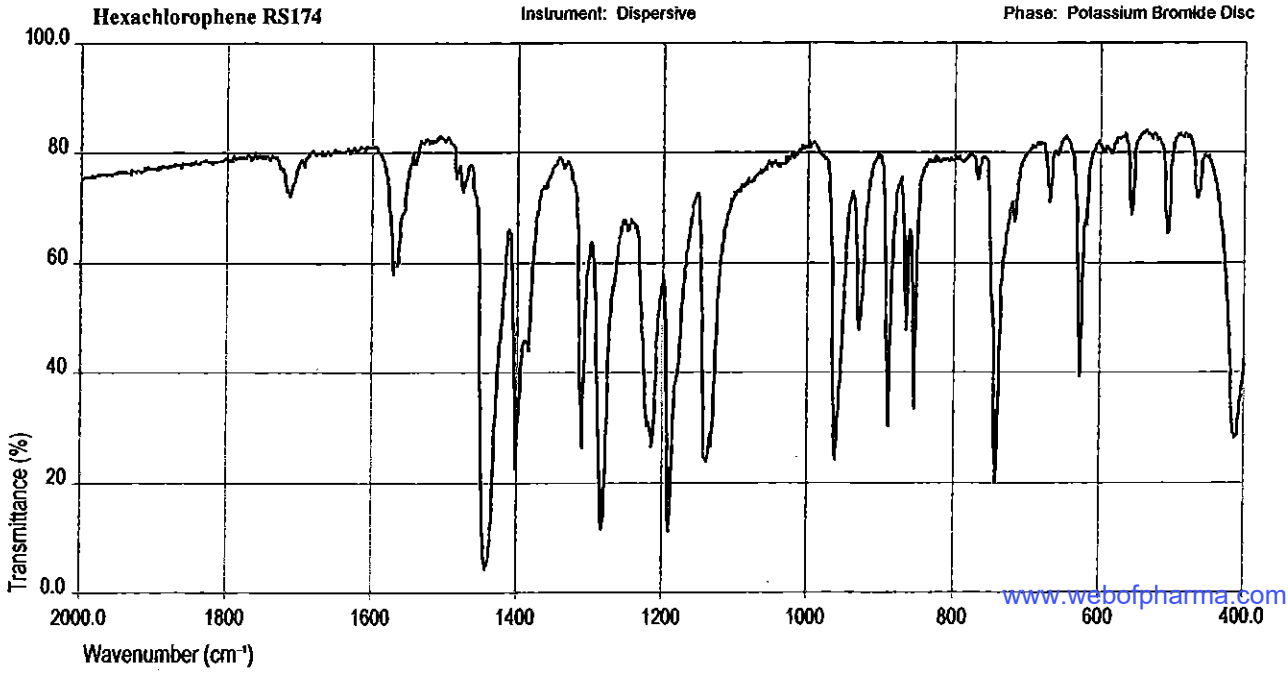
Instrument: Dispersive

Phase: Potassium Bromide Disc

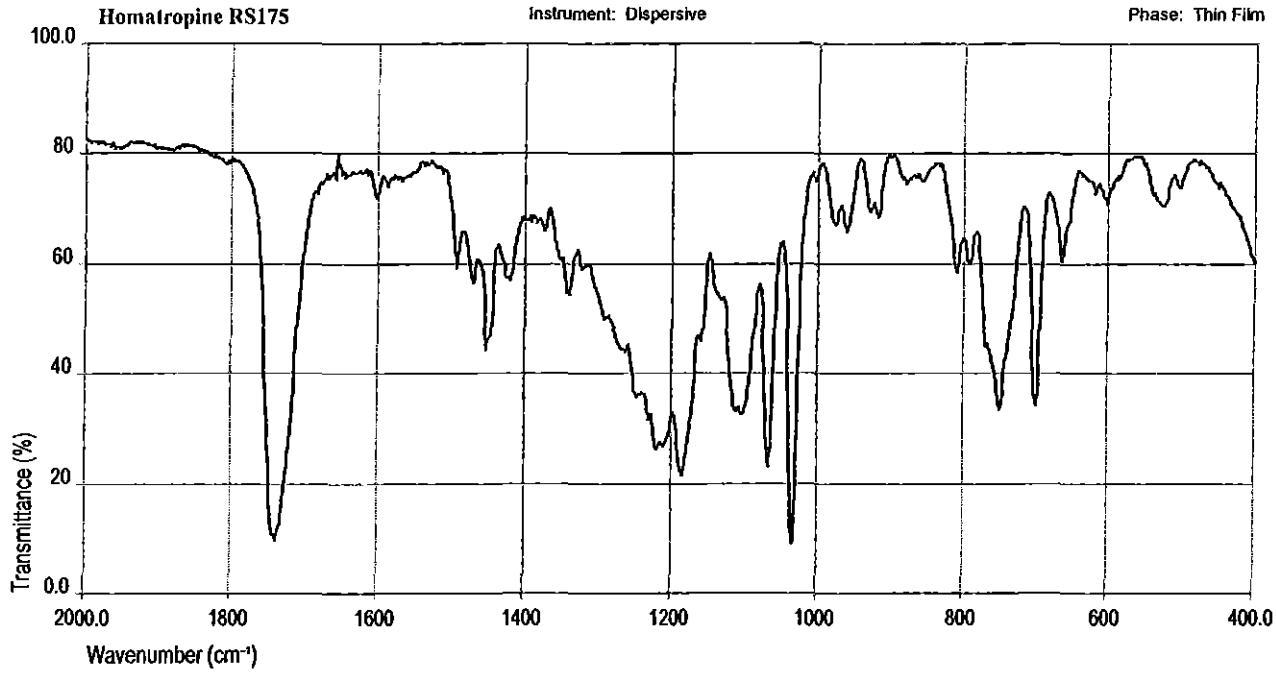




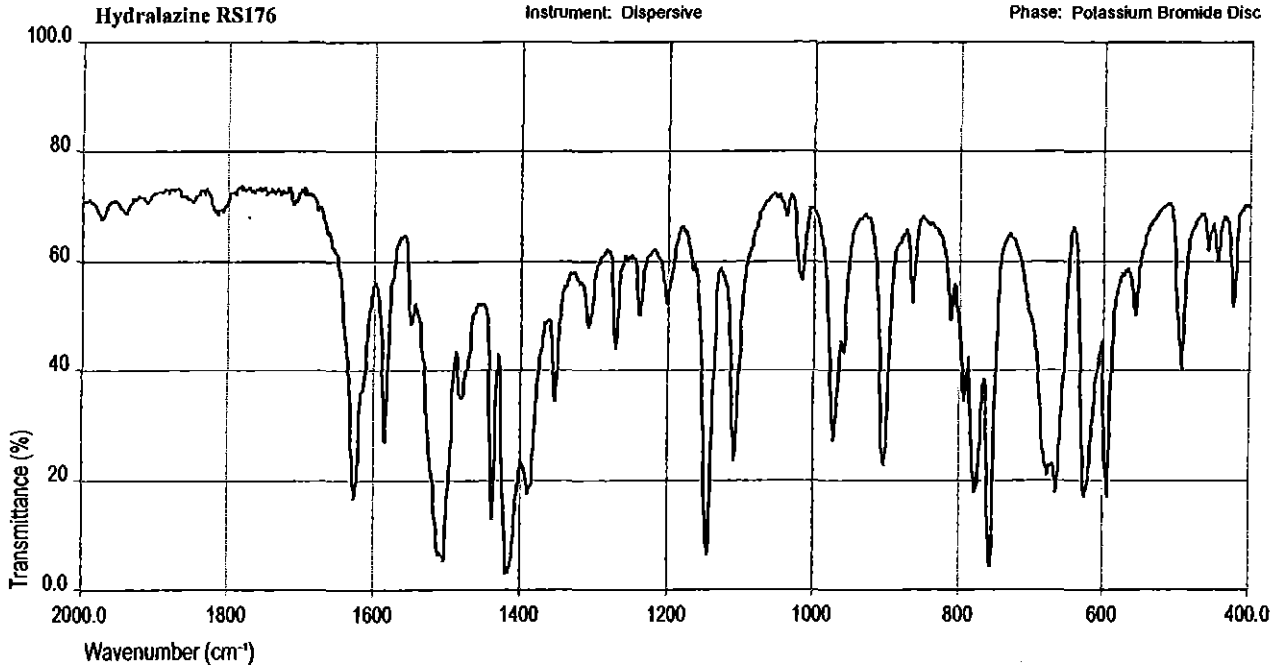




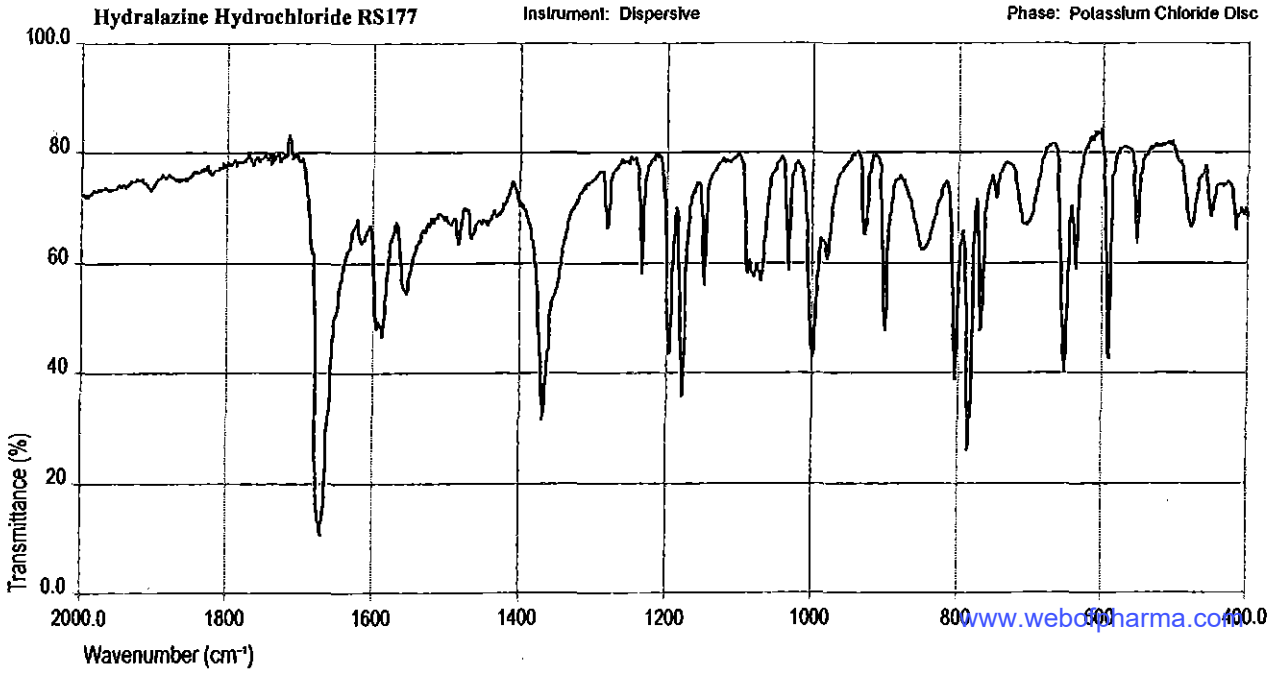
Homatropine RS175

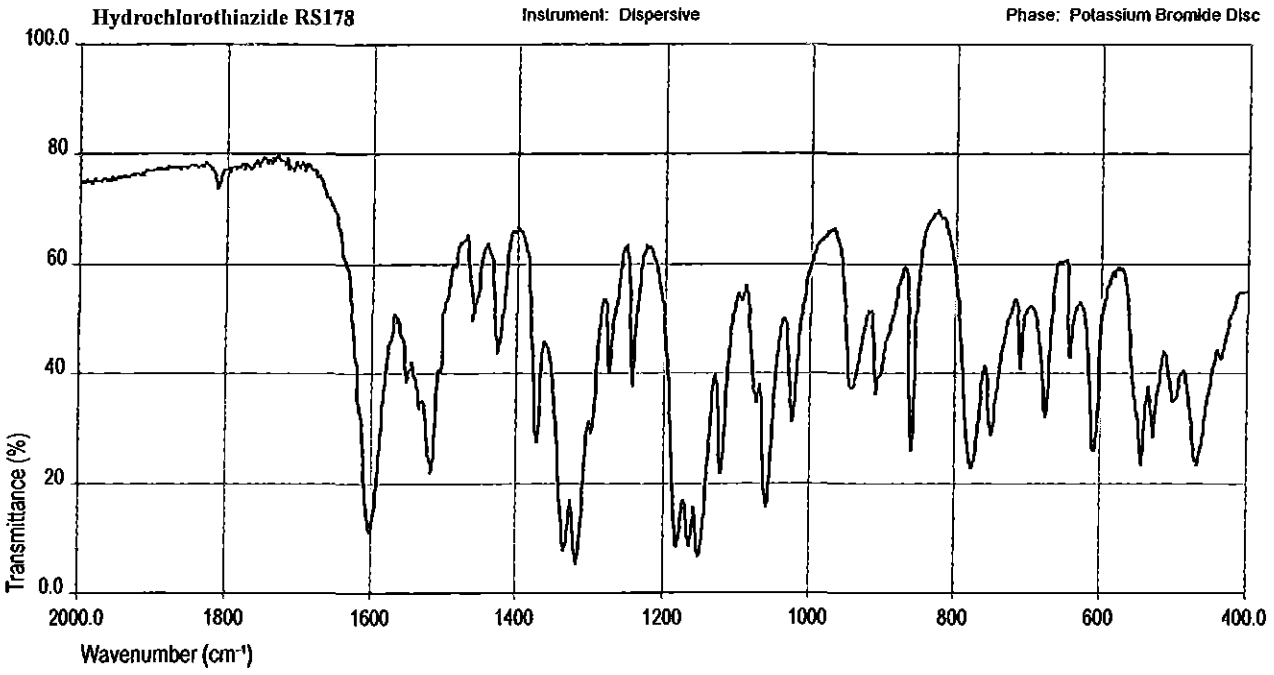


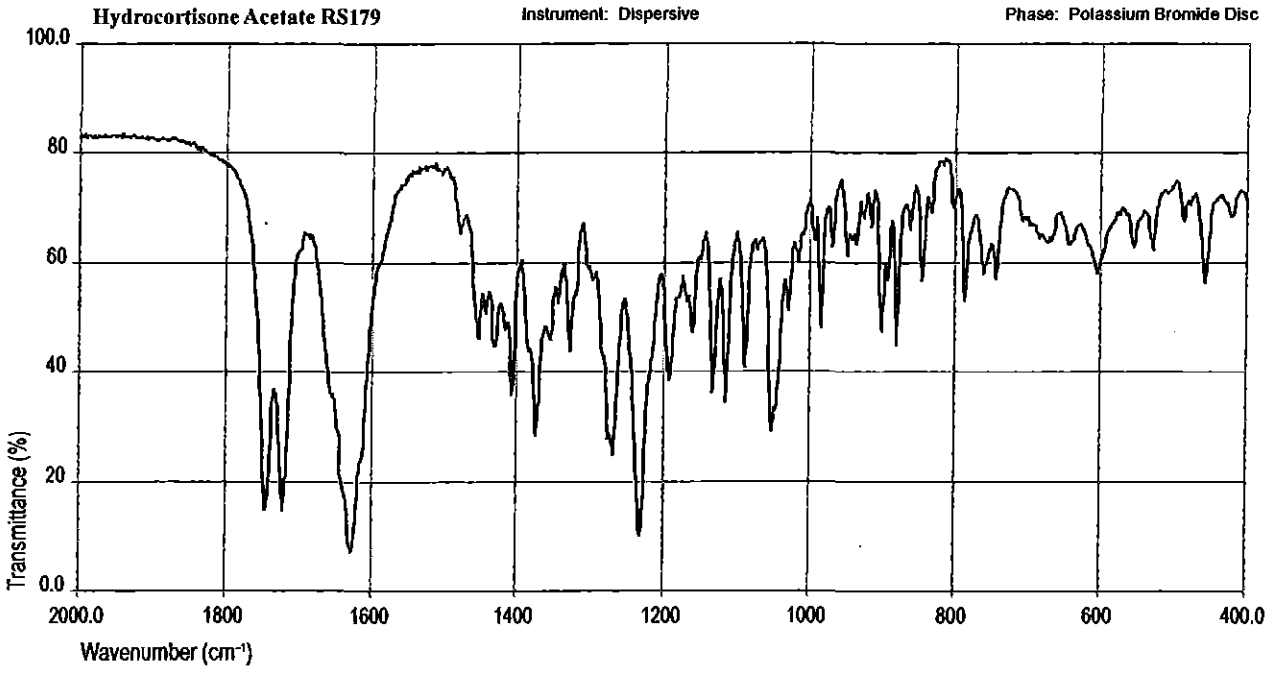
Hydralazine RS176

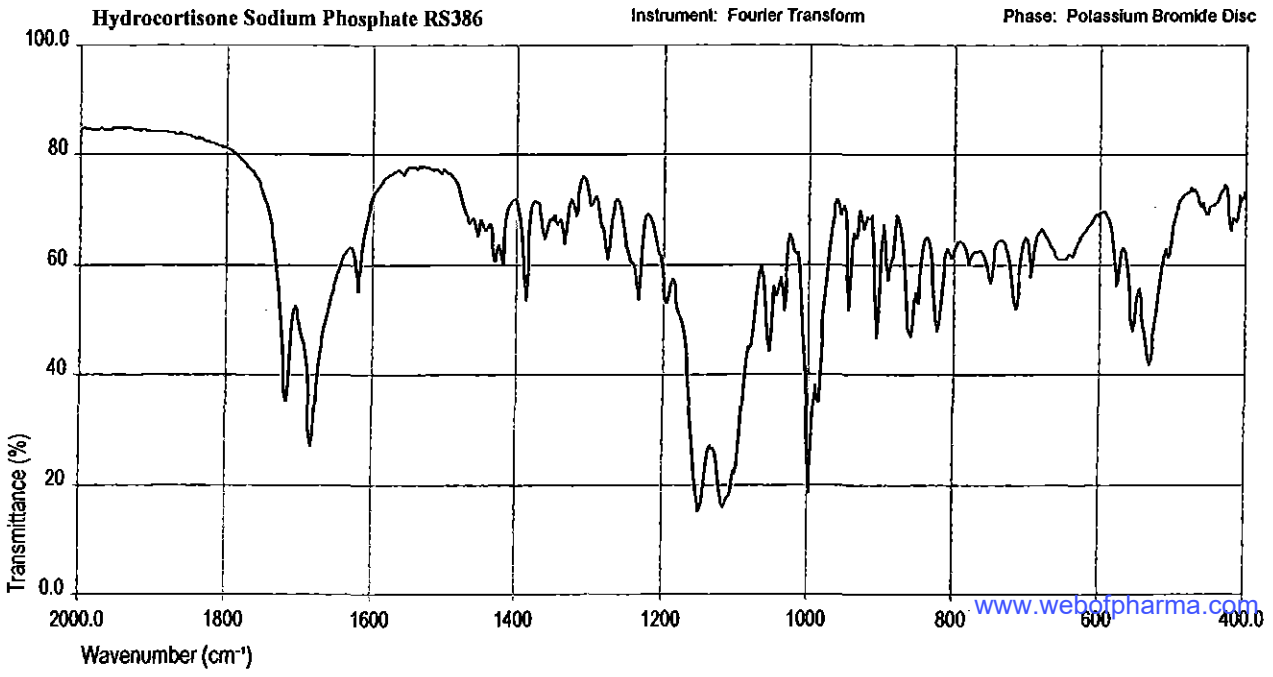


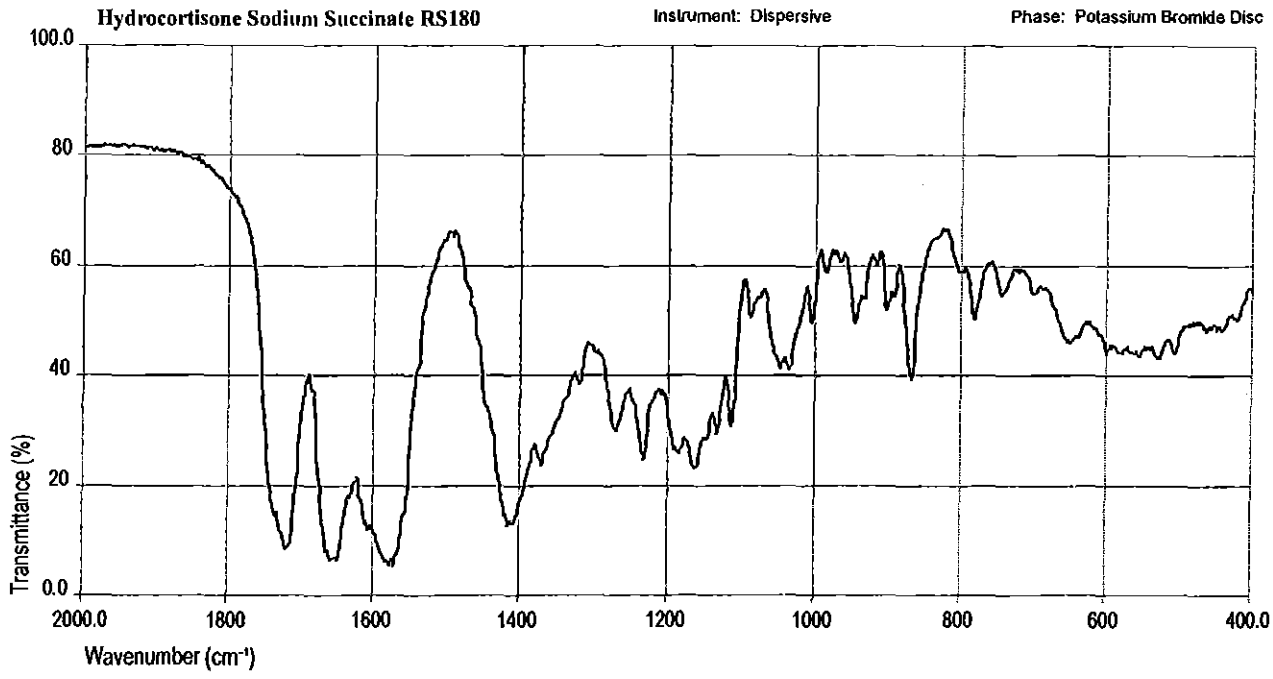
Hydralazine Hydrochloride RS177

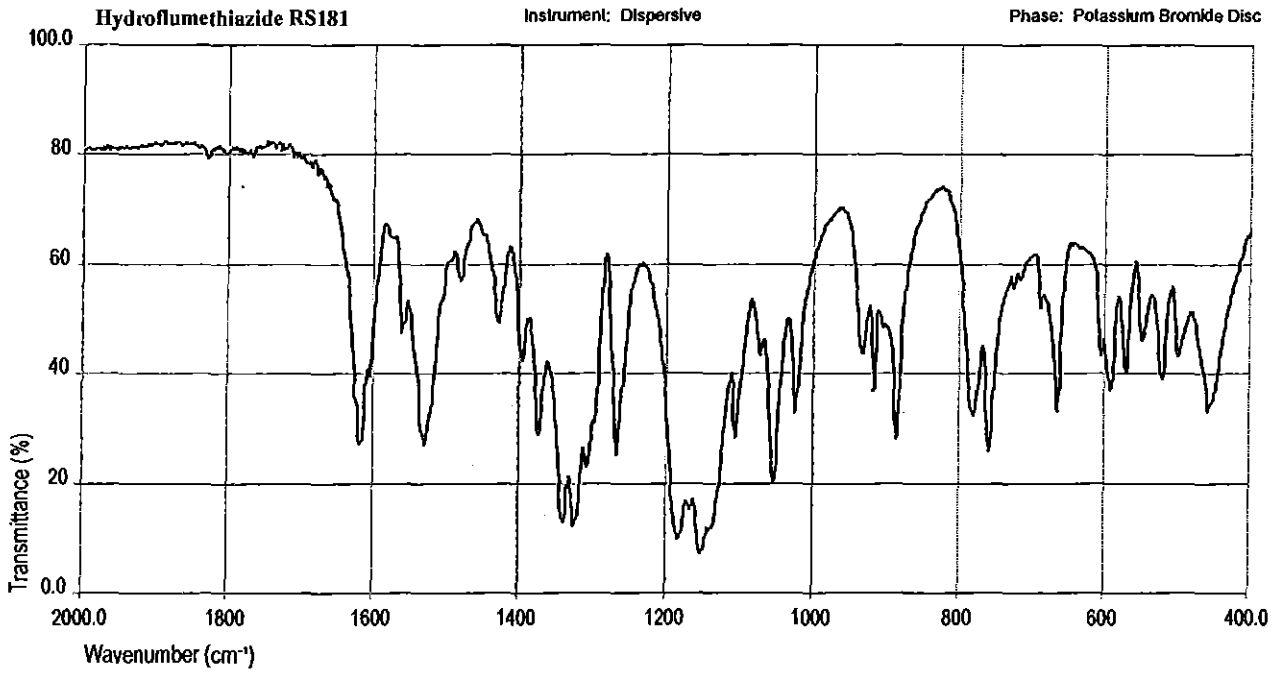


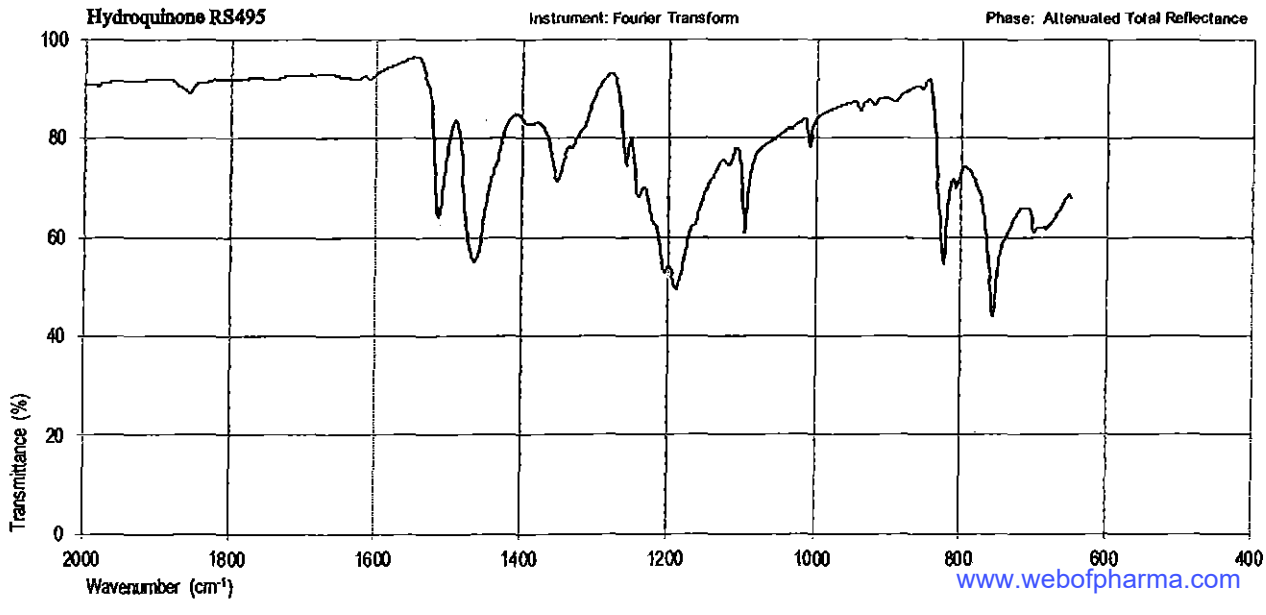


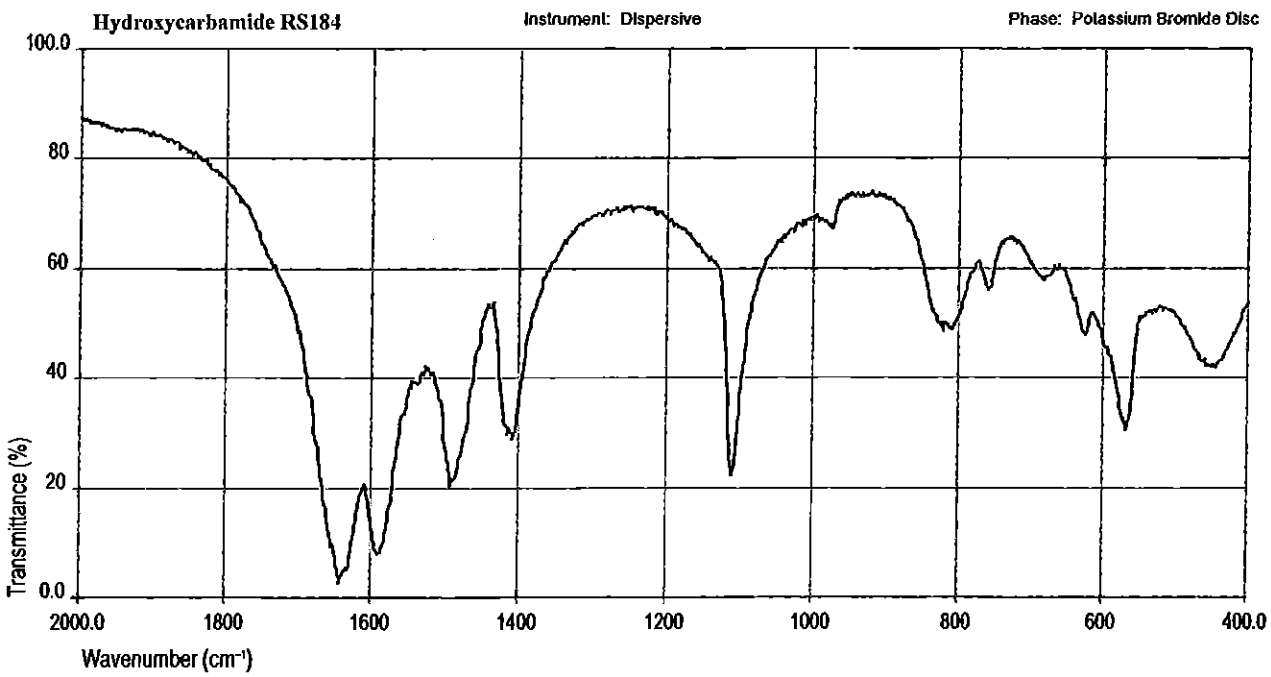


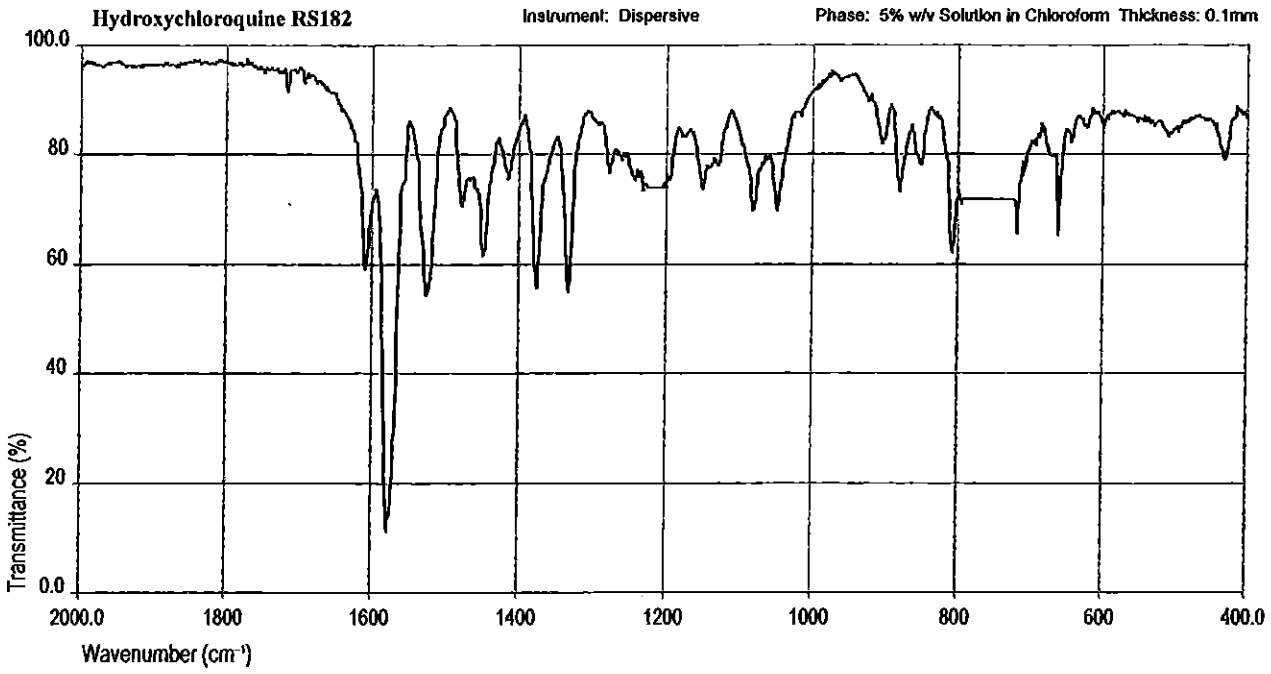


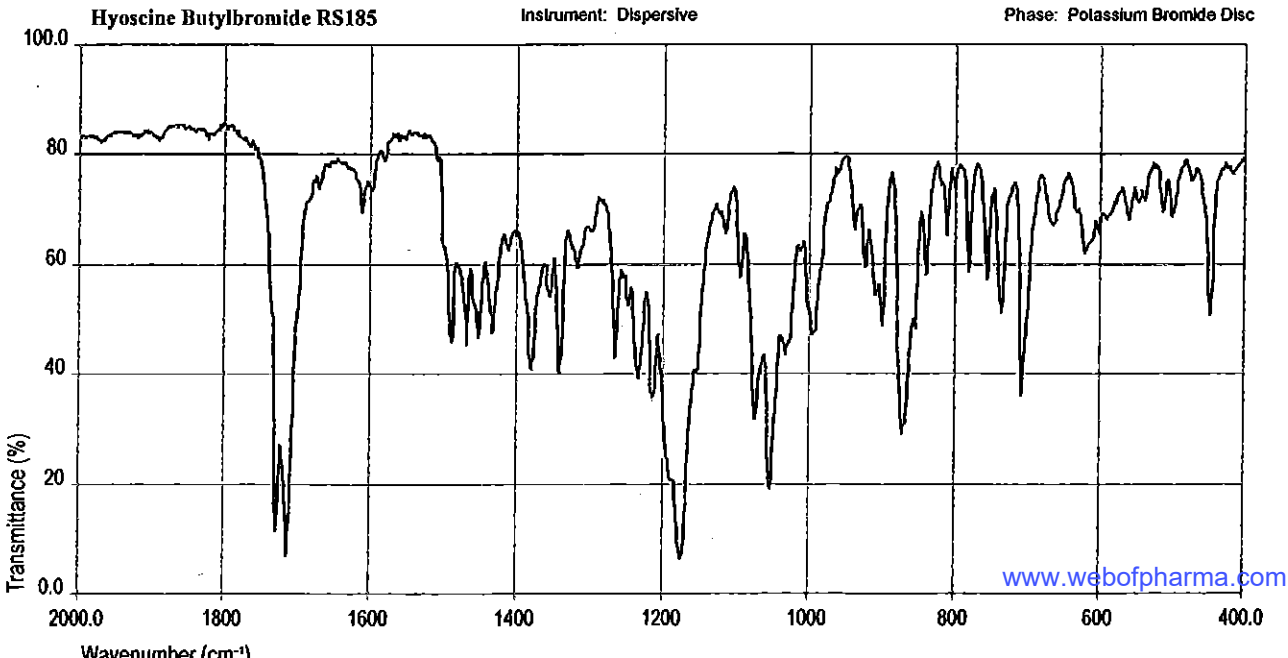


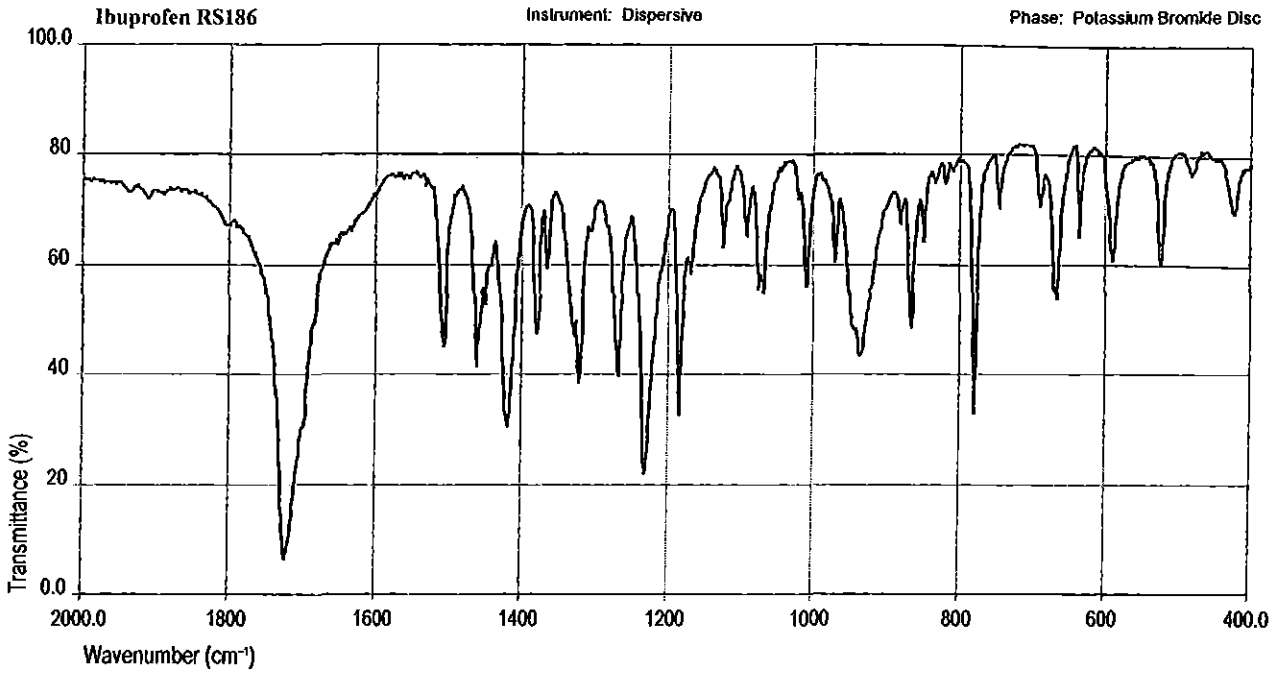


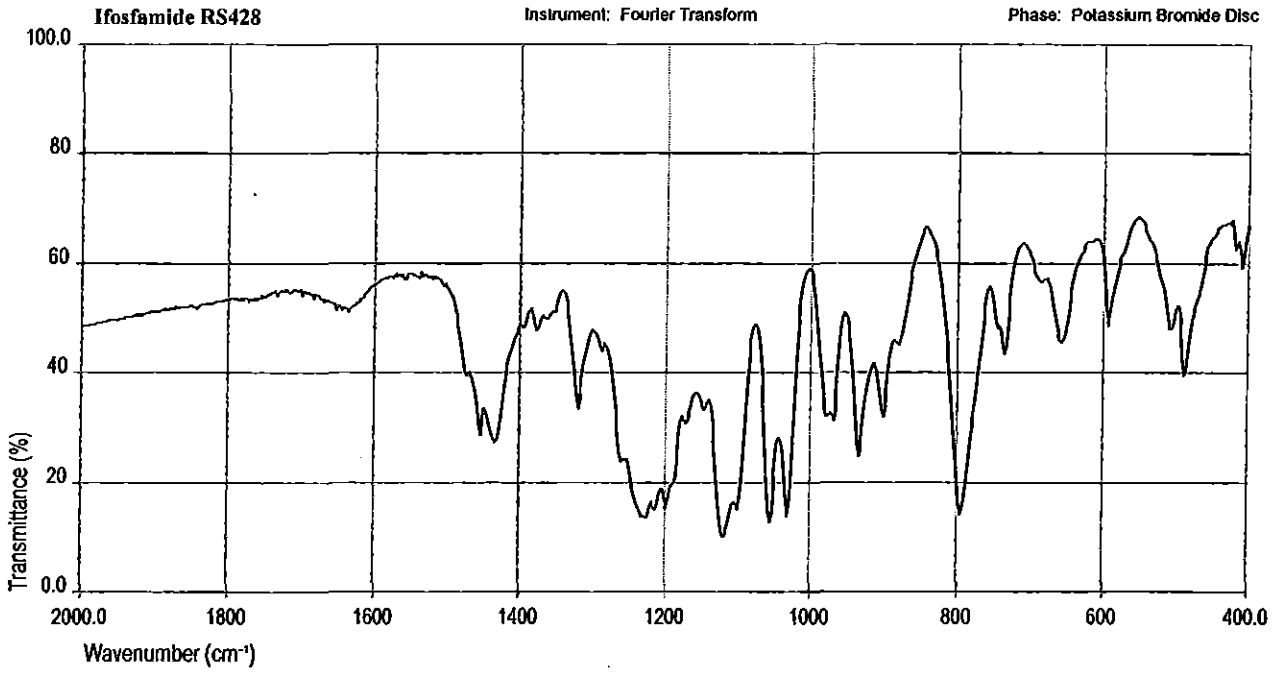


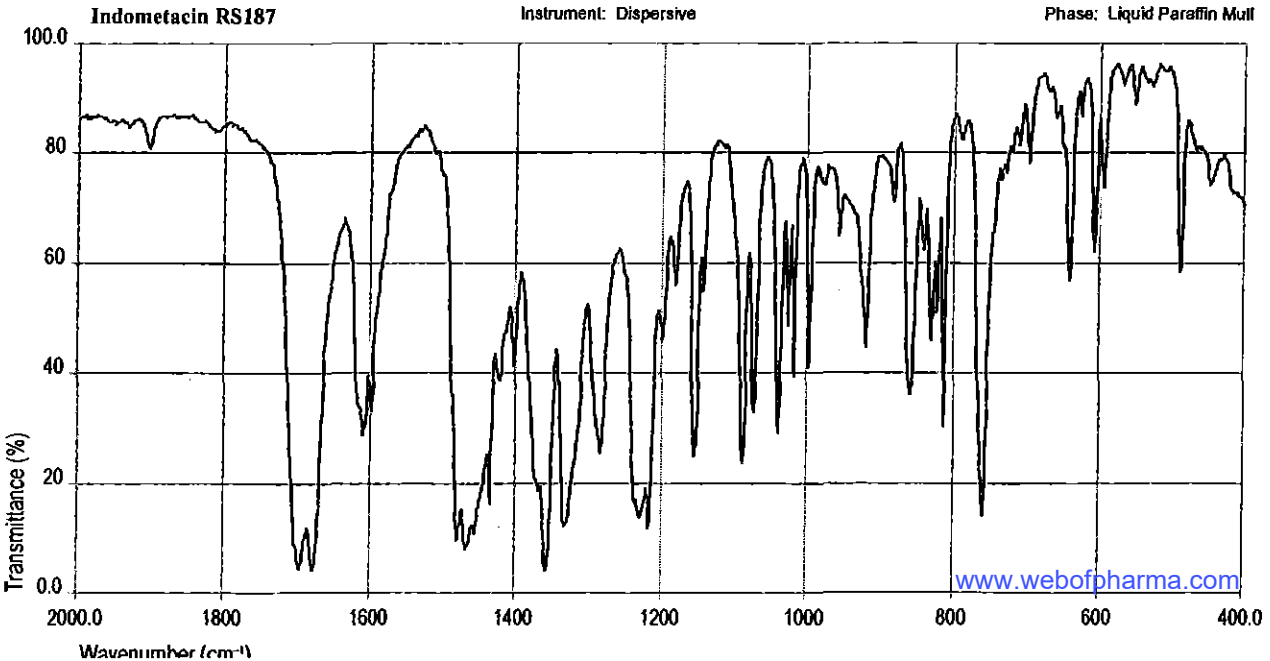


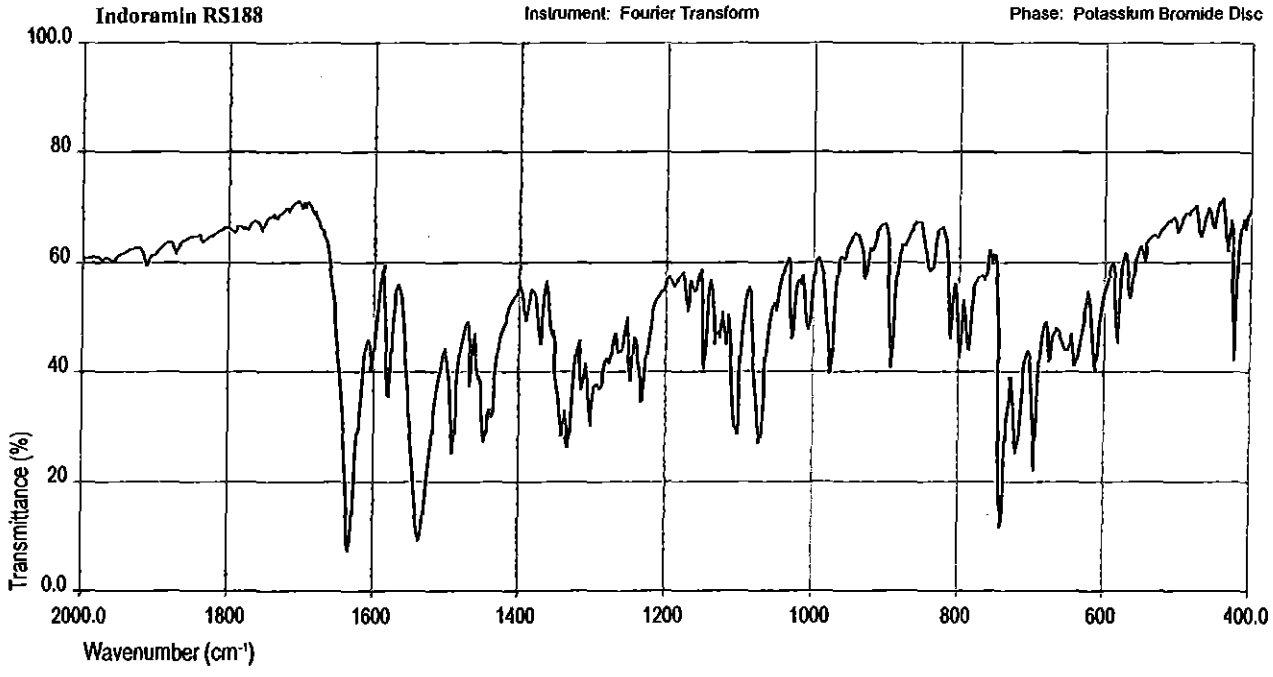


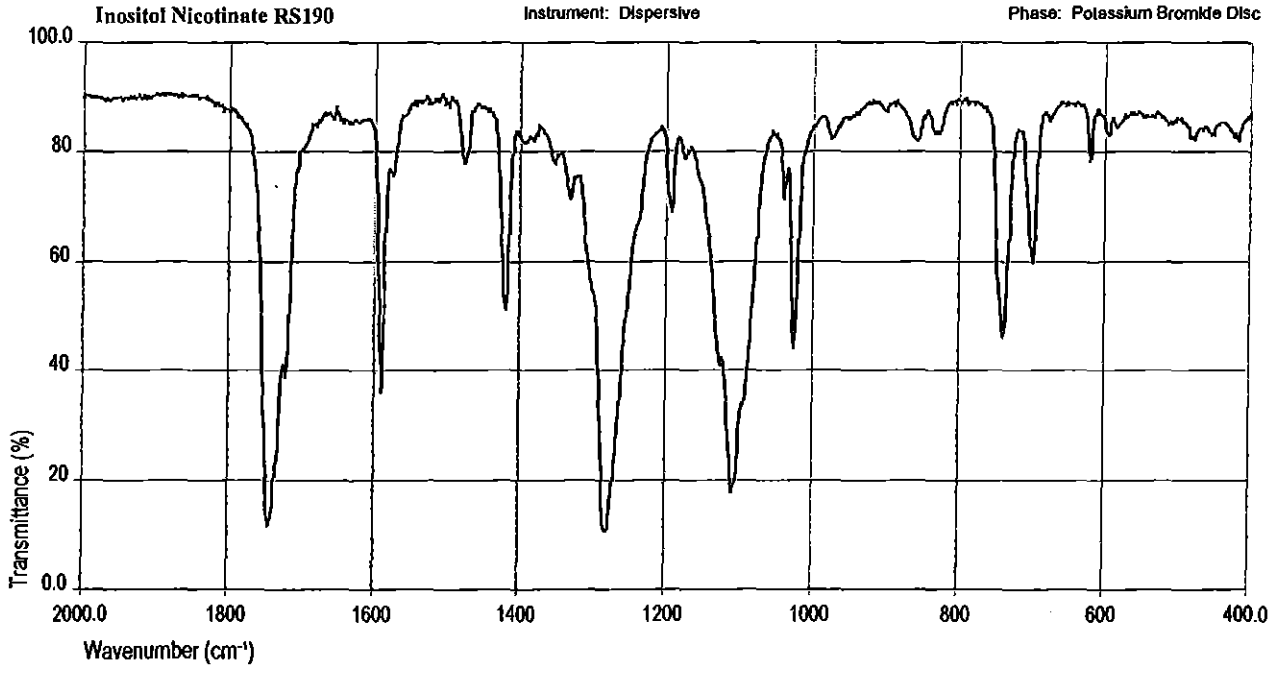


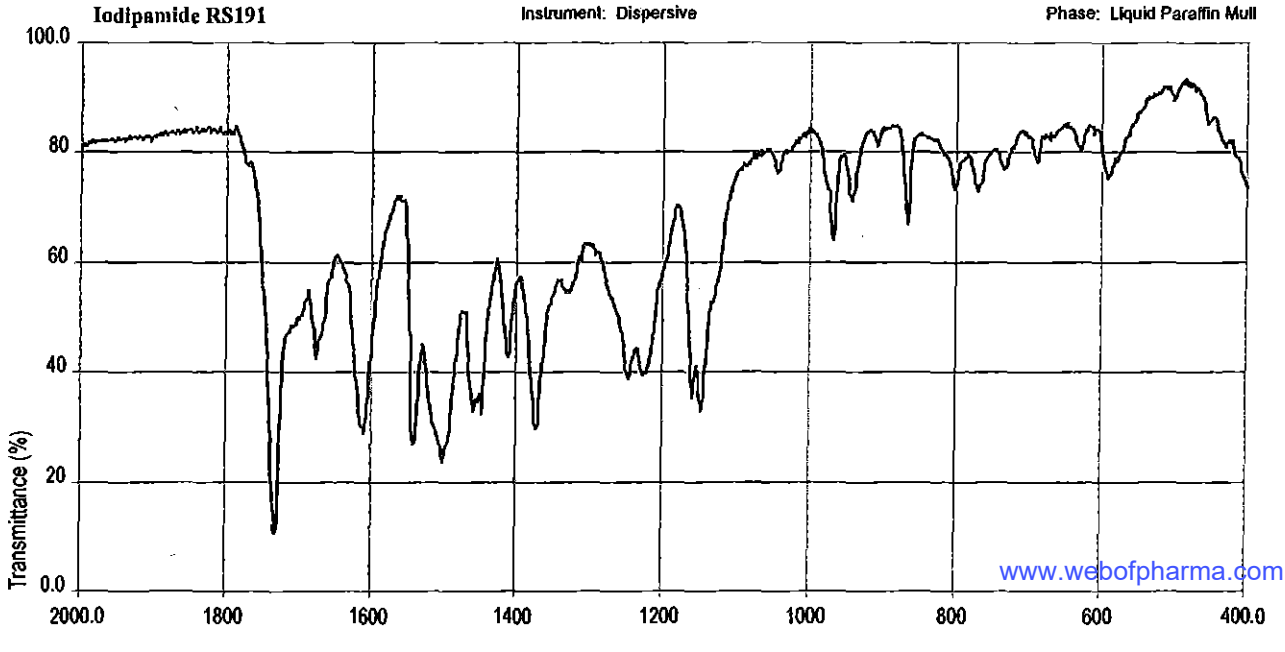


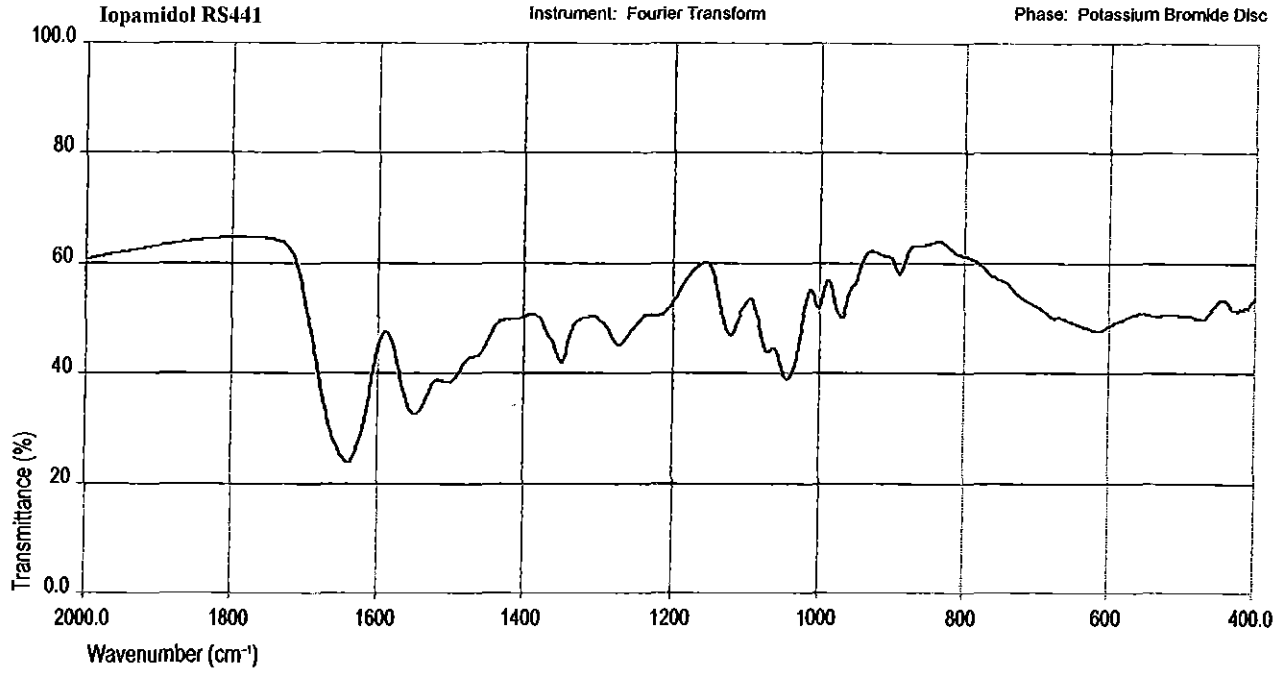


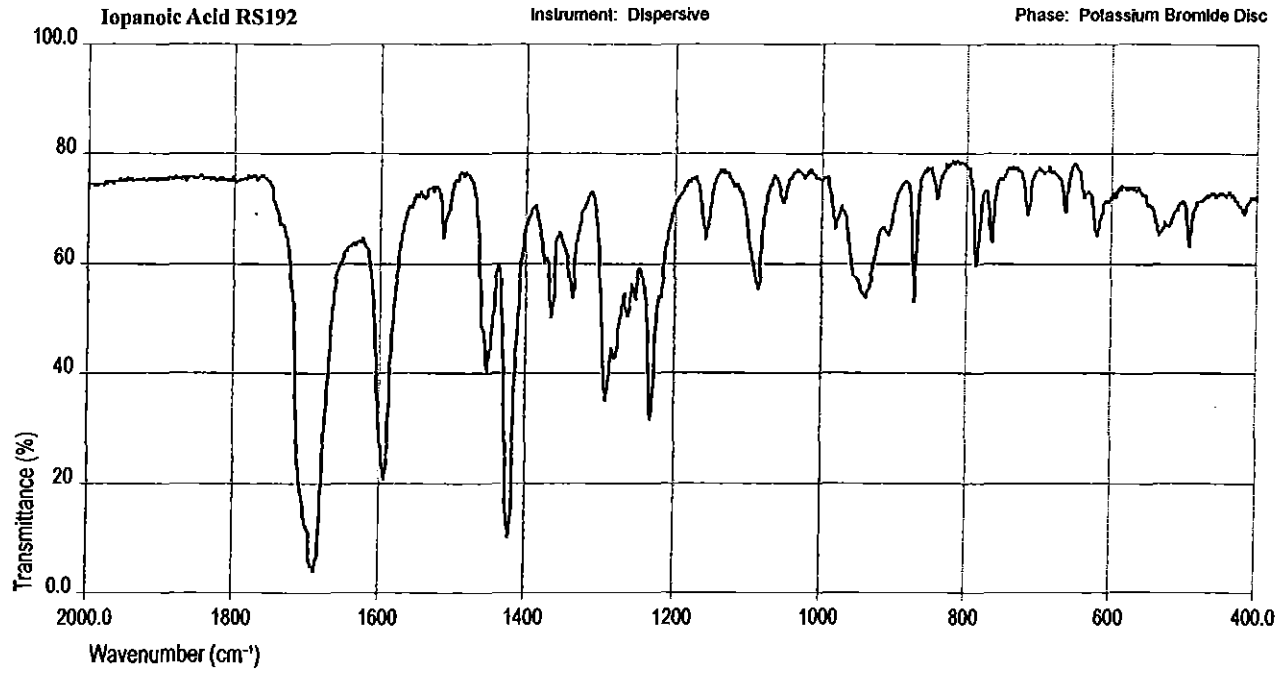


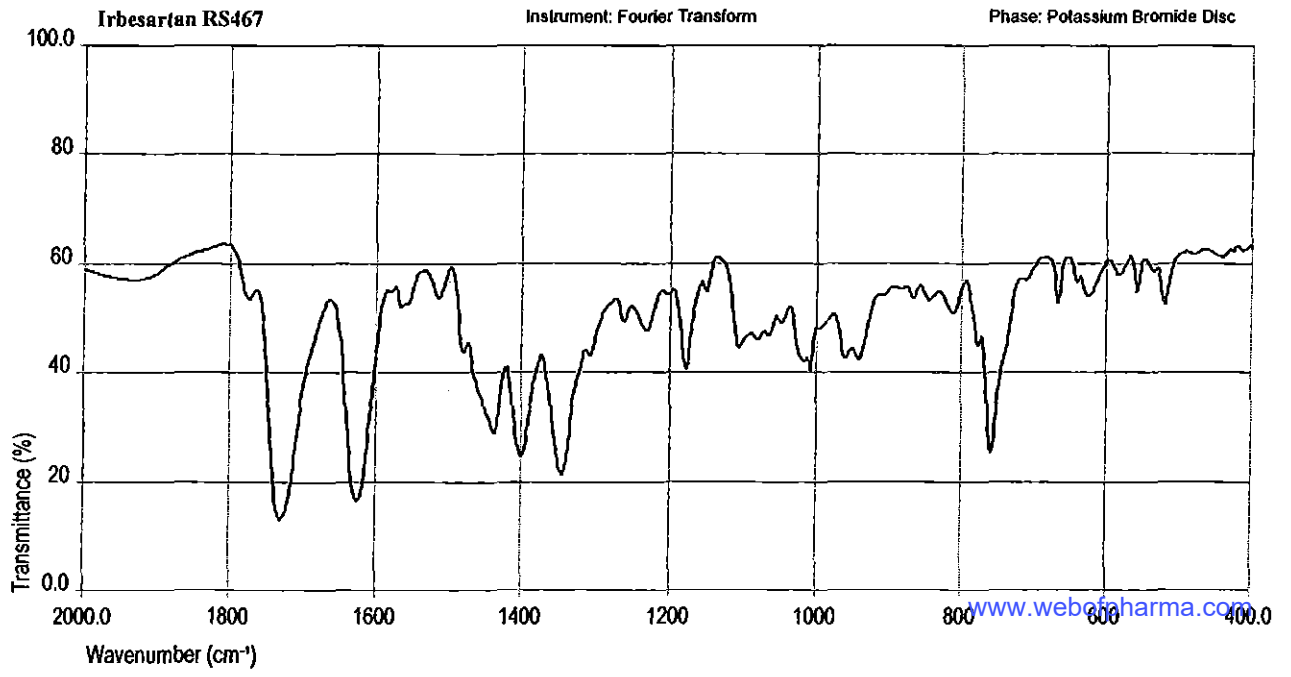


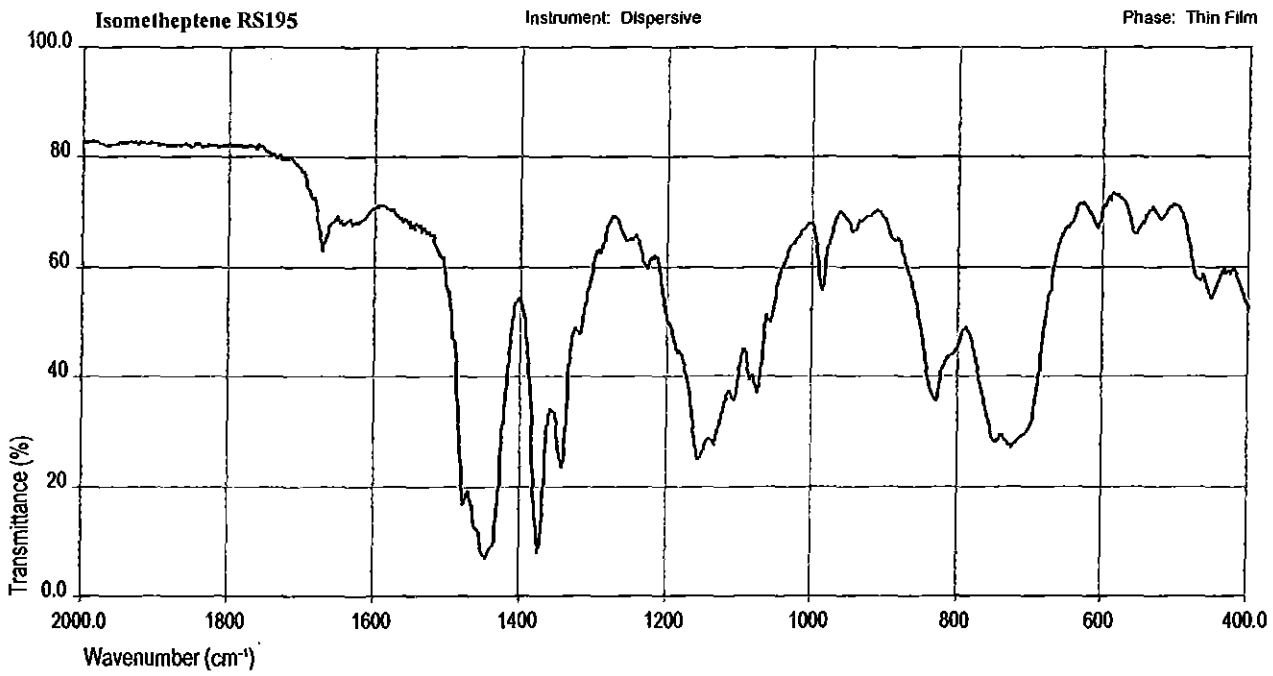


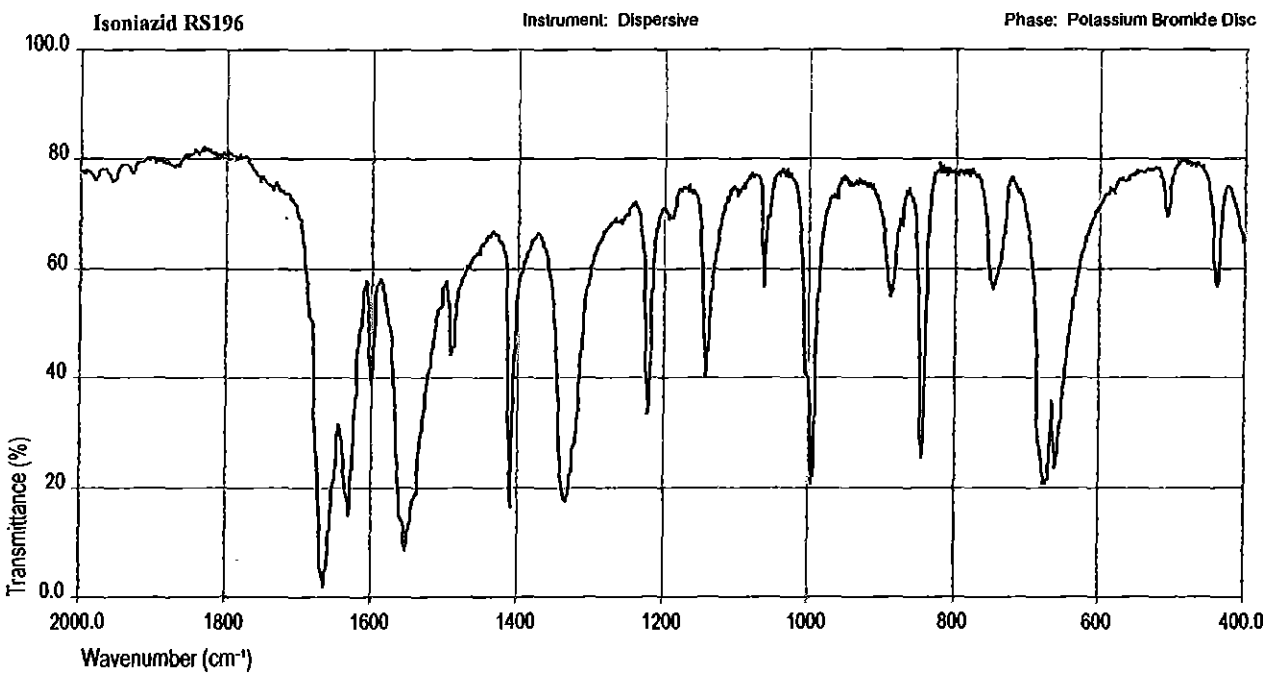


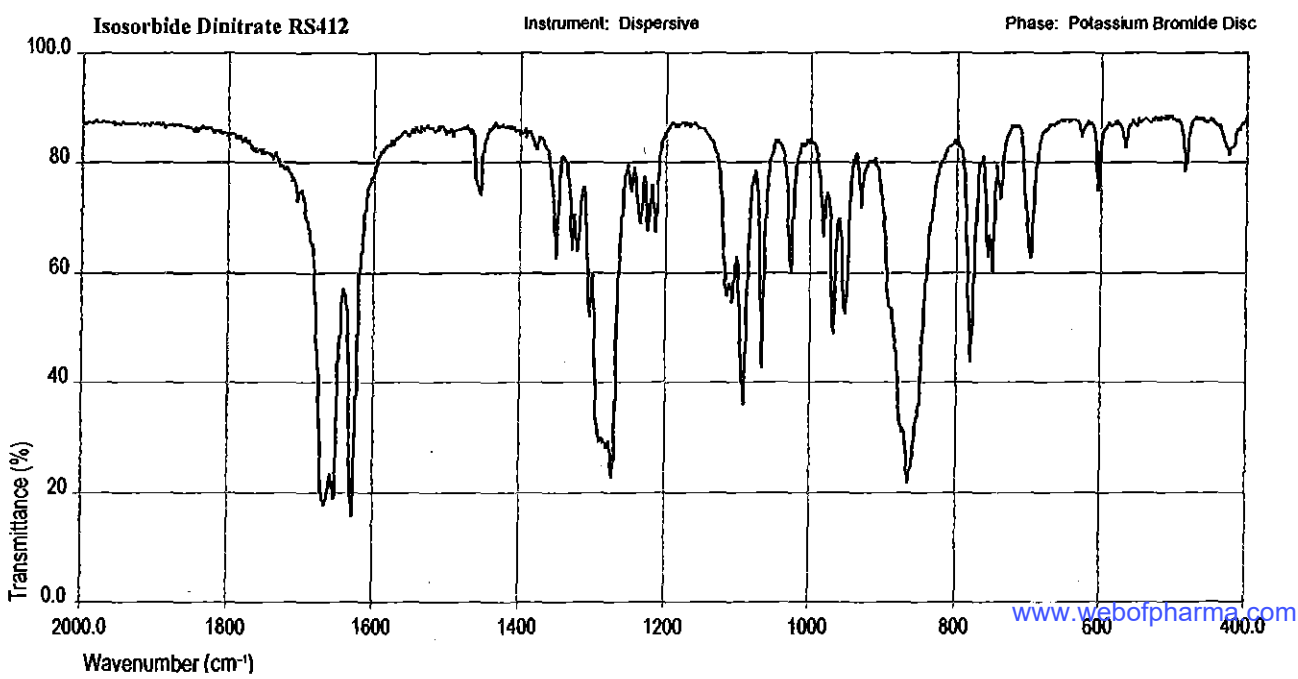


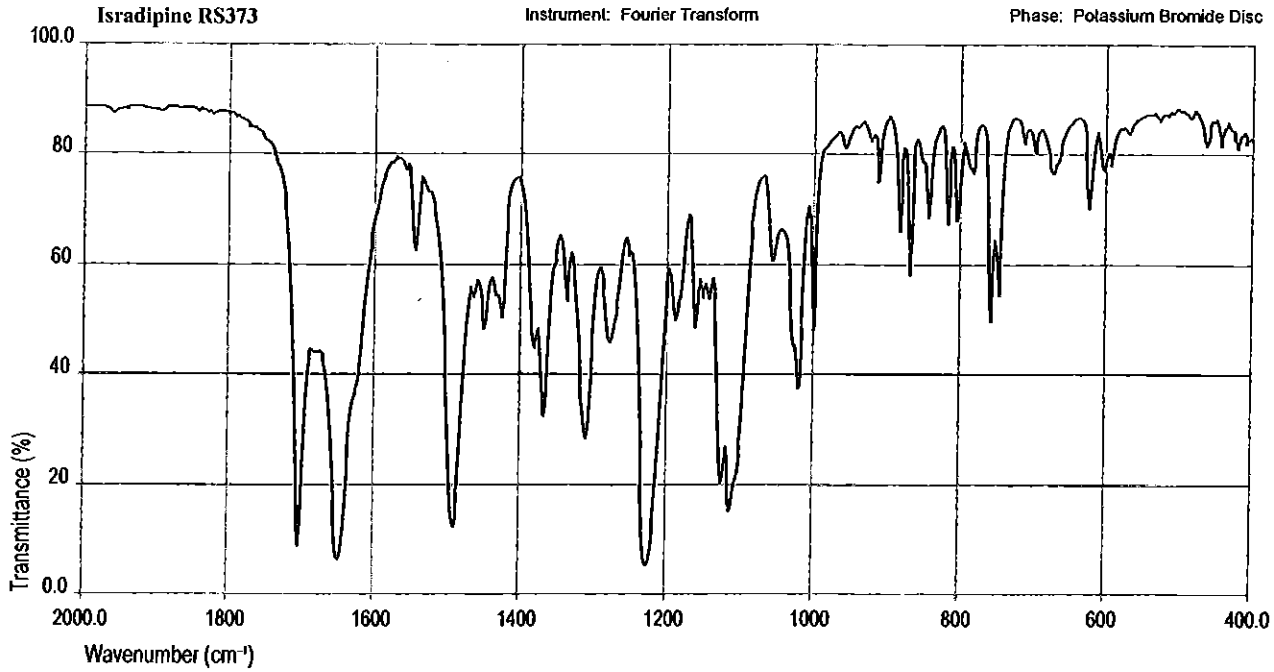


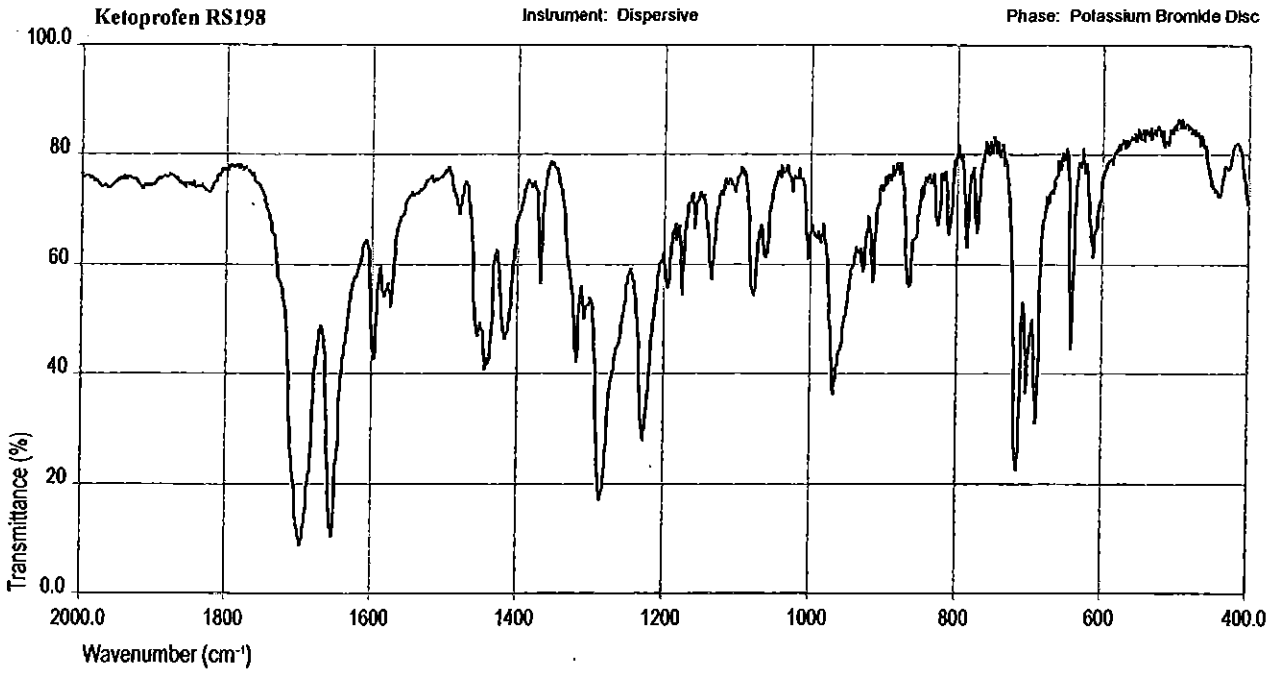


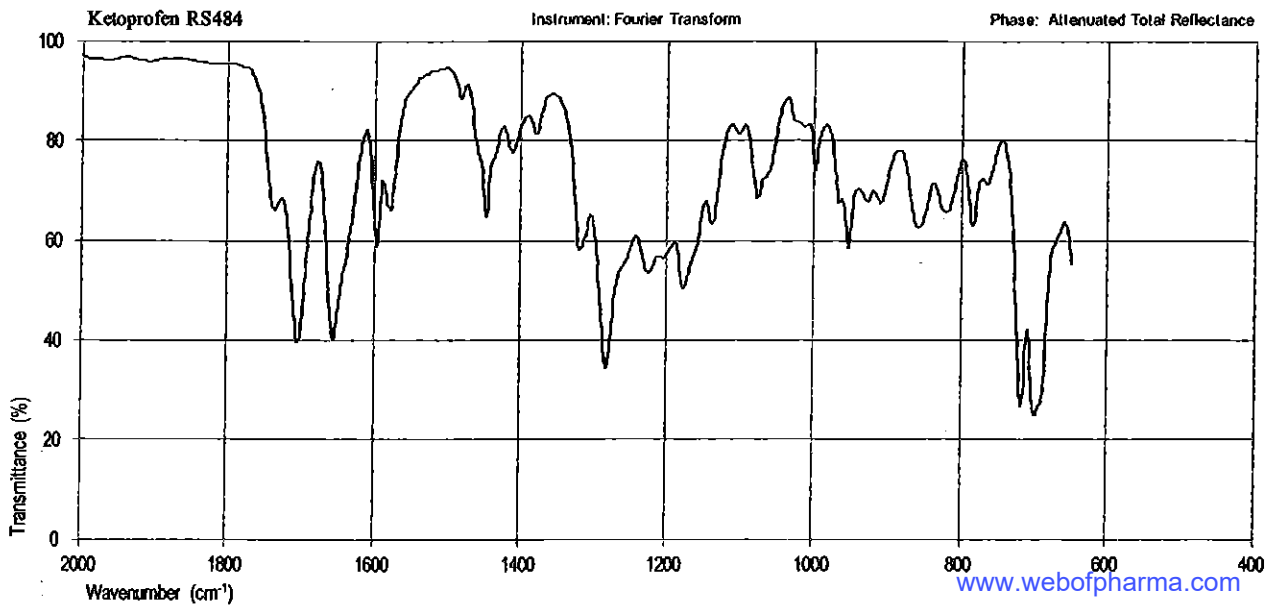


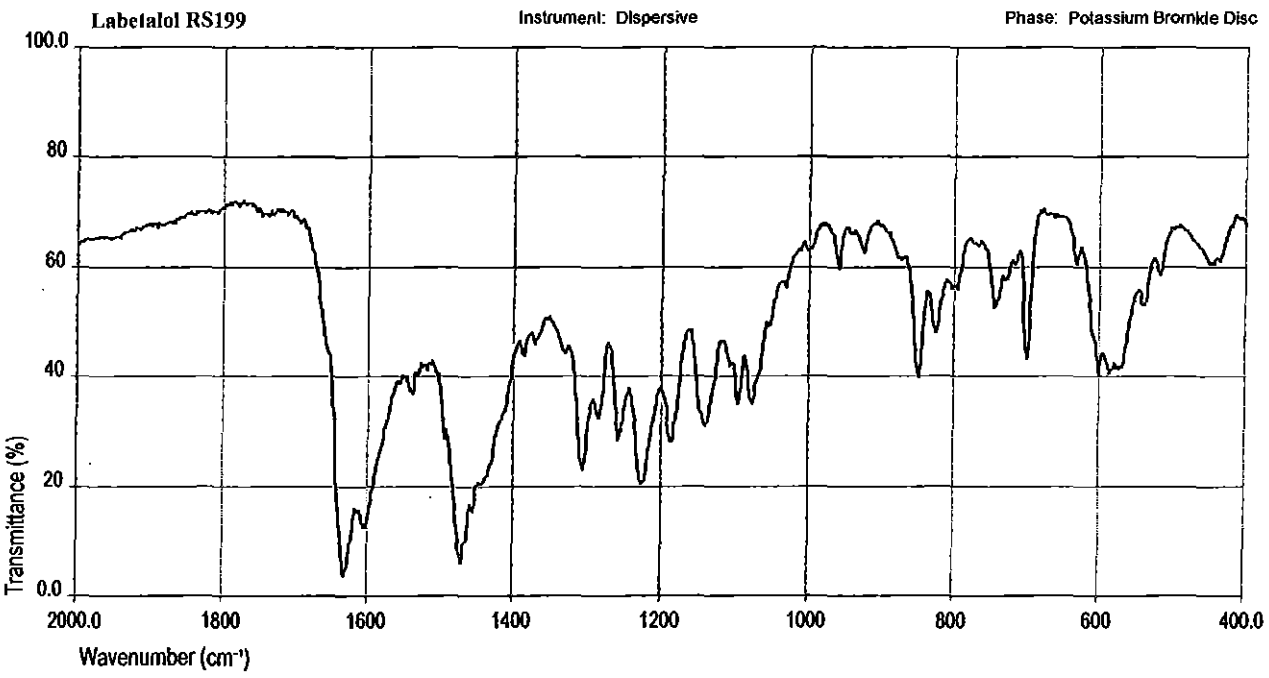


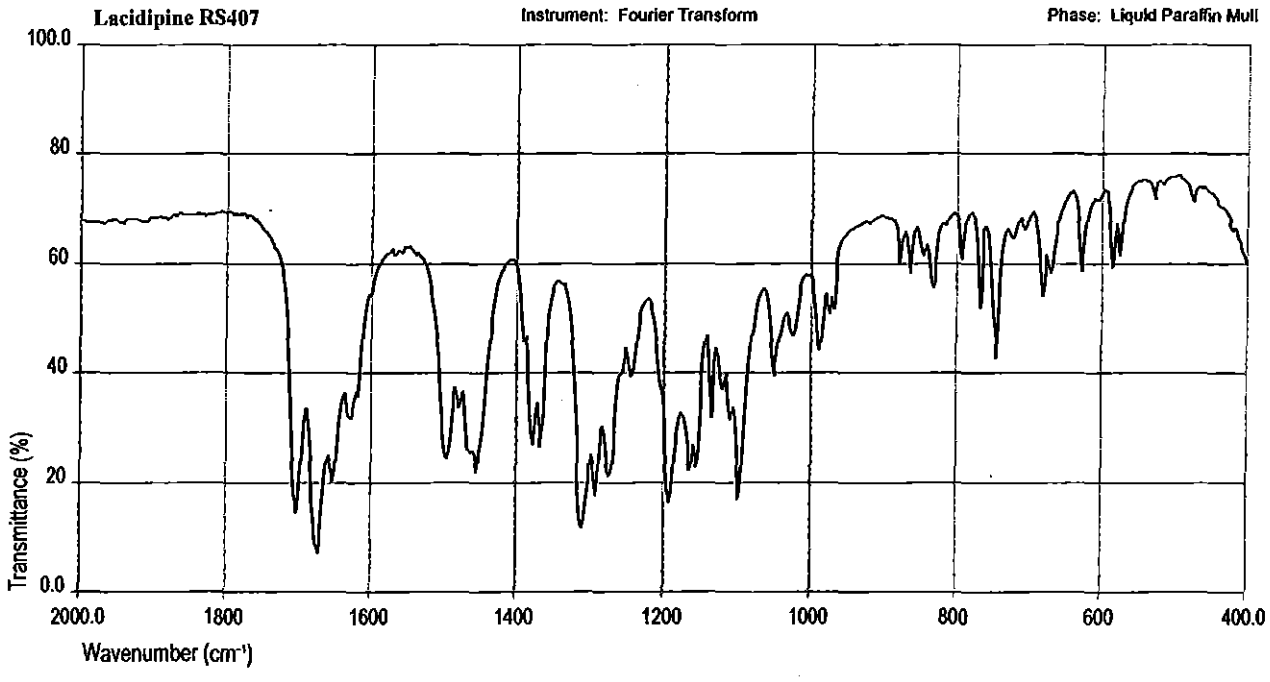


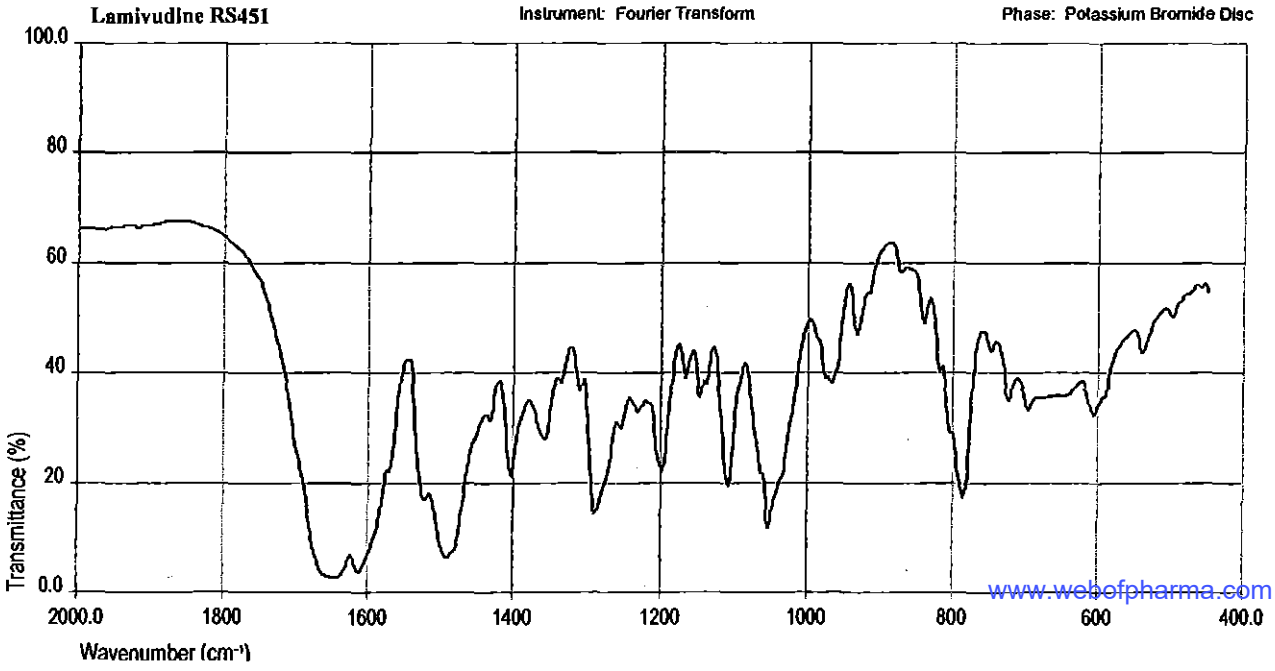


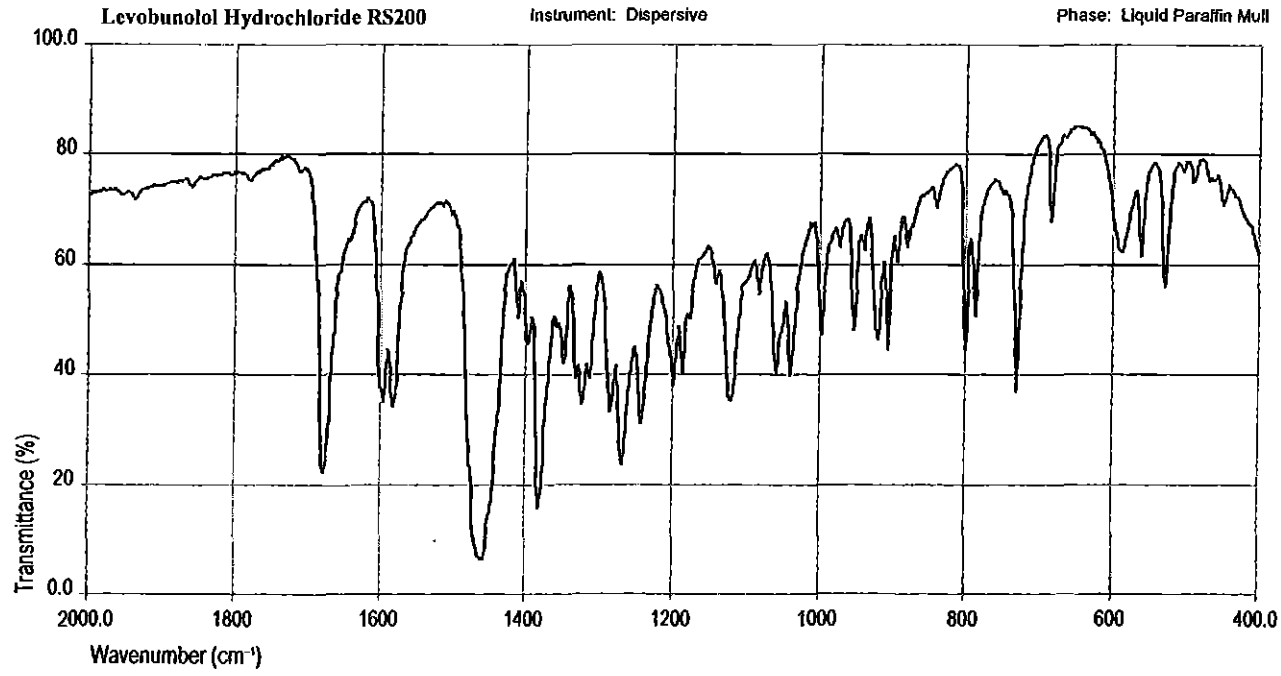


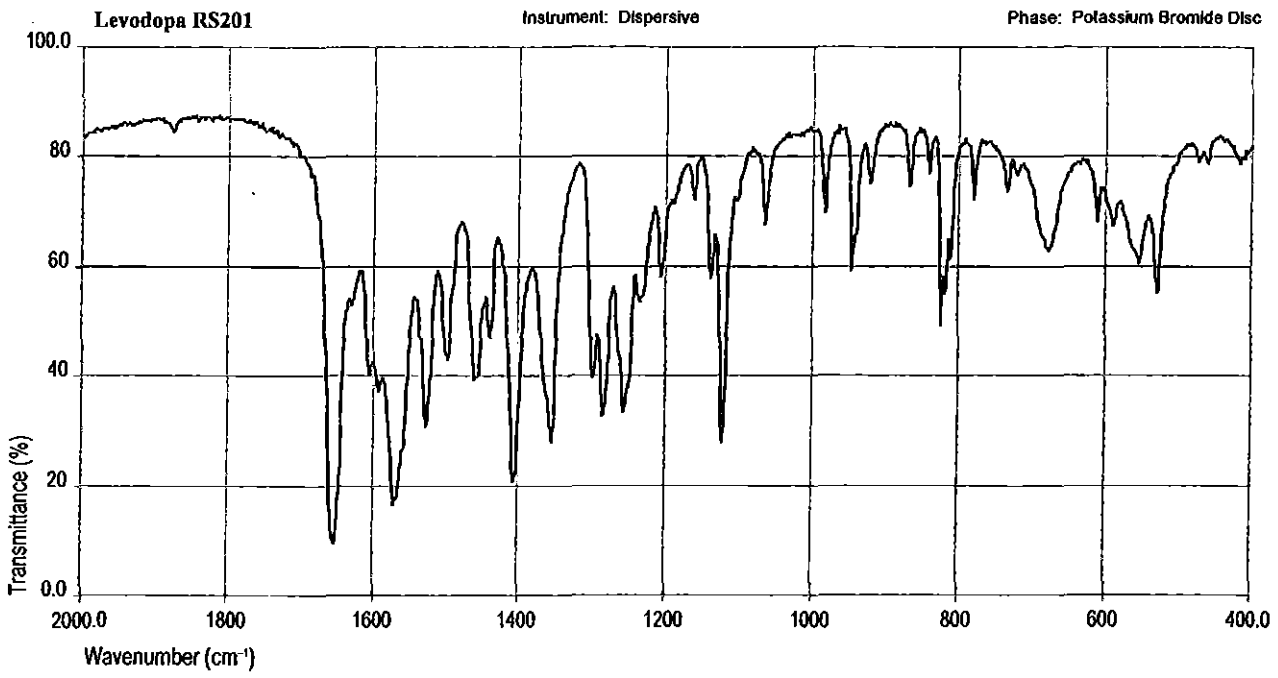


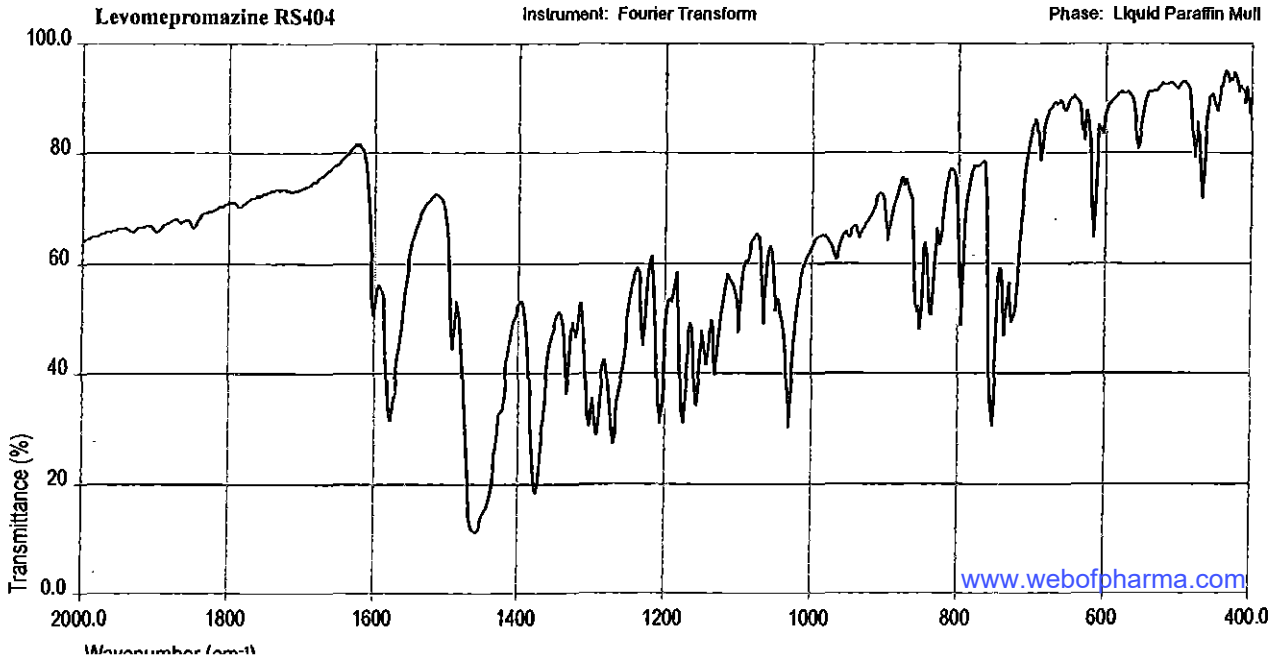


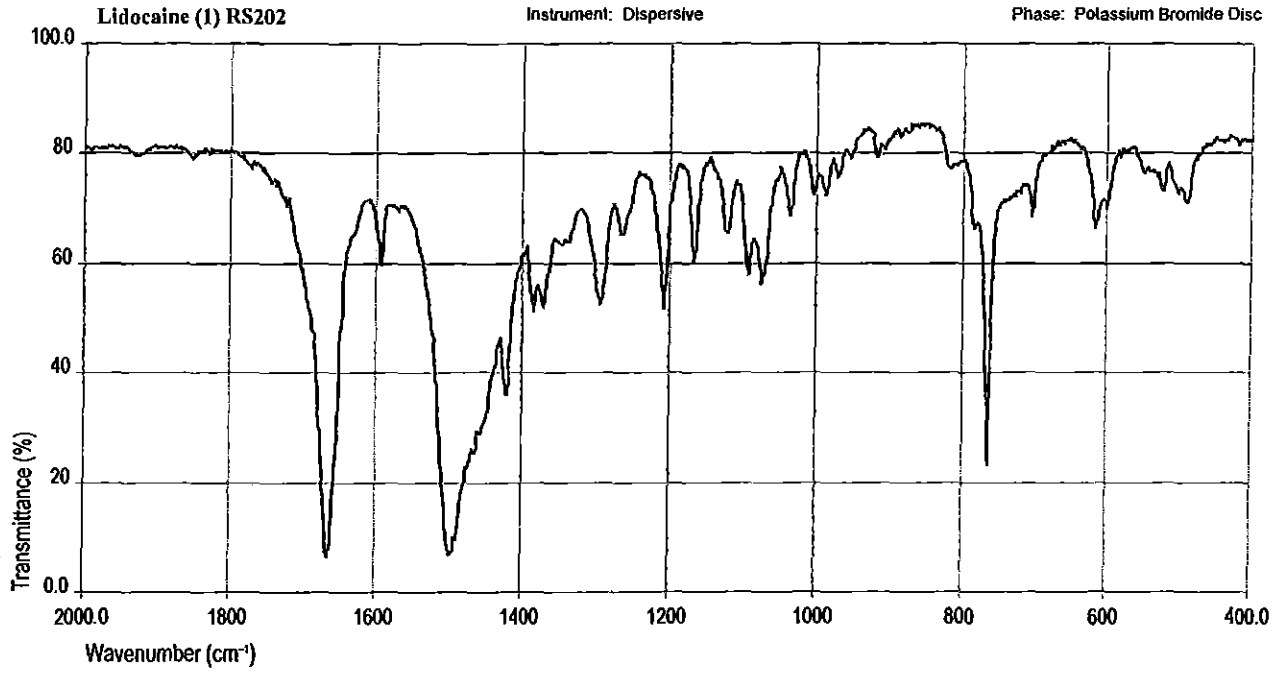


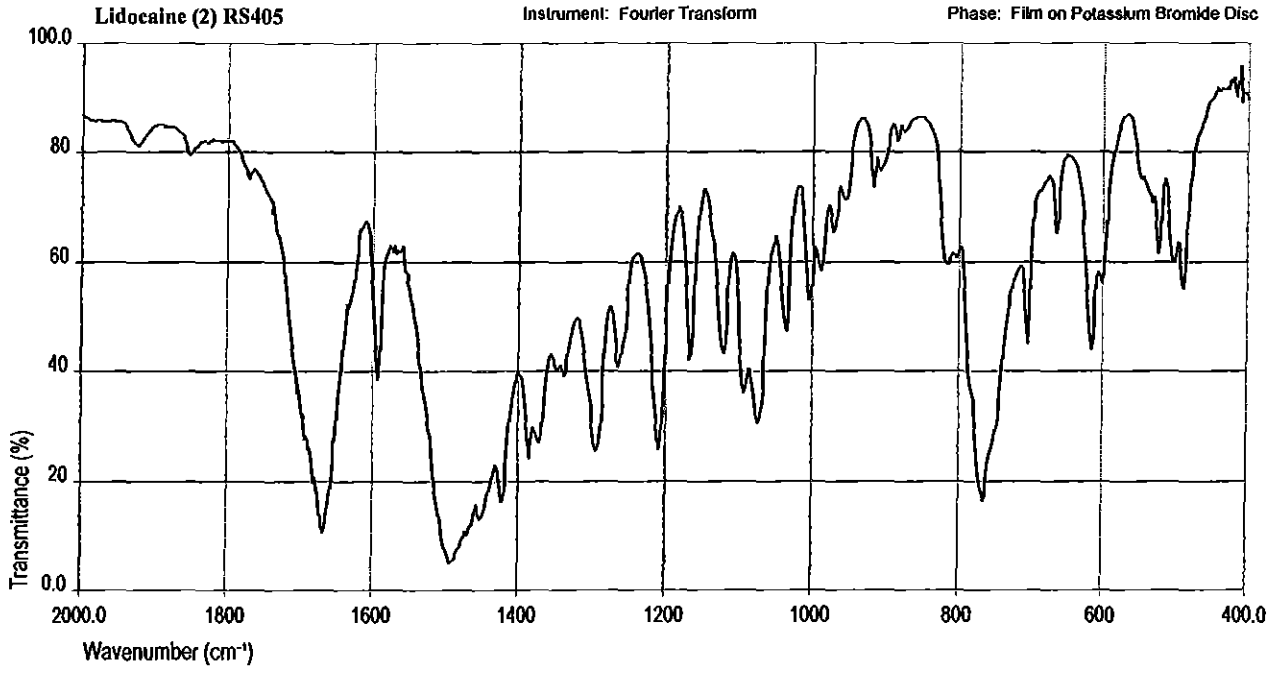


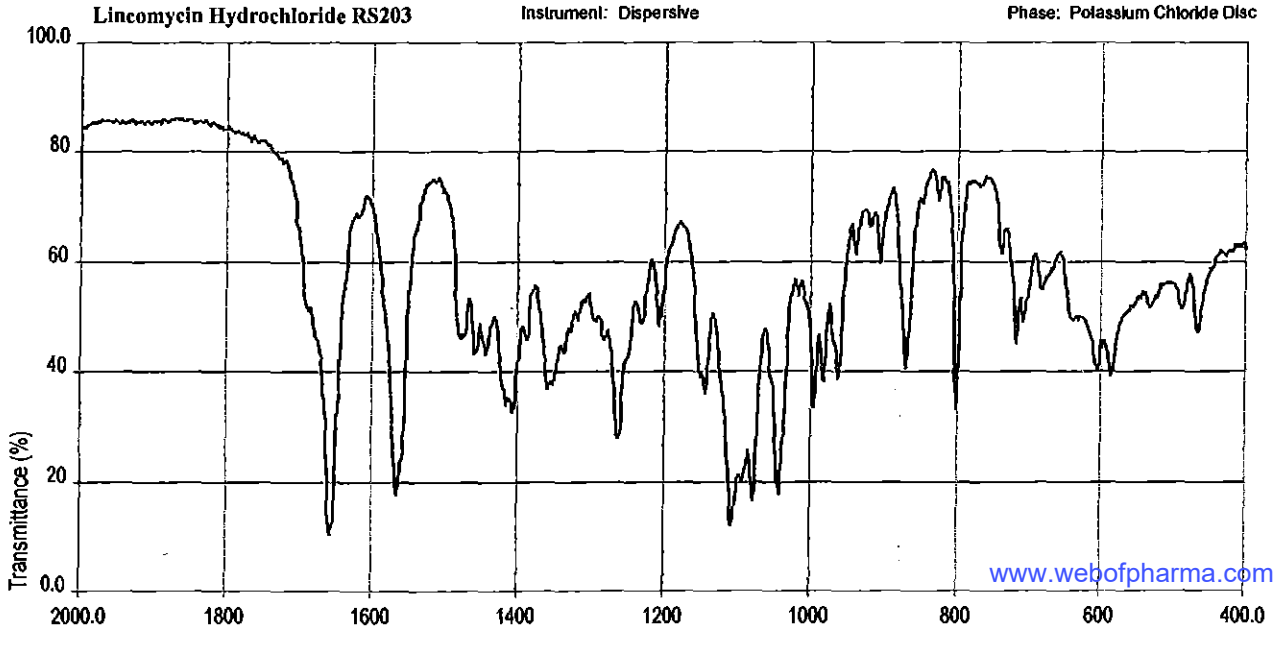

Levobunolol Hydrochloride RS200
Instrument: Dispersive
Phase: Liquid Paraffin Mull
100.0
80
60
40
20
0.0
Transmittance (%)
2000.0
1800
1600
1400
1200
1000
800
600
400.0
Wavenumber (cm⁻¹)


Levodopa RS201
Instrument: Dispersive
Phase: Potassium Bromide Disc
100.0
80
60
40
20
0.0
Transmittance (%)
2000.0
1800
1600
1400
1200
1000
800
600
400.0
Wavenumber (cm⁻¹)

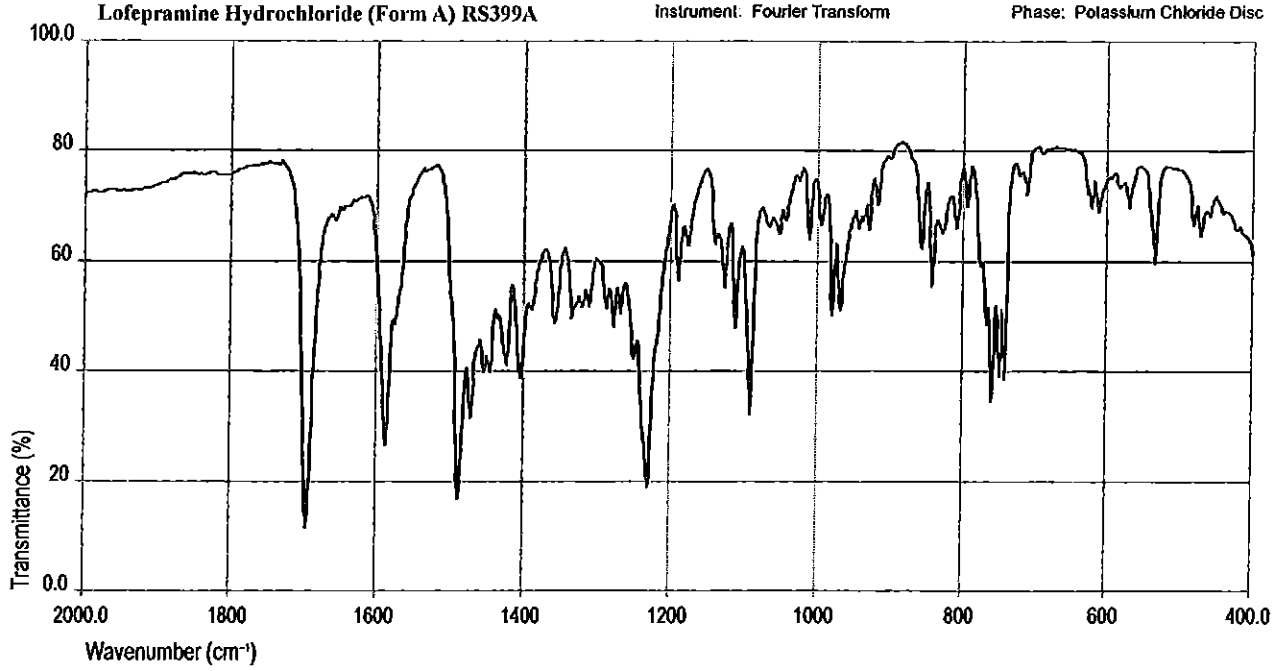

Levomepromazine RS404
Instrument: Fourier Transform
Phase: Liquid Paraffin Mull
100.0
80
60
40
20
0.0
Transmittance (%)
2000.0
1800
1600
1400
1200
1000
800
600
400.0
www.webofpharma.com



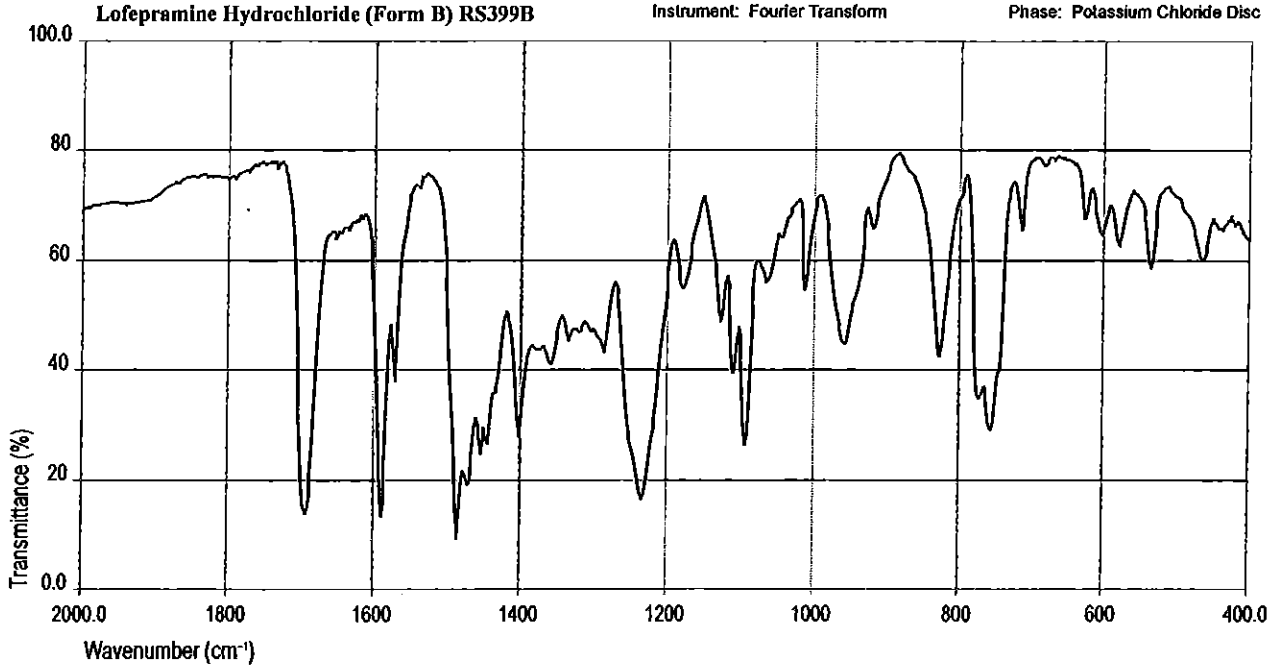




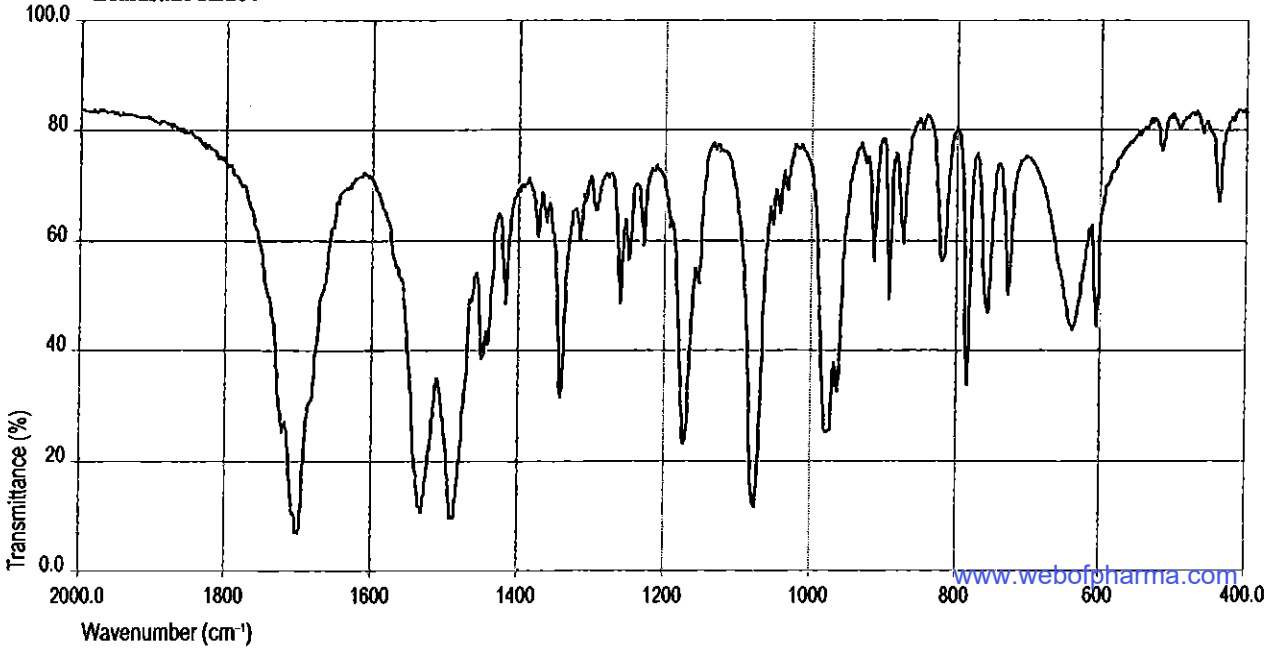
Lofepramine Hydrochloride (Form A) RS399A Instrument: Fourier Transform Phase: Potassium Chloride Disc

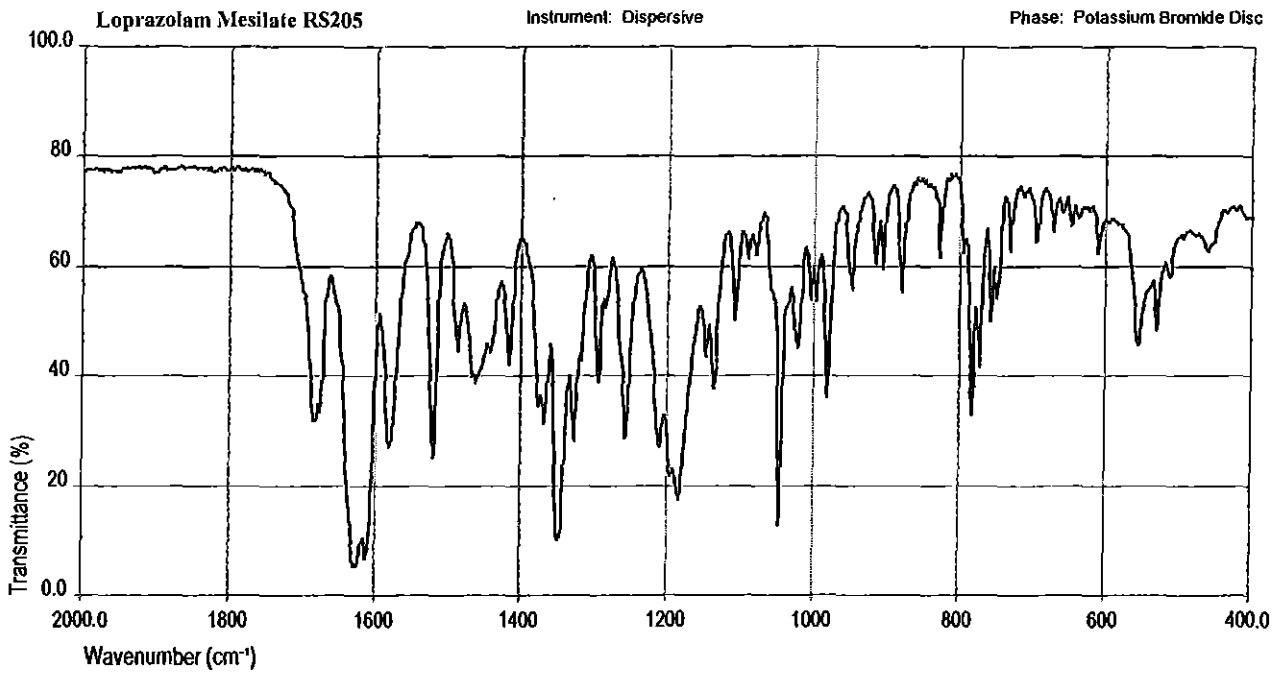


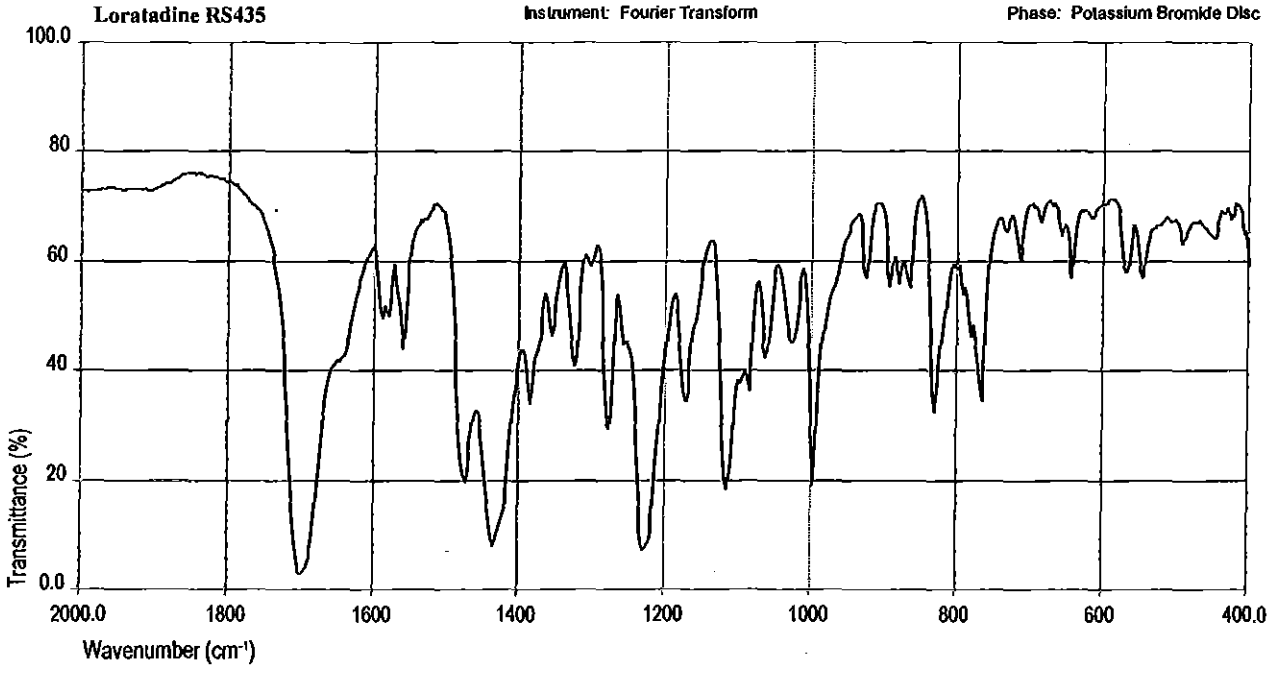
Lofepramine Hydrochloride (Form B) RS399B Instrument: Fourier Transform Phase: Potassium Chloride Disc

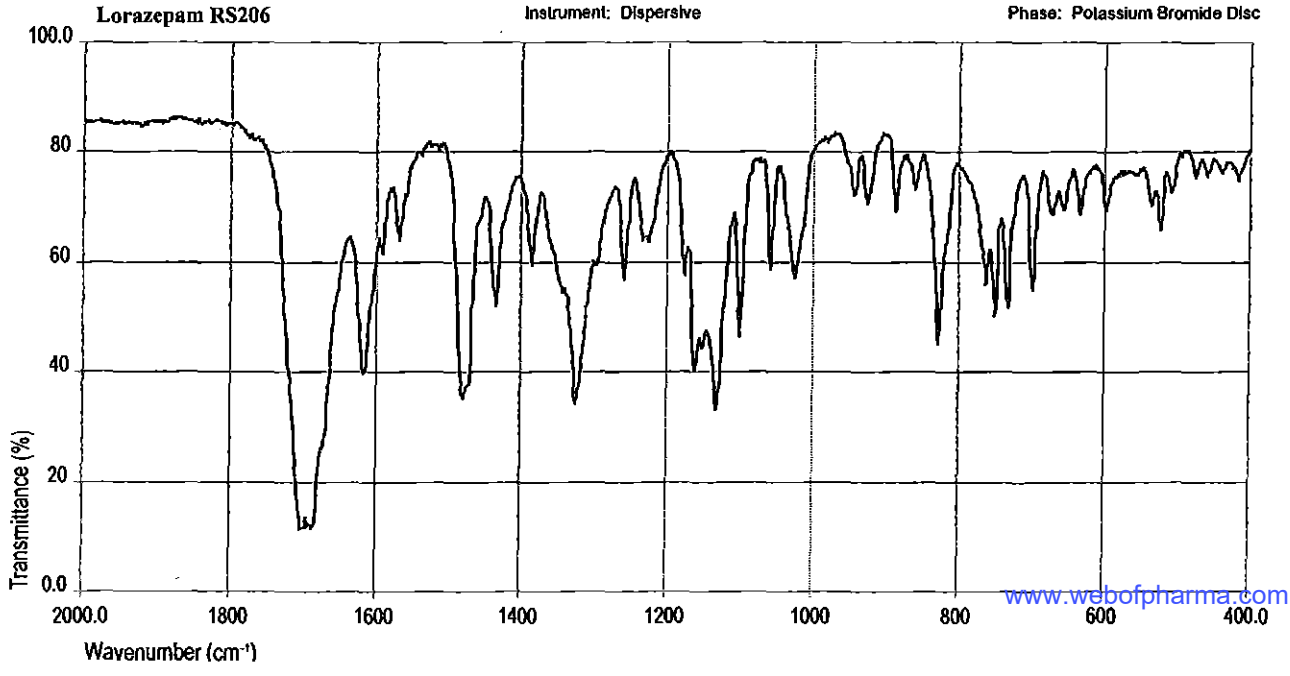


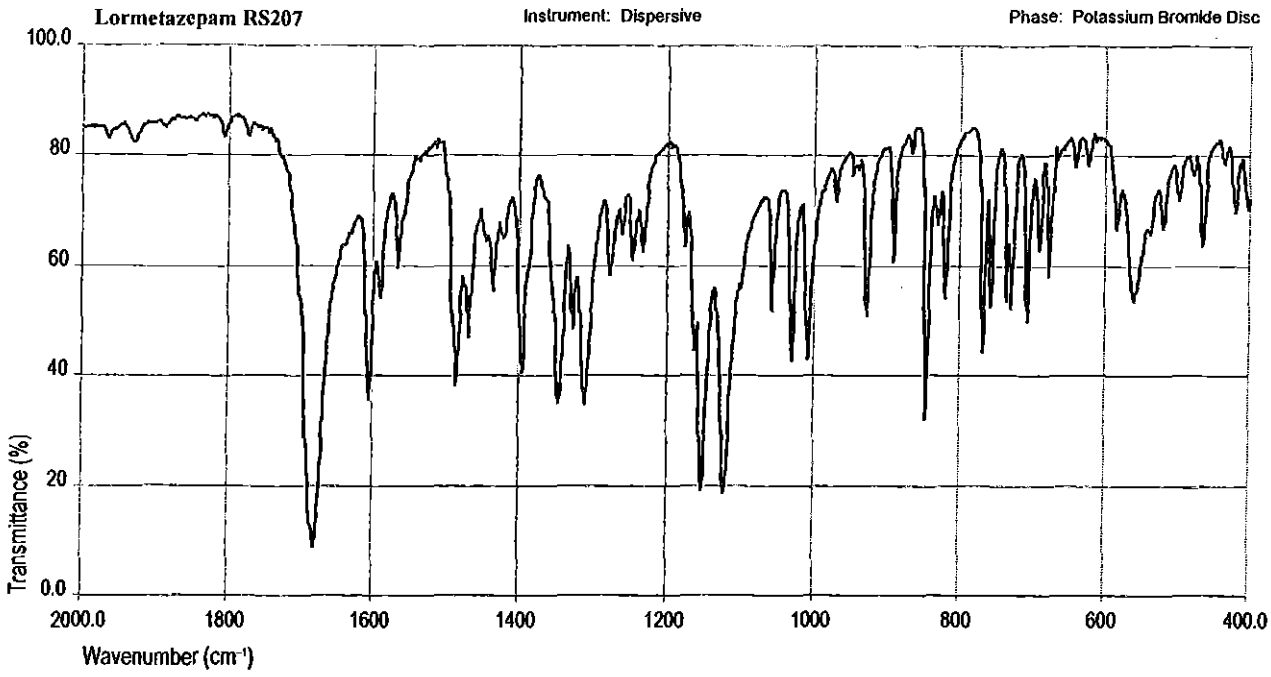
Lomustine RS204 Instrument: Dispersive Phase: Potassium Bromide Disc

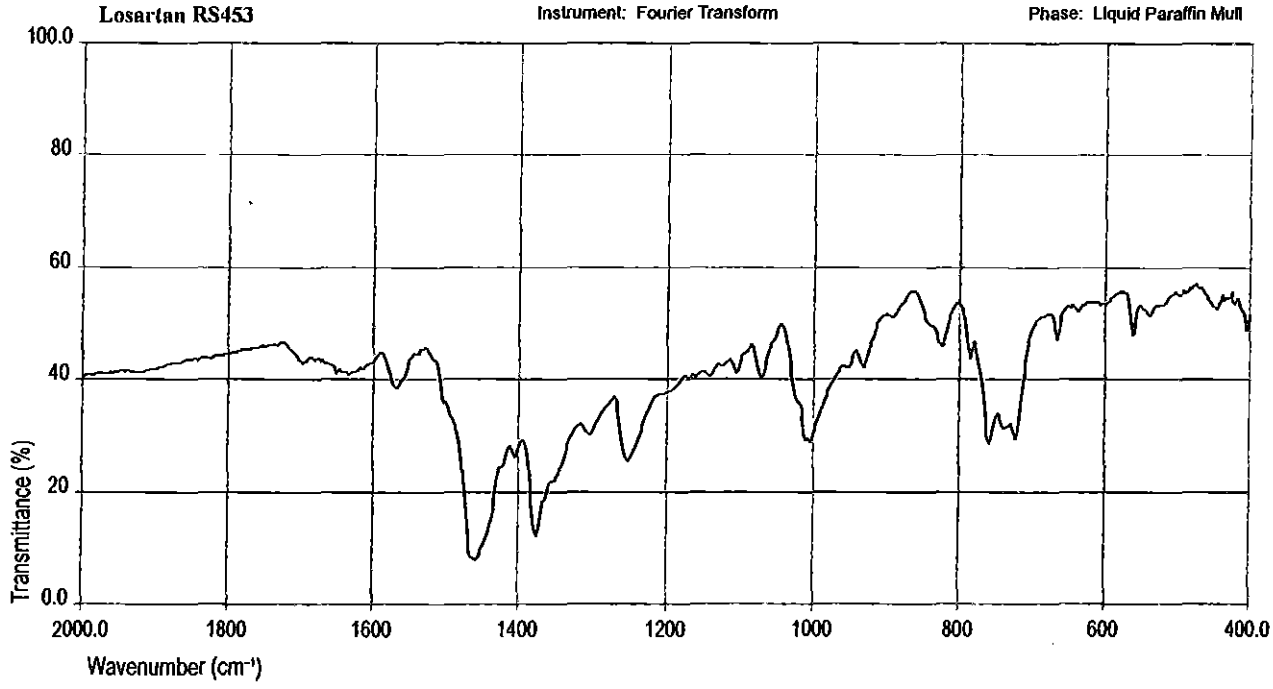


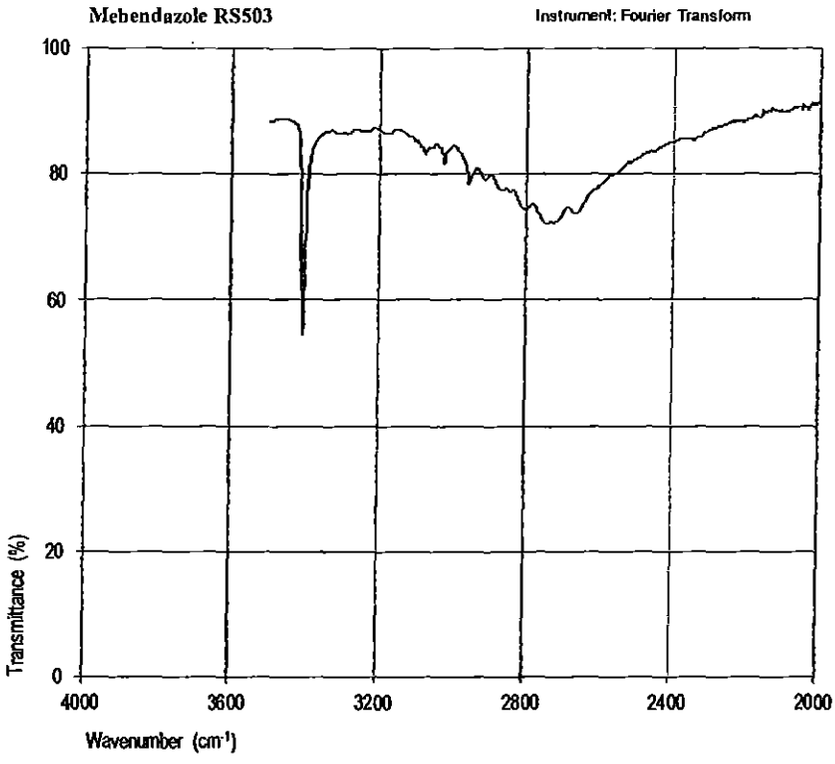


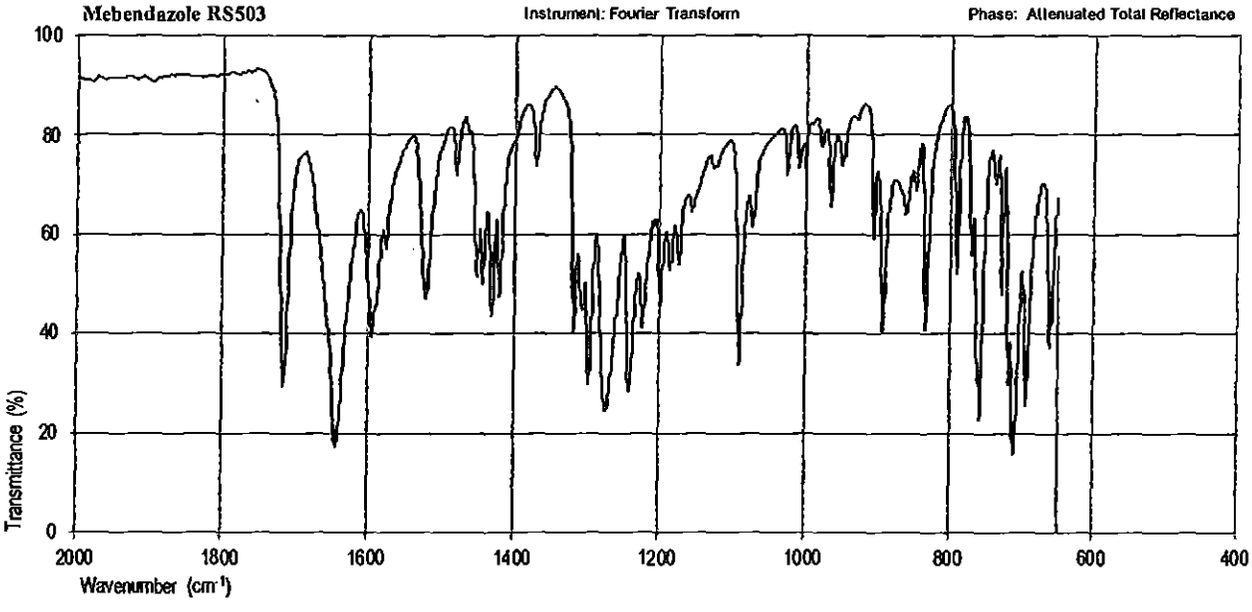


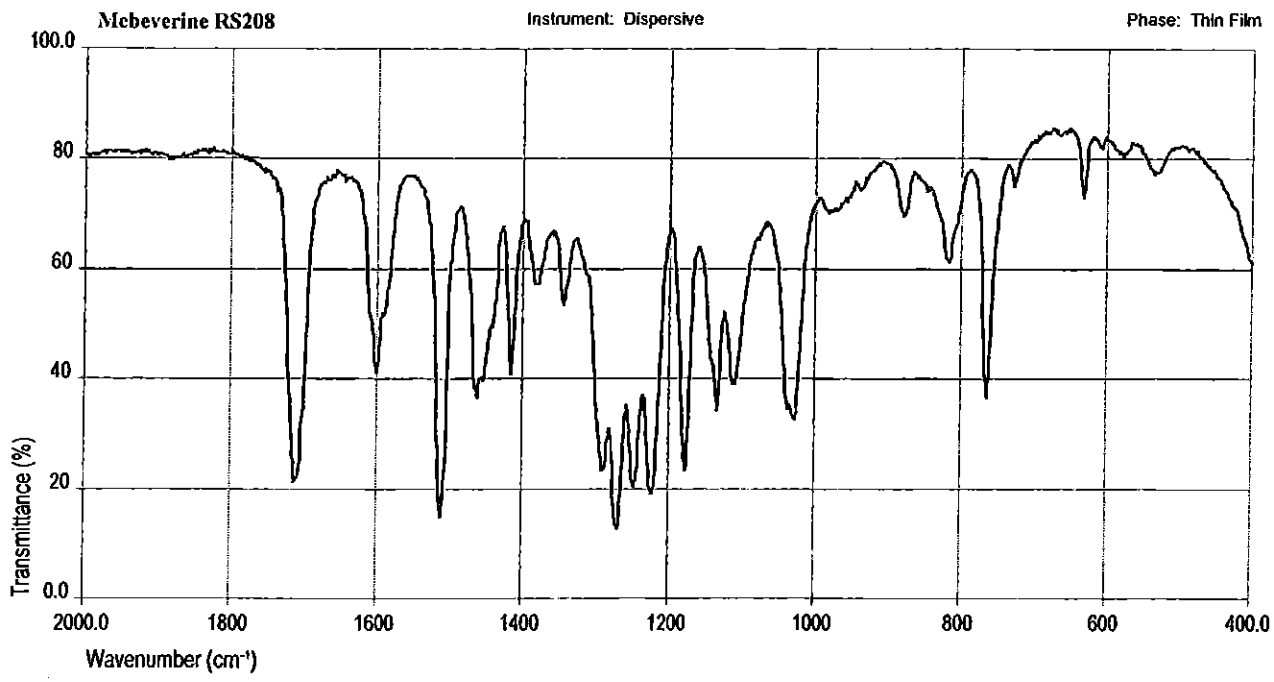


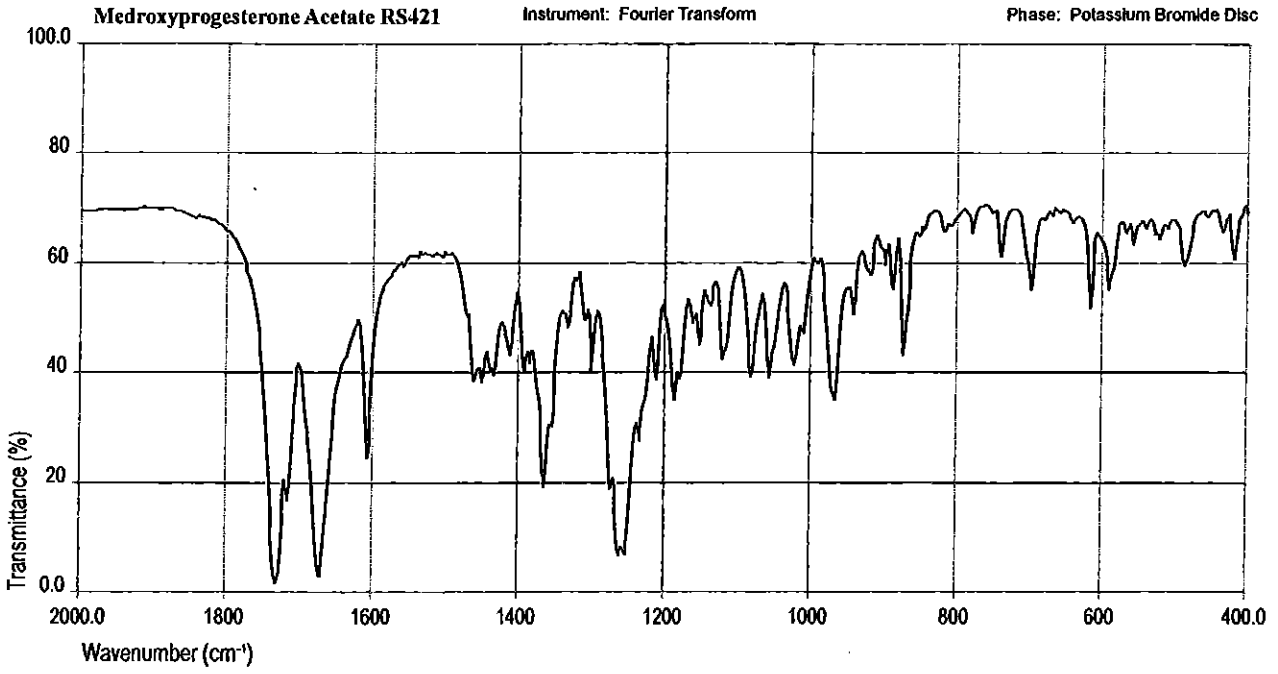


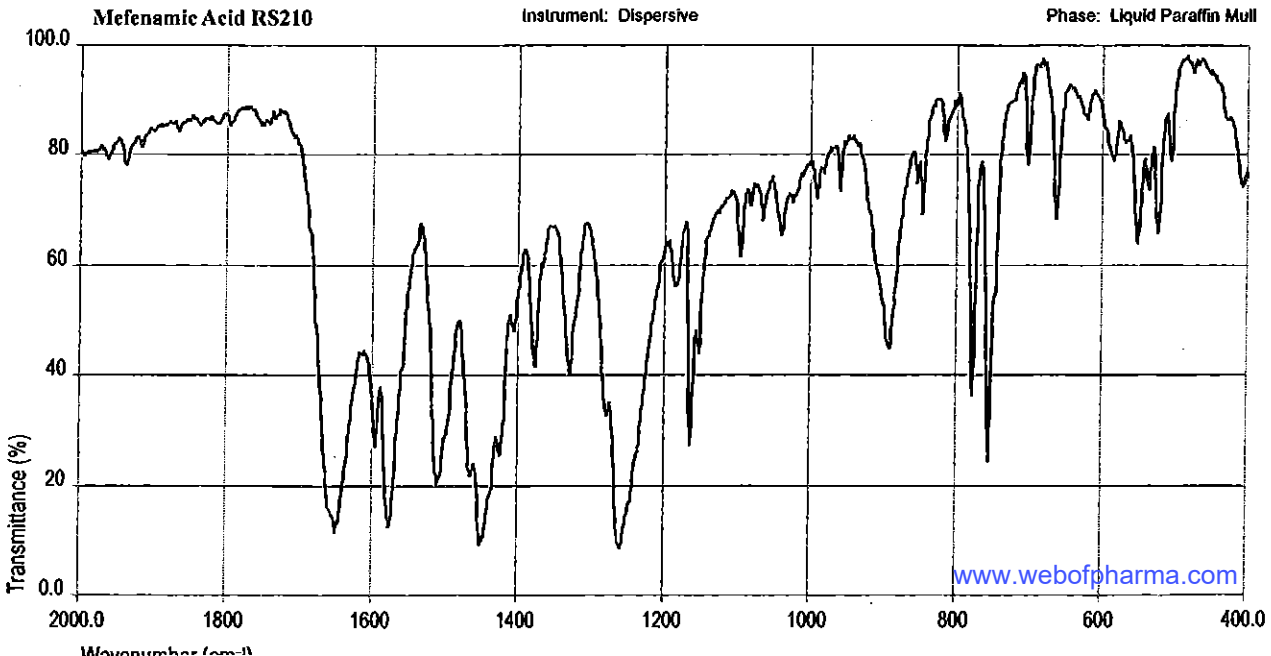


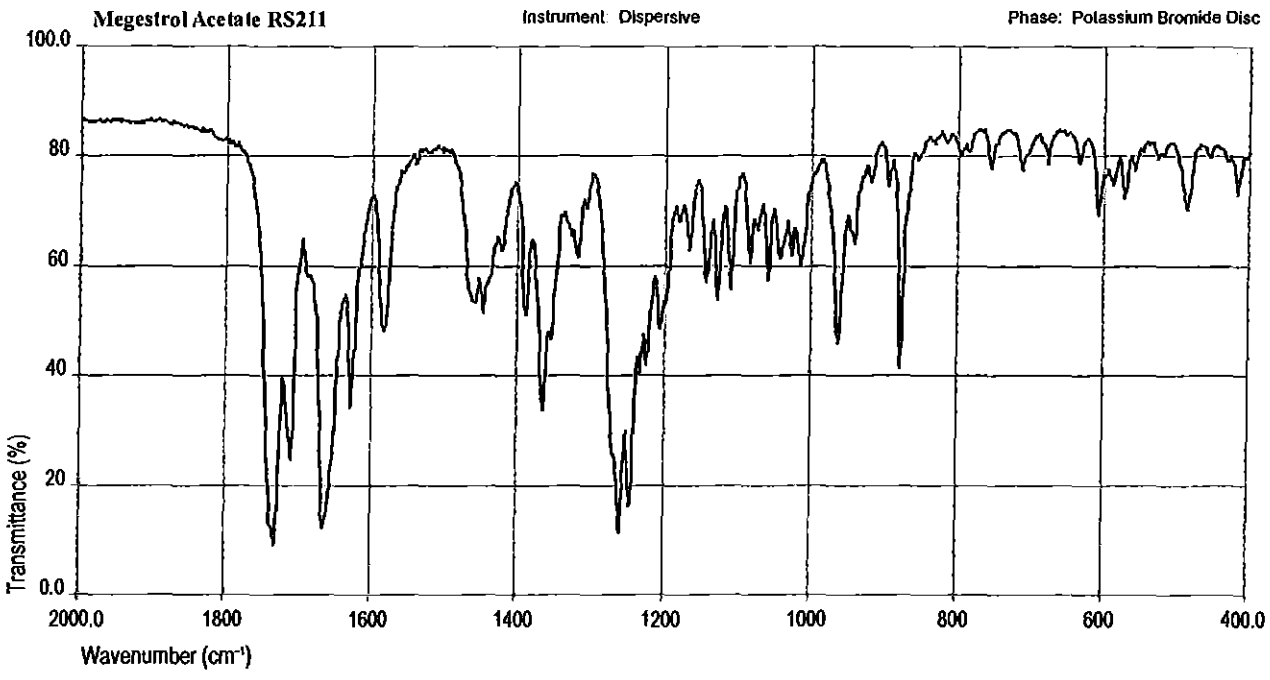


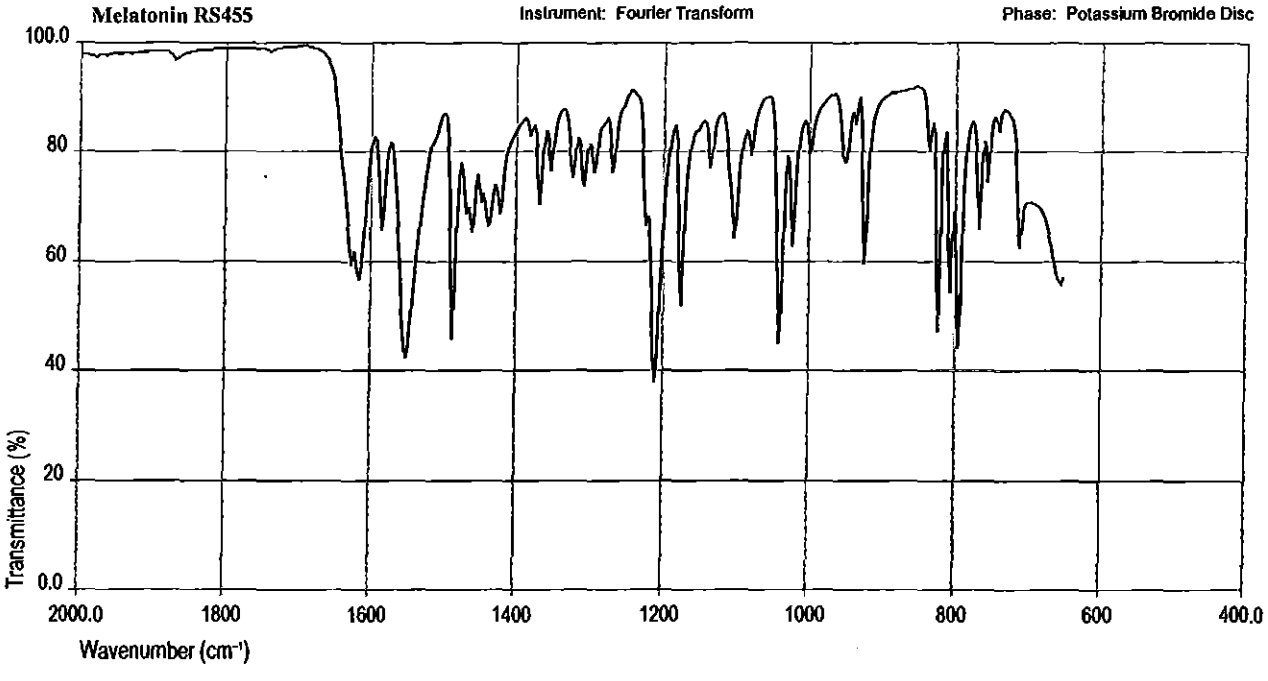


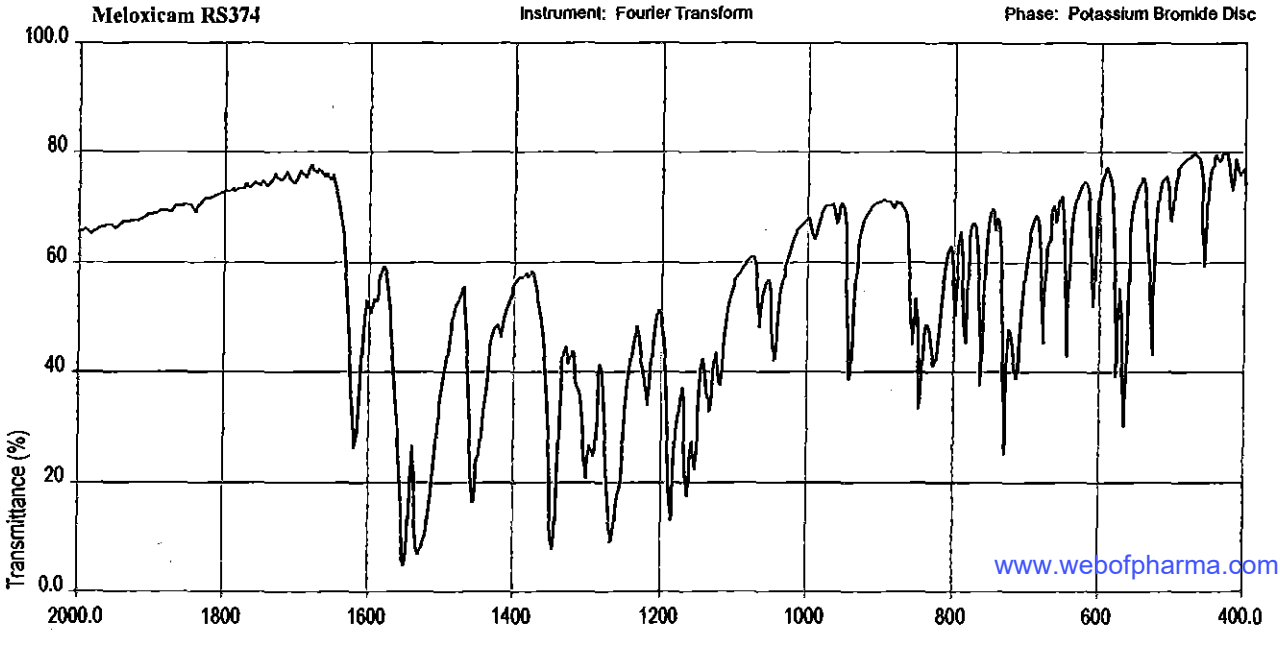

Mebeverine RS208
Instrument: Dispersive
Phase: Thin Film
100.0
80
60
40
20
0.0
Transmittance (%)
2000.0
1800
1600
1400
1200
1000
800
600
400.0
Wavenumber (cm⁻¹)


Medroxyprogesterone Acetate RS421
Instrument: Fourier Transform
Phase: Potassium Bromide Disc
100.0
80
60
40
20
0.0
Transmittance (%)
2000.0
1800
1600
1400
1200
1000
800
600
400.0
Wavenumber (cm⁻¹)

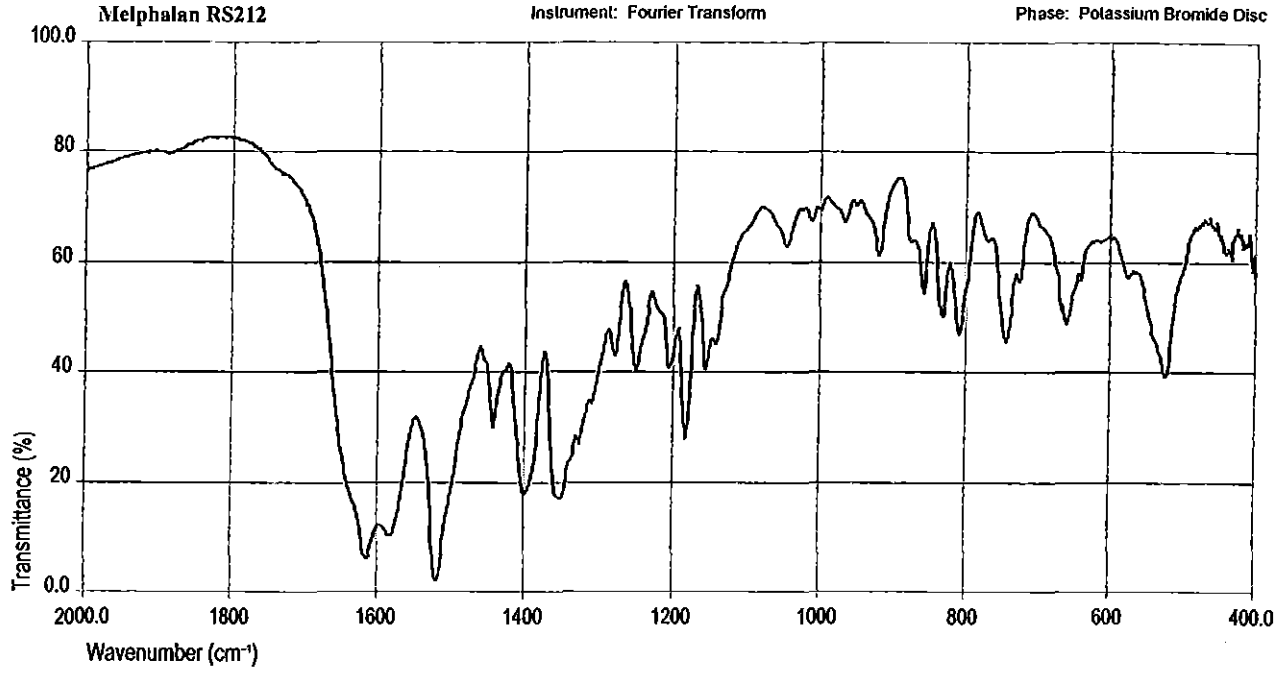

Mefenamic Acid RS210
Instrument: Dispersive
Phase: Liquid Paraffin Mull
100.0
80
60
40
20
0.0
Transmittance (%)
2000.0
1800
1600
1400
1200
1000
800
600
400.0
www.webofpharma.com



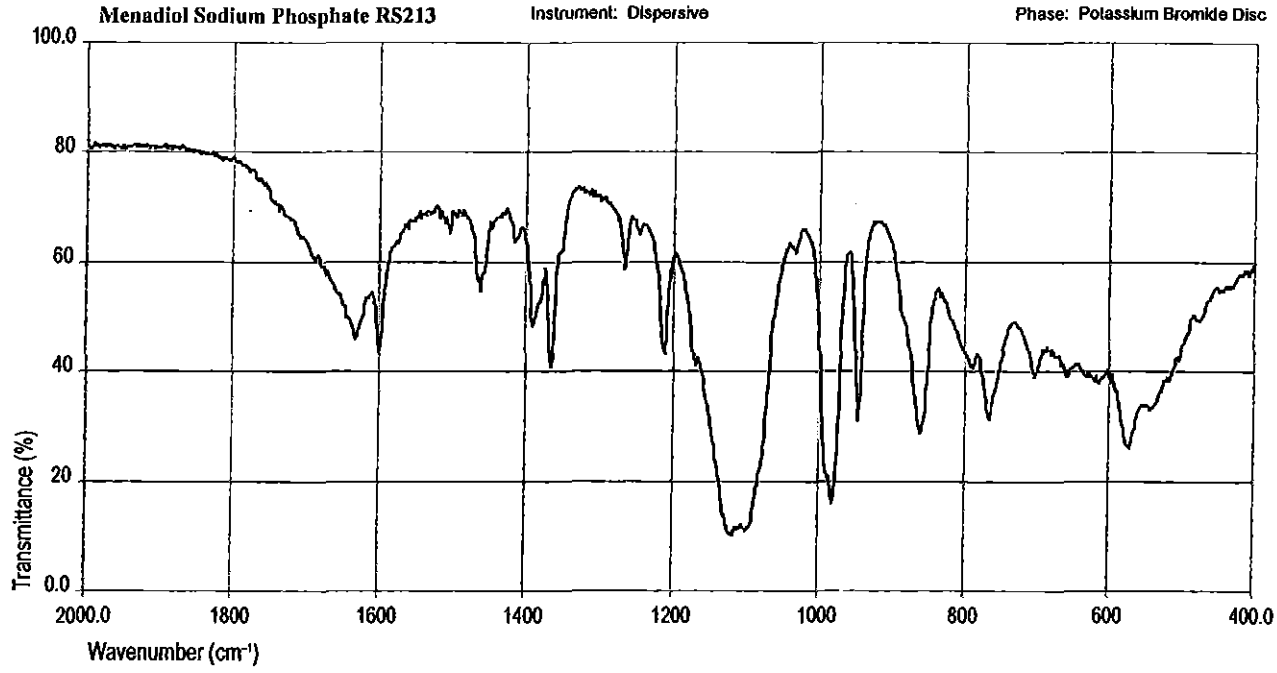




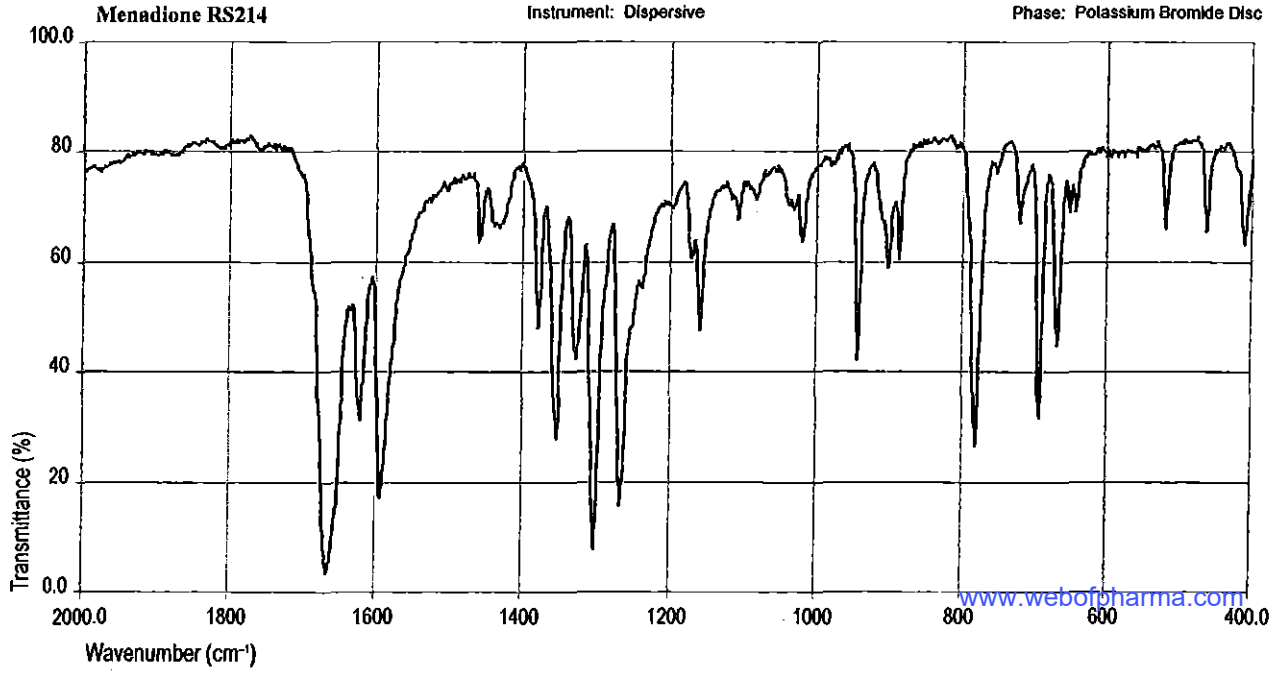
Melphalan RS212 Instrument: Fourier Transform Phase: Potassium Bromide Disc



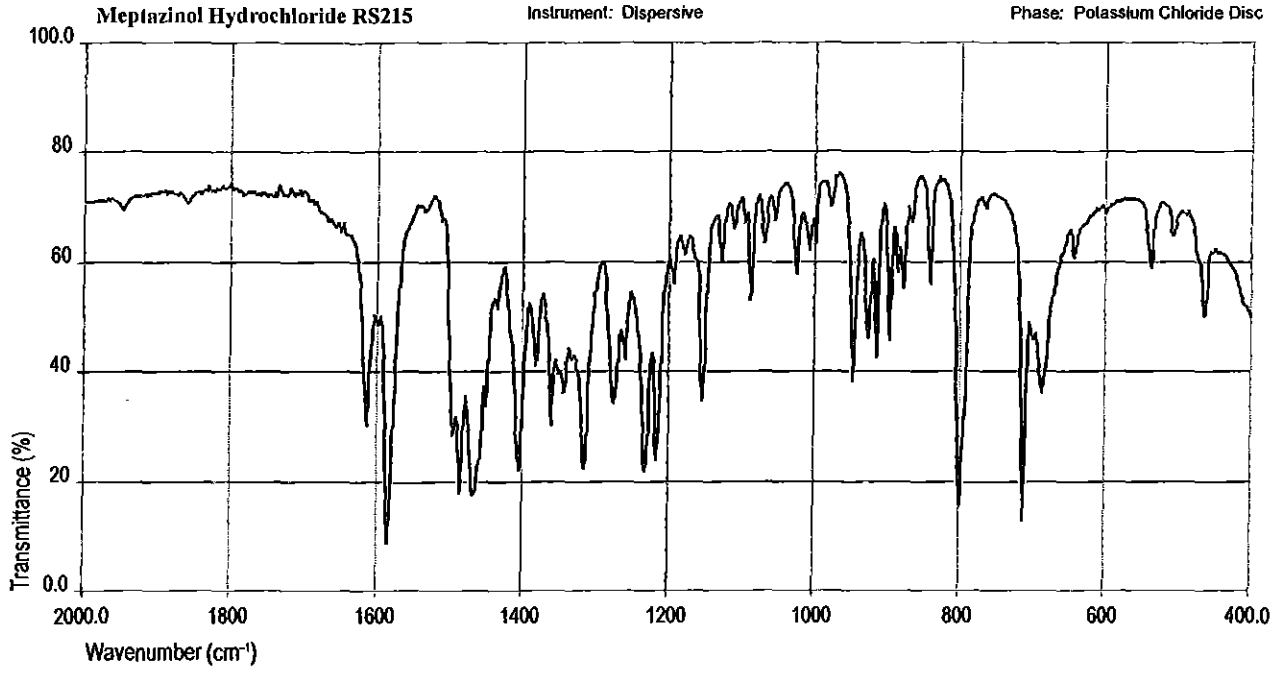
Menadiol Sodium Phosphate RS213 Instrument: Dispersive Phase: Potassium Bromide Disc



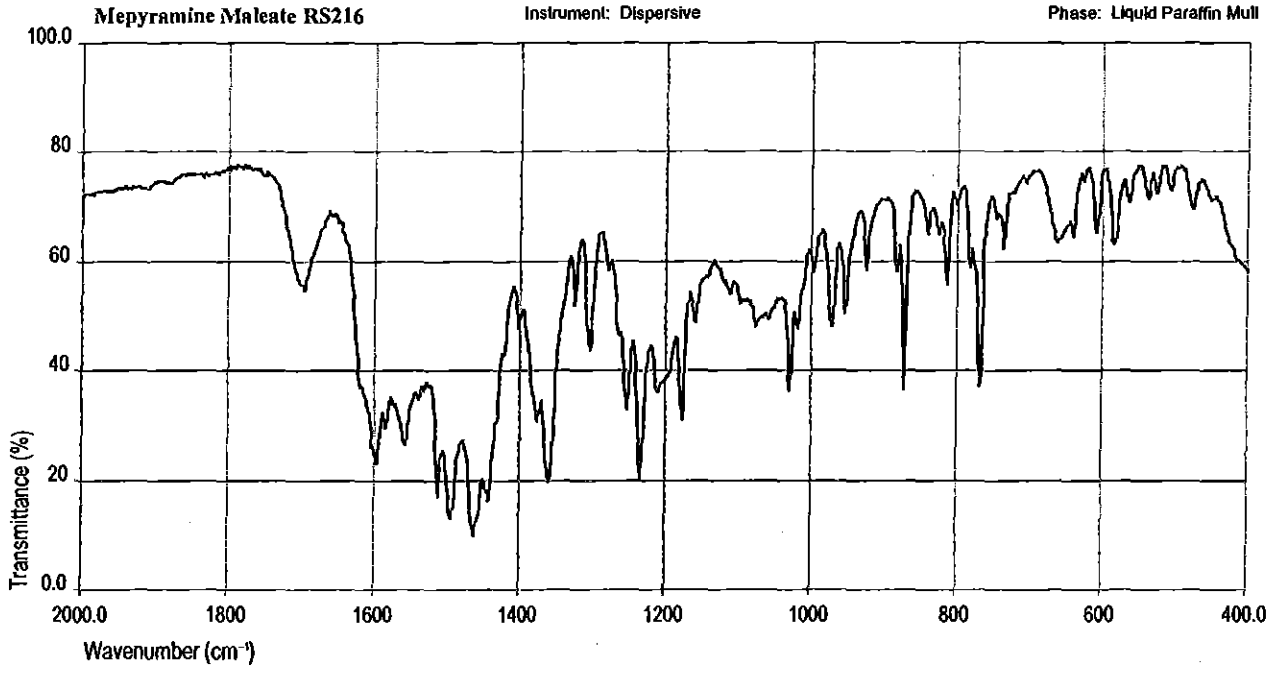
Menadione RS214 Instrument: Dispersive Phase: Potassium Bromide Disc



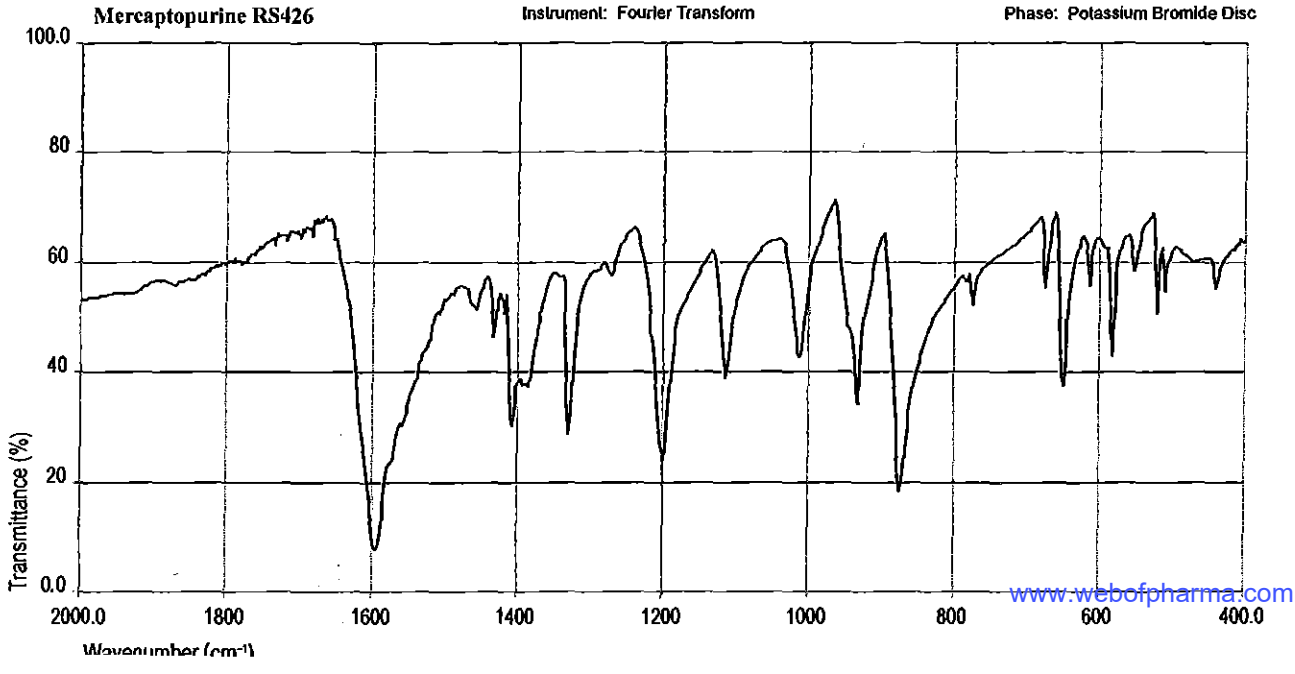
Meptazinol Hydrochloride RS215 Instrument: Dispersive Phase: Potassium Chloride Disc



Mepyramine Maleate RS216 Instrument: Dispersive Phase: Liquid Paraffin Mull



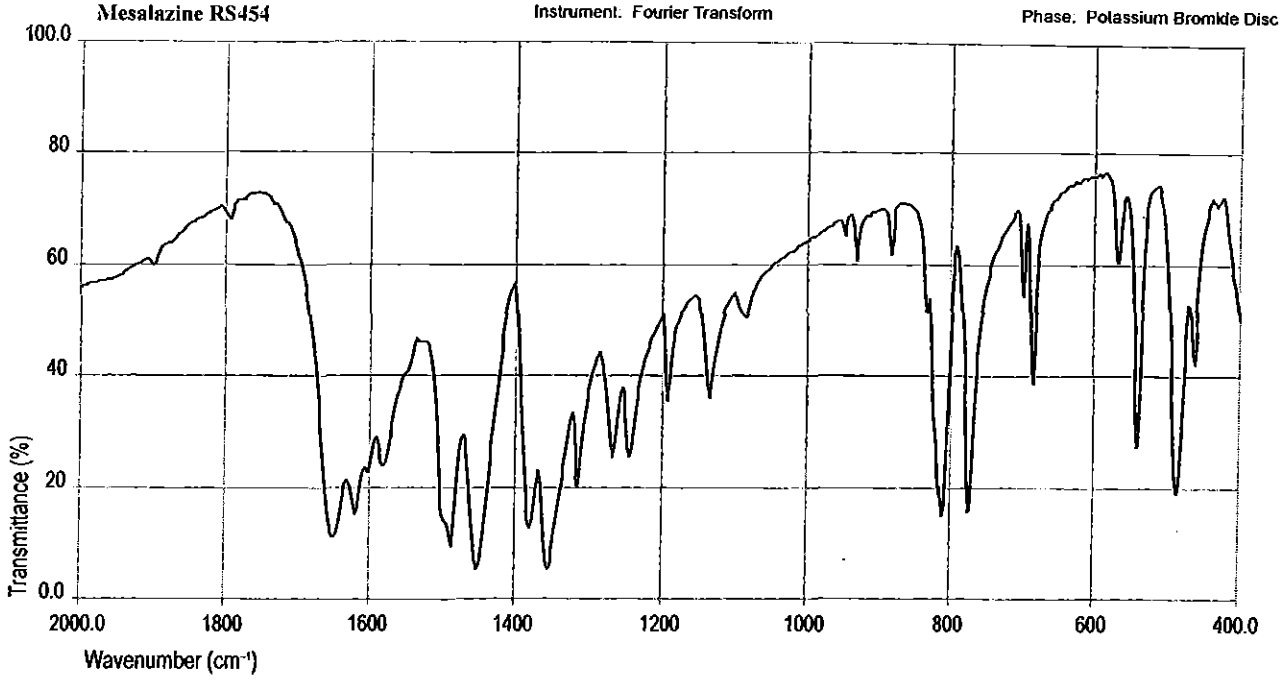
Mercaptopurine RS426 Instrument: Fourier Transform Phase: Potassium Bromide Disc



Mesalazine RS454

Instrument: Fourier Transform

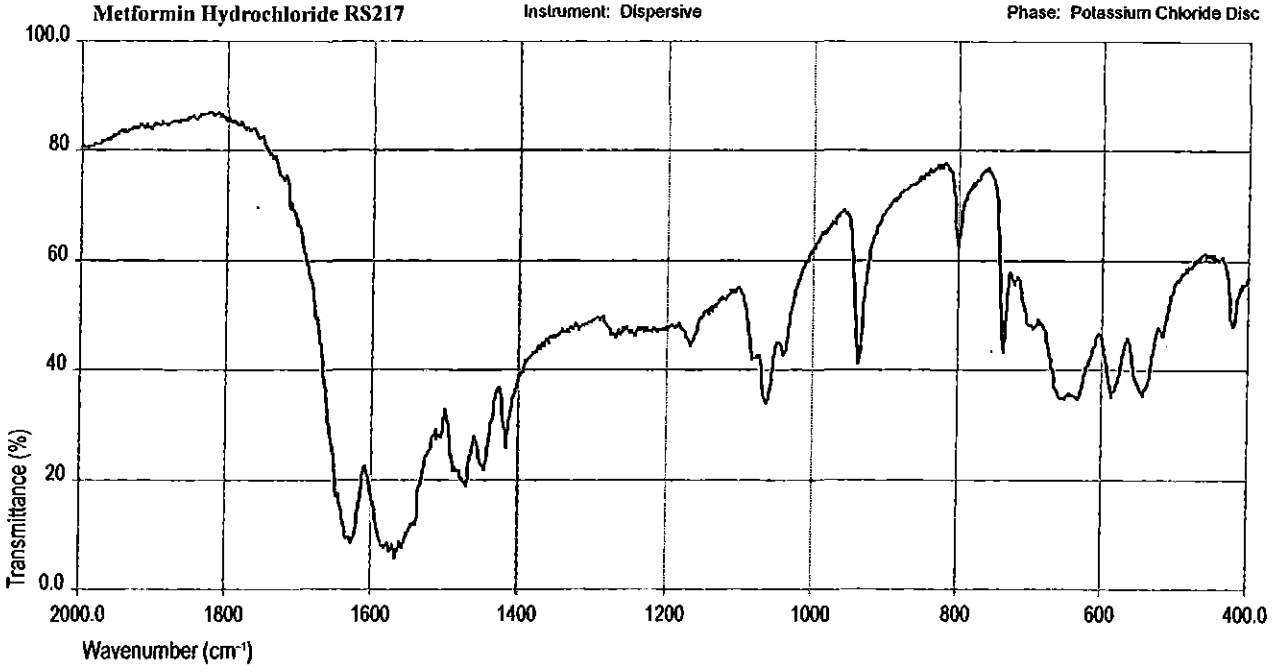
Phase: Potassium Bromide Disc



Metformin Hydrochloride RS217

Instrument: Dispersive

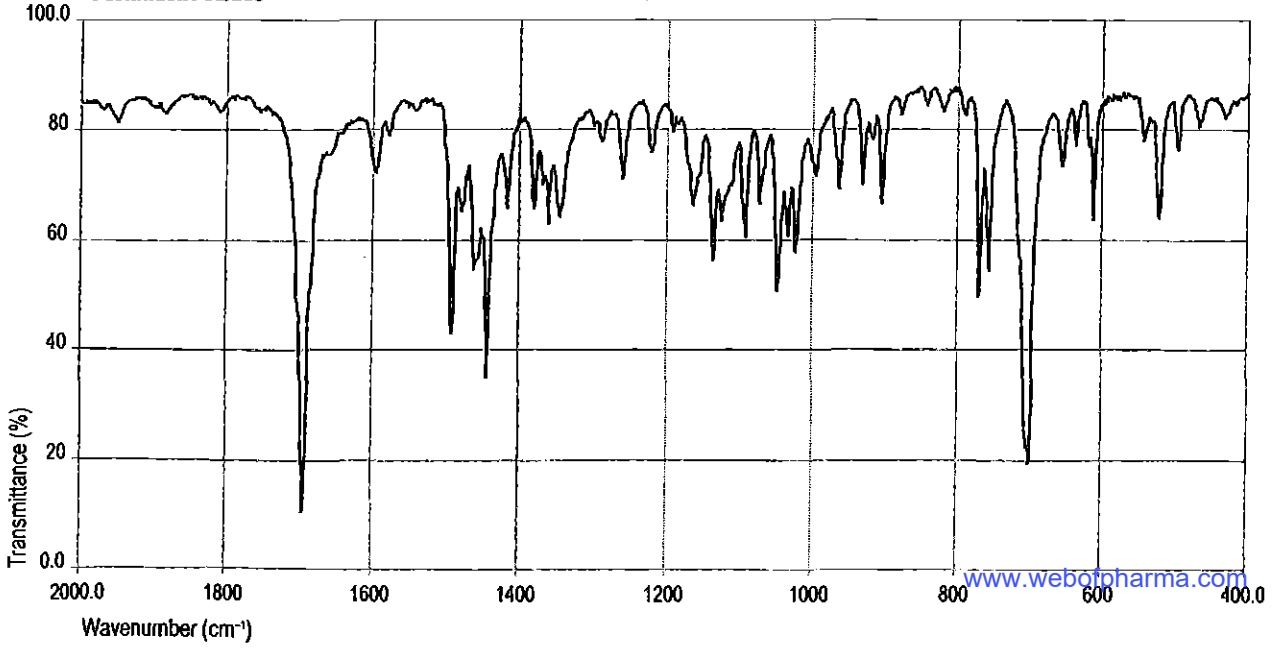
Phase: Potassium Chloride Disc



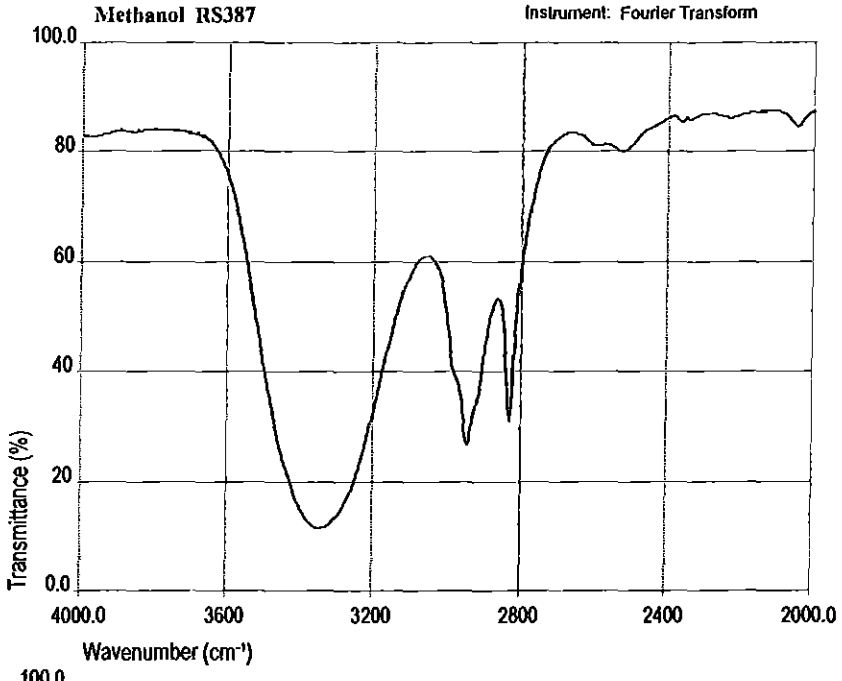
Methadone RS218

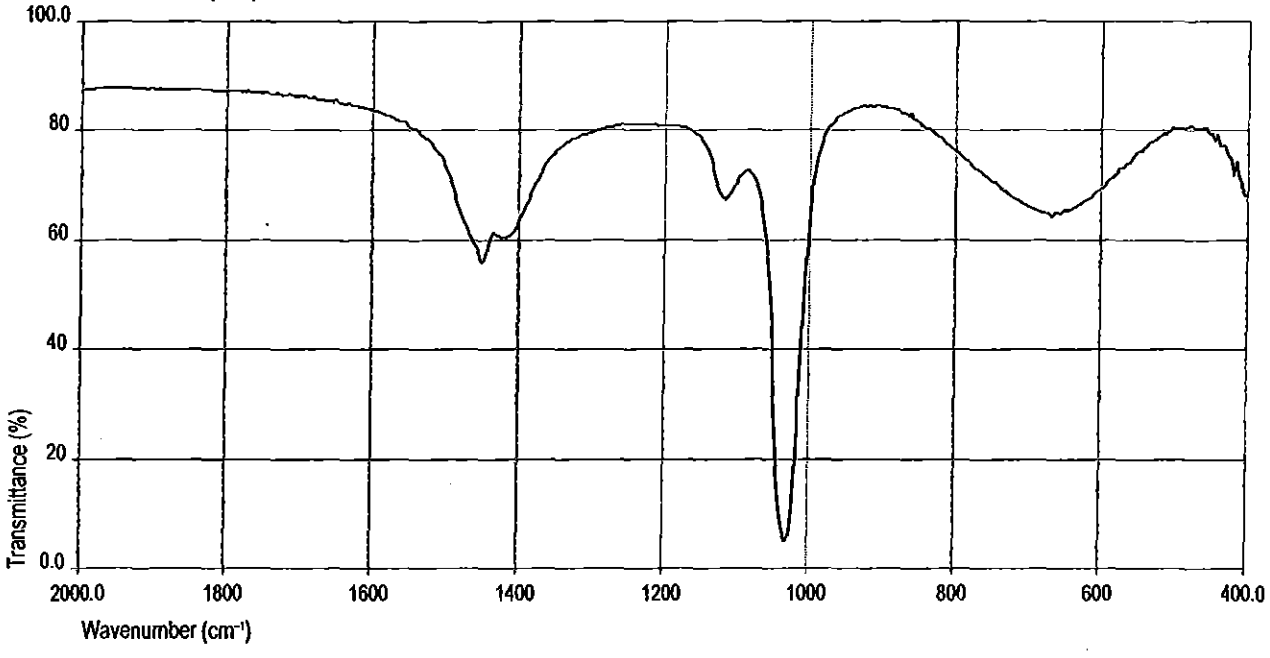
Instrument: Dispersive

Phase: Potassium Bromide Disc

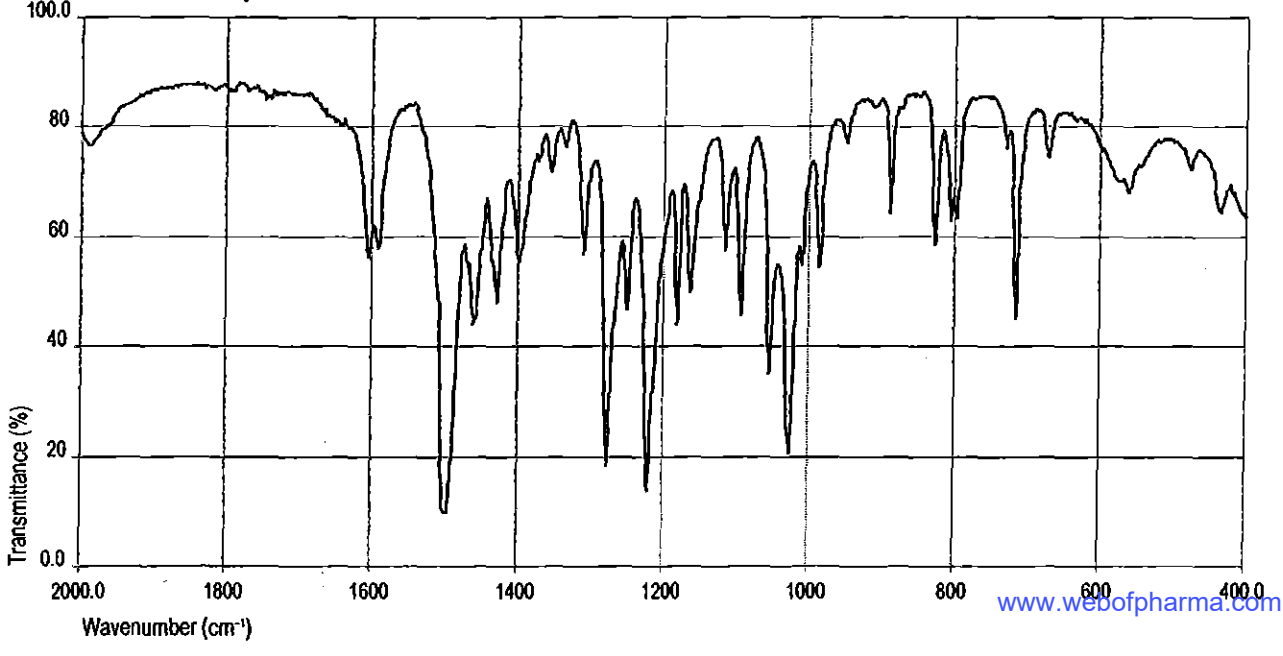


Methanol RS387 Instrument: Fourier Transform Phase: Thin Film

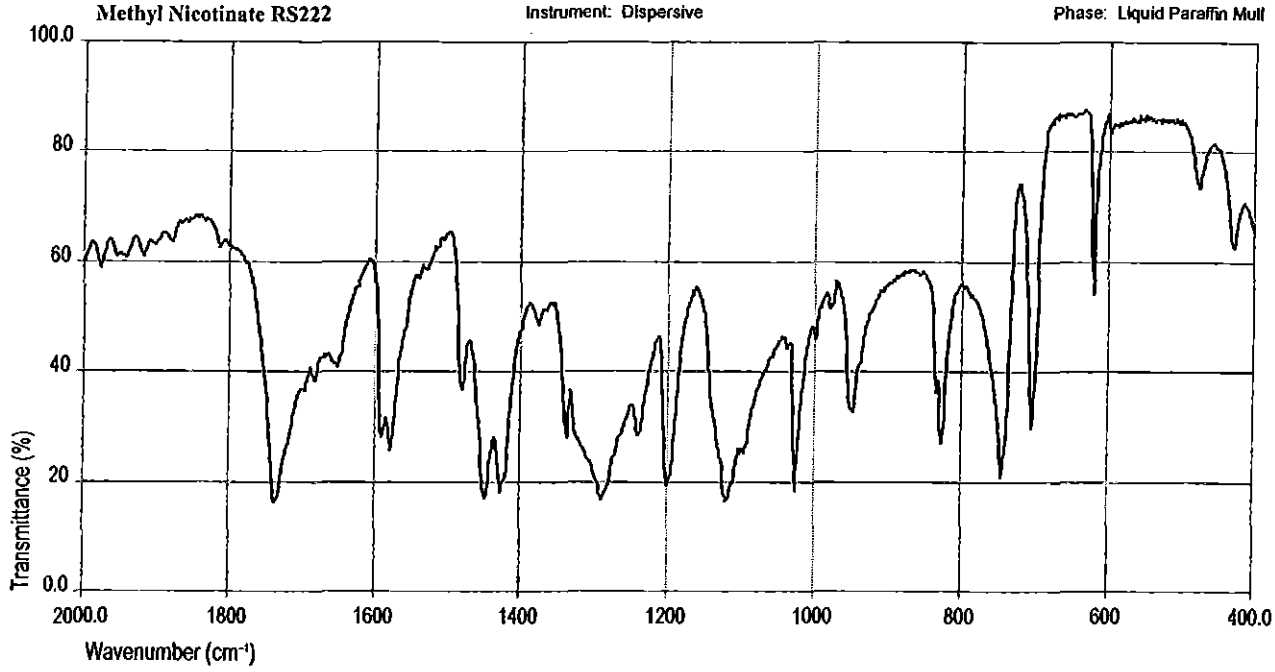




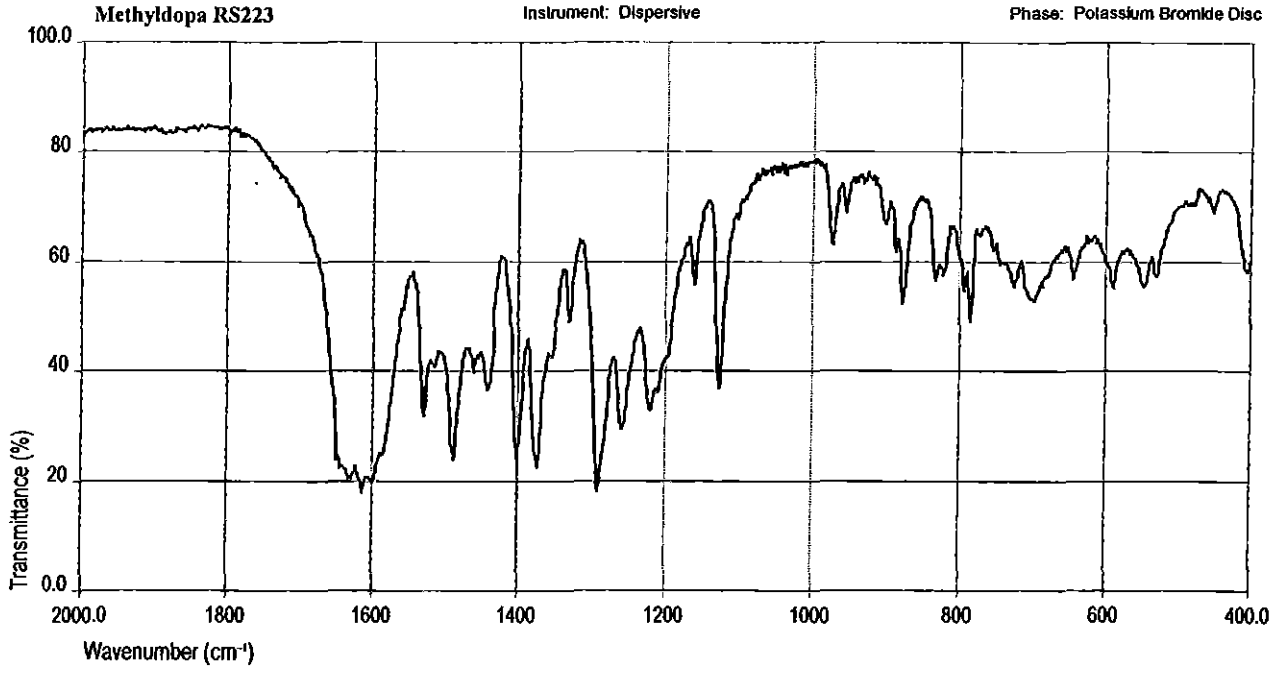
Methoxamine Hydrochloride RS220 Instrument: Dispersive Phase: Potassium Chloride Disc



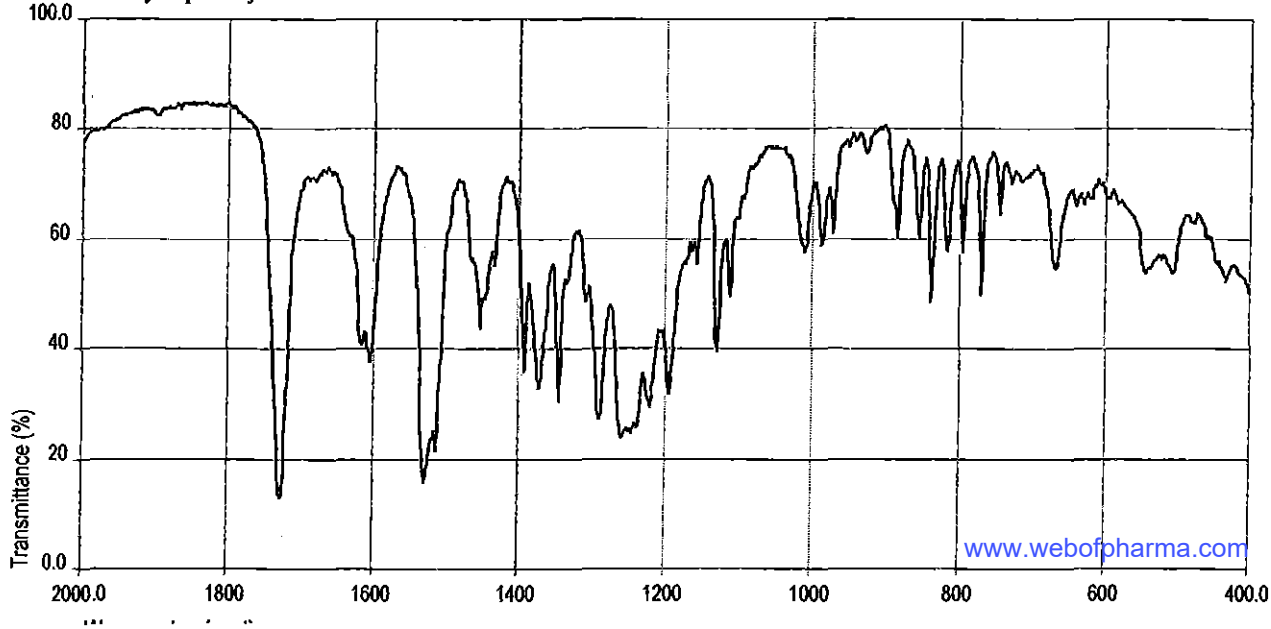
Methyl Nicotinate RS222 Instrument: Dispersive Phase: Liquid Paraffin Mull

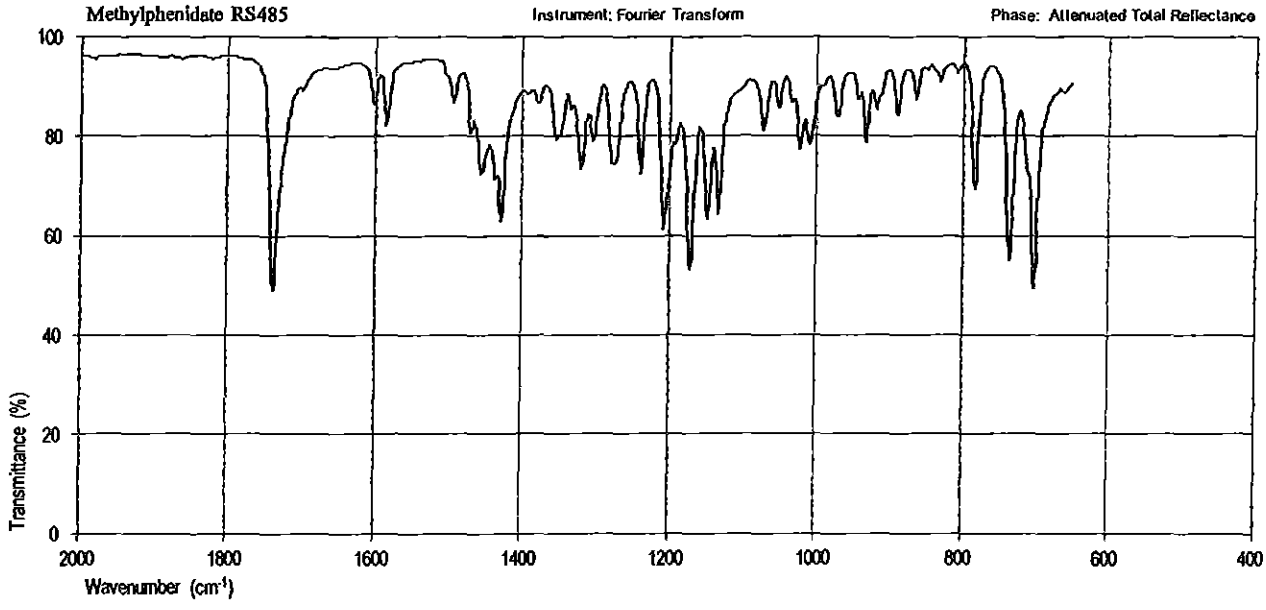


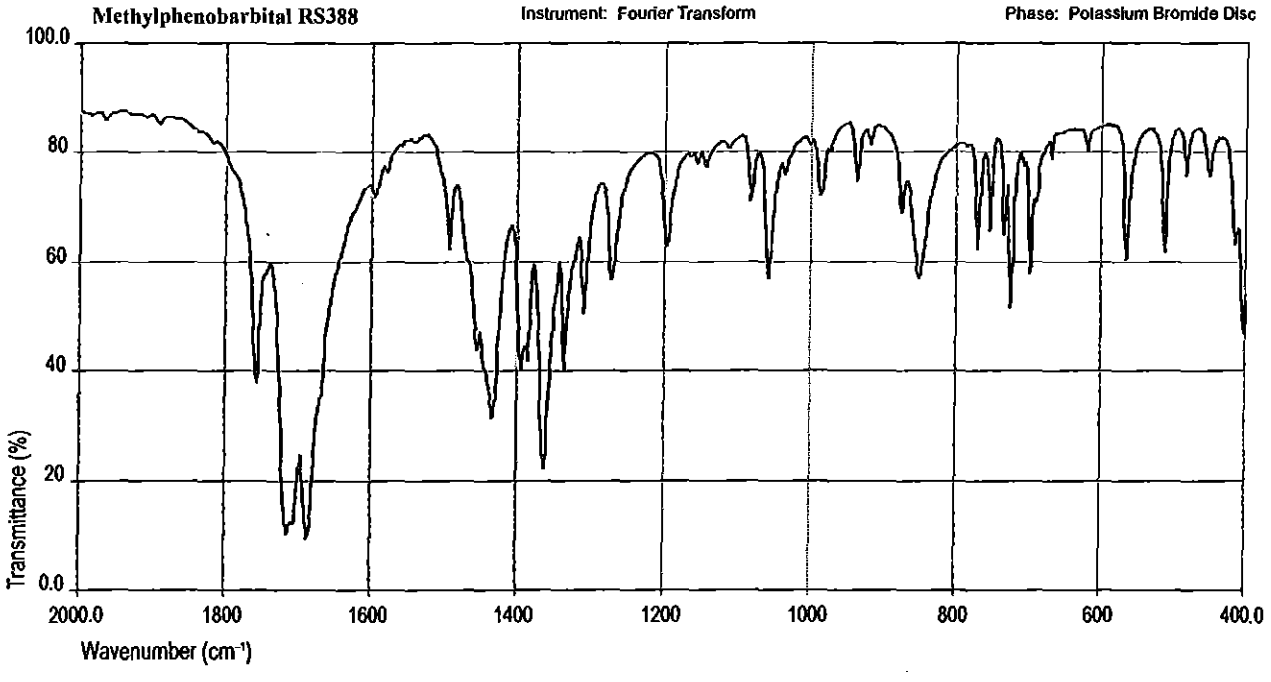
Methyldopa RS223 Instrument: Dispersive Phase: Potassium Bromide Disc

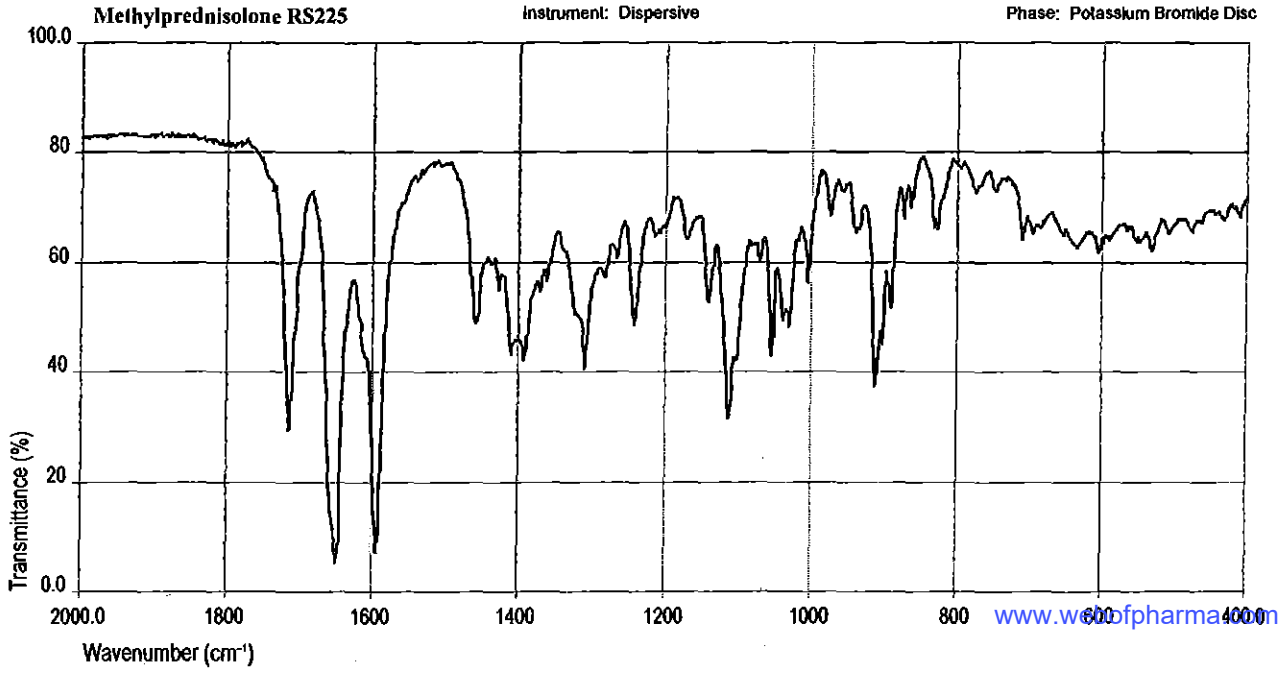


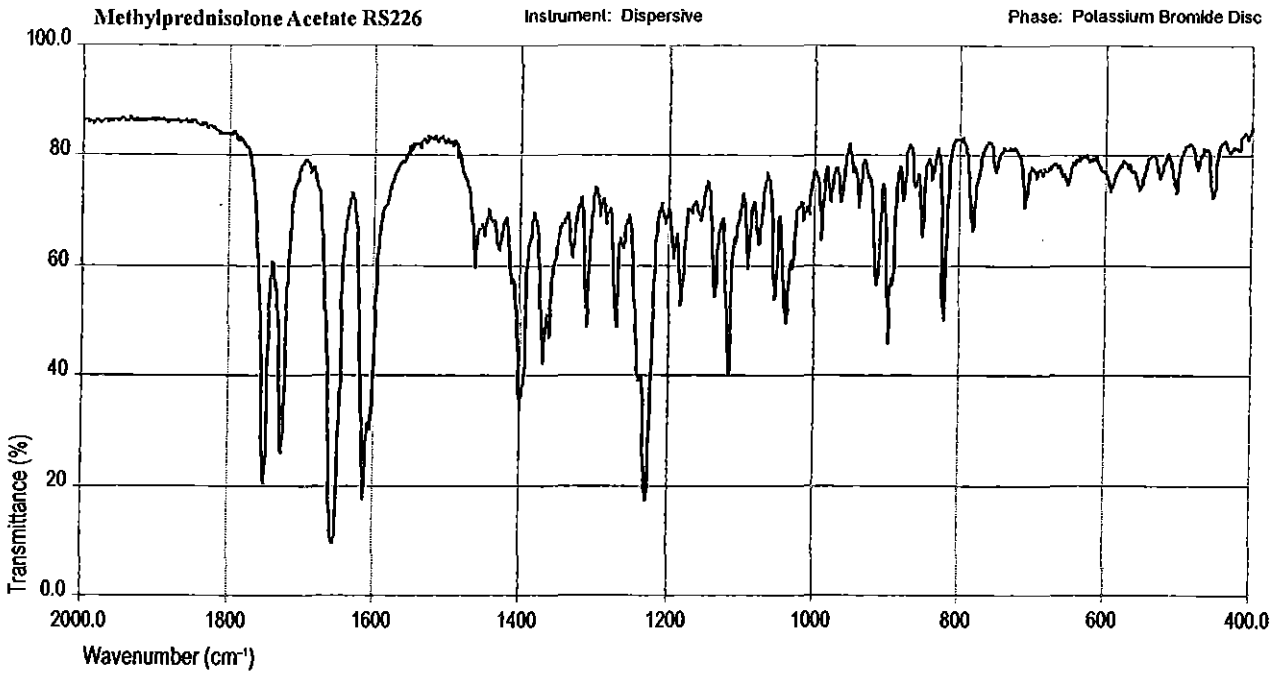
Methyldopate Hydrochloride RS224 Instrument: Dispersive Phase: Potassium Chloride Disc

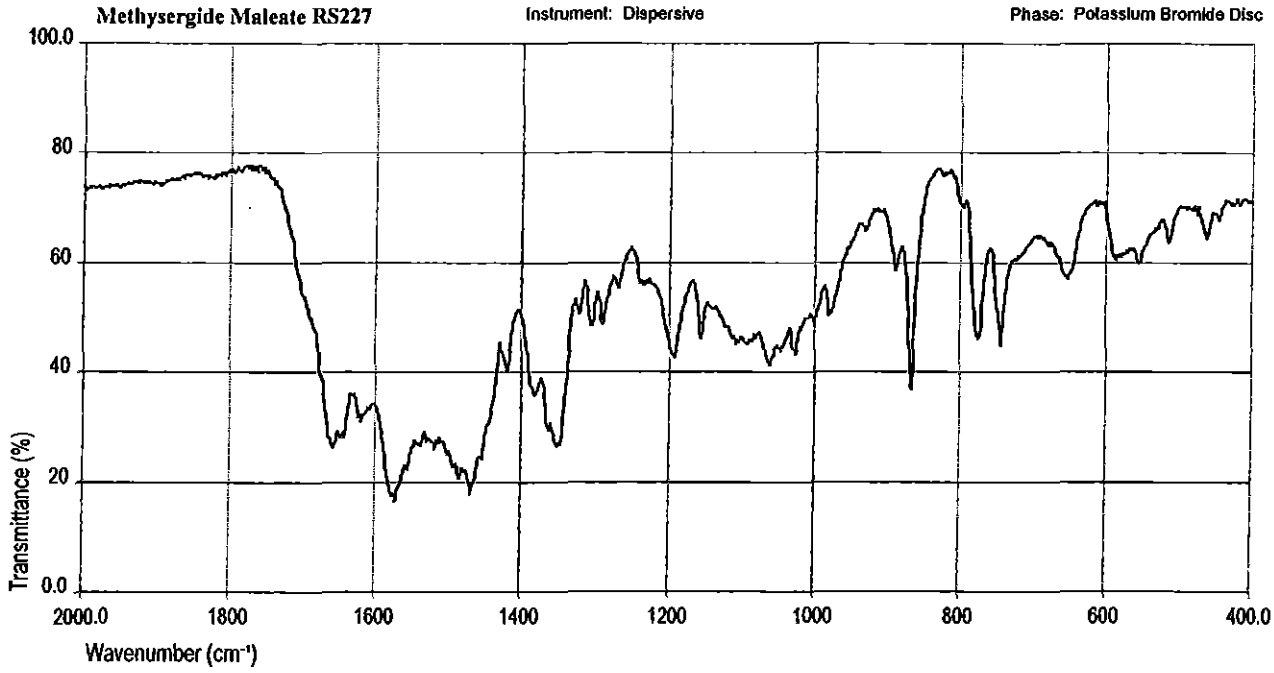


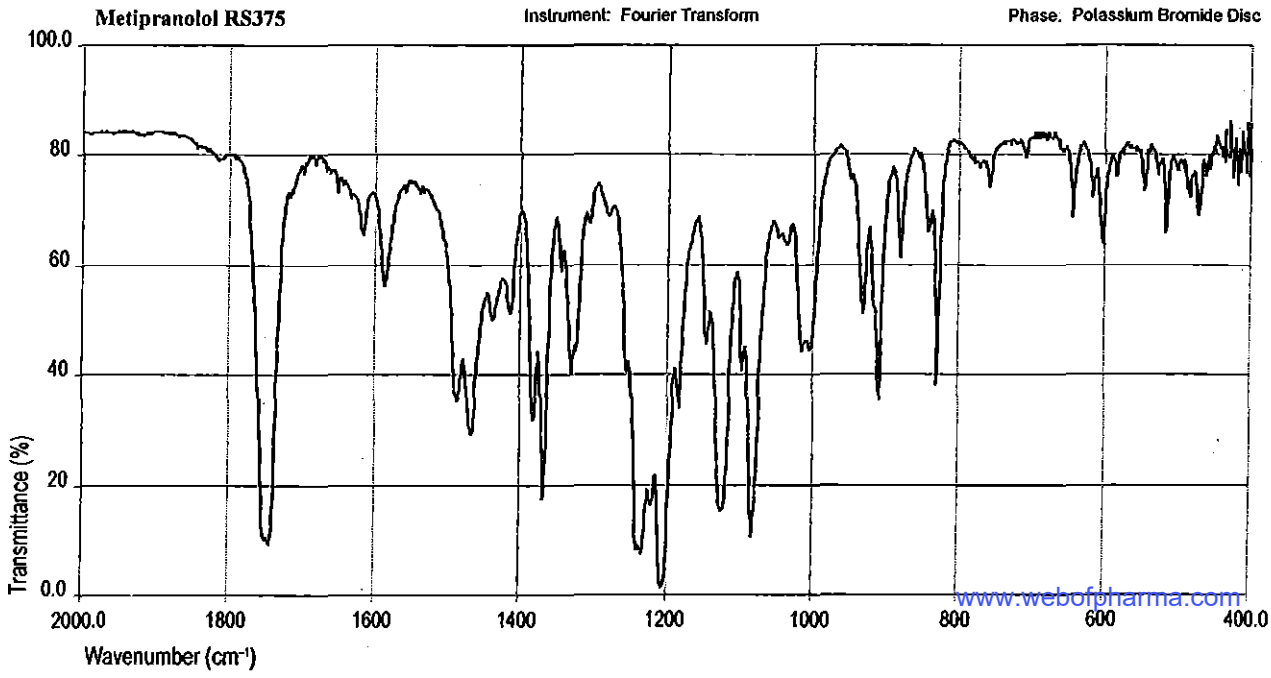


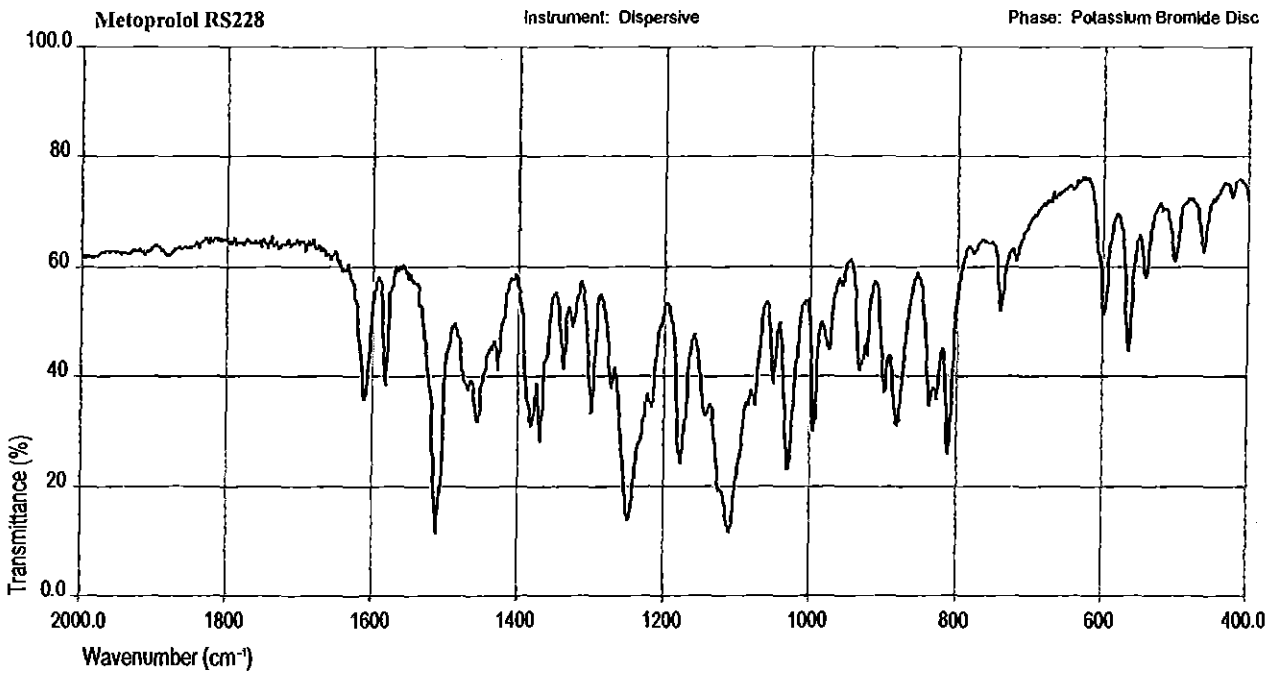


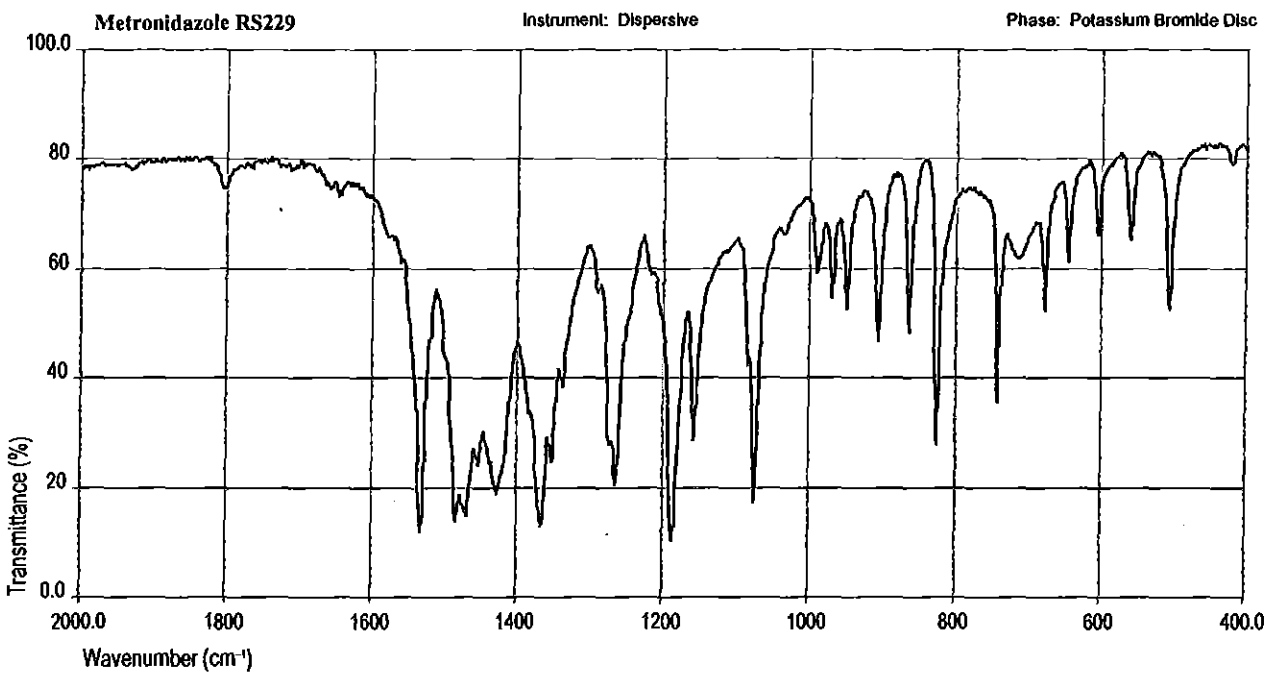


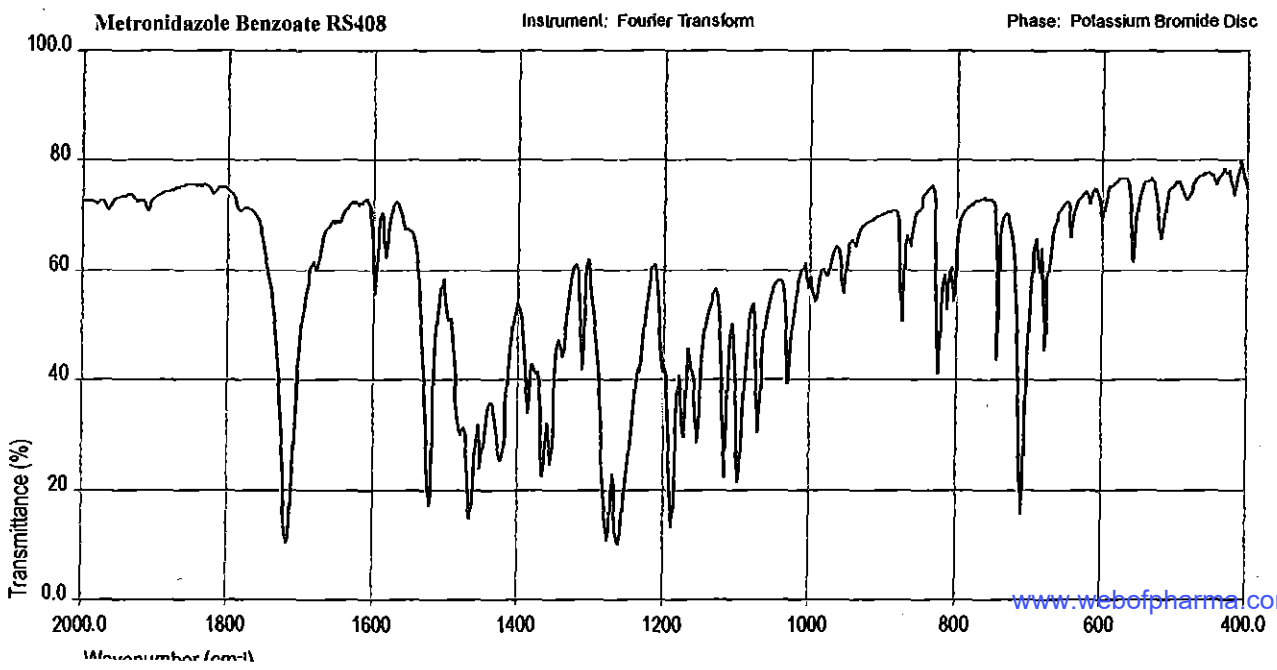


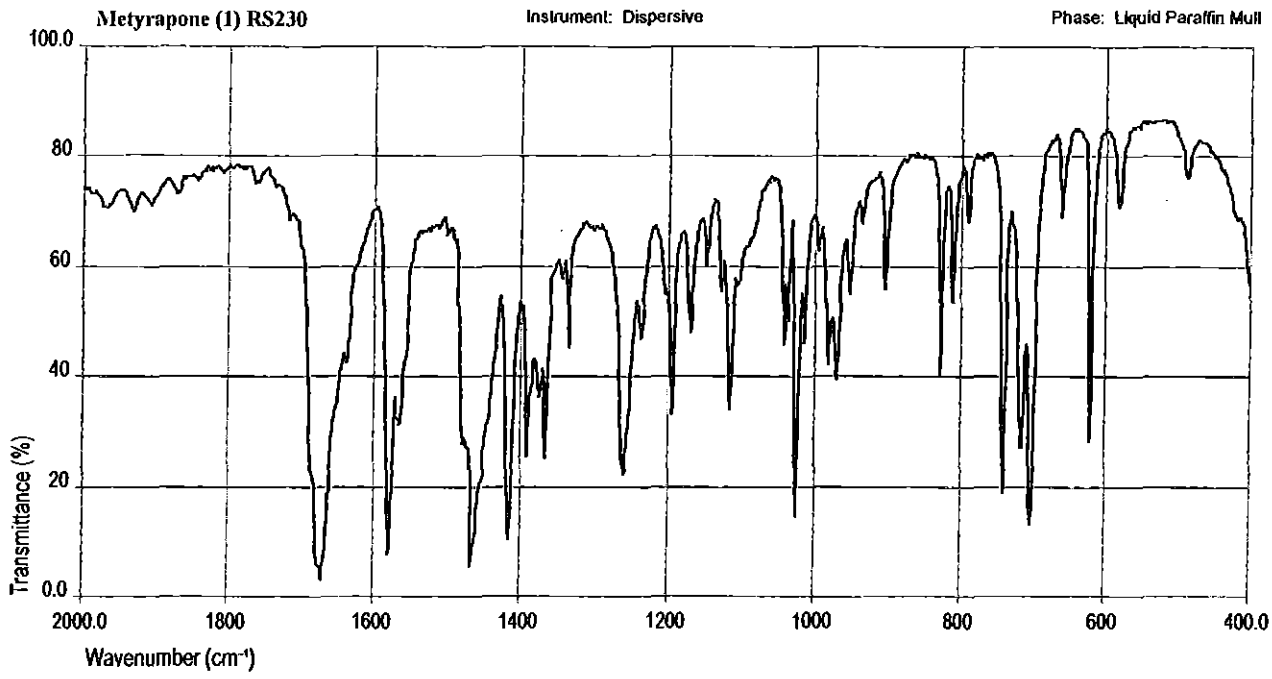


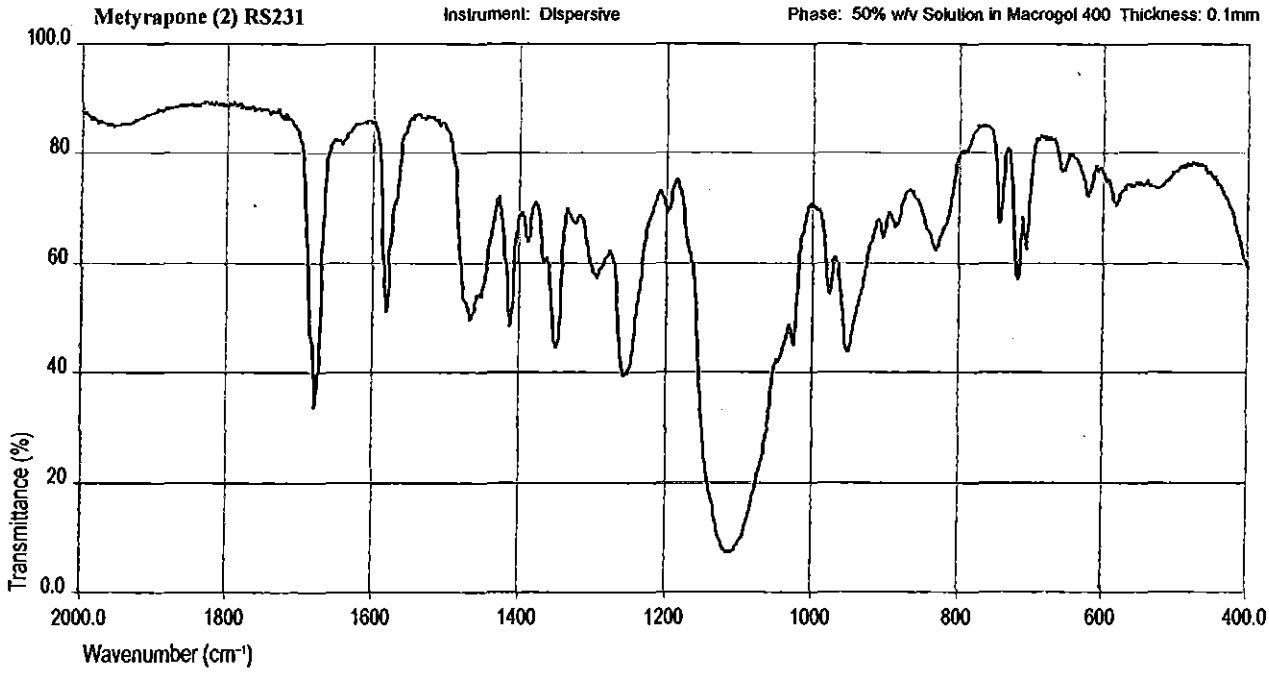


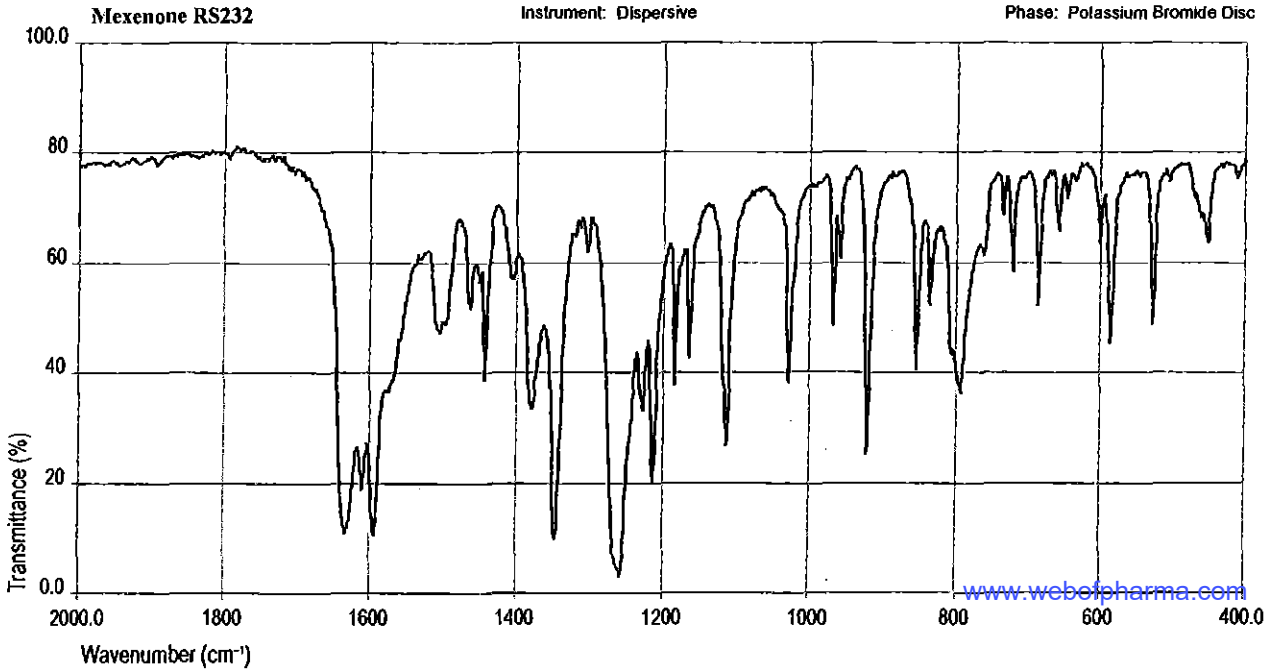


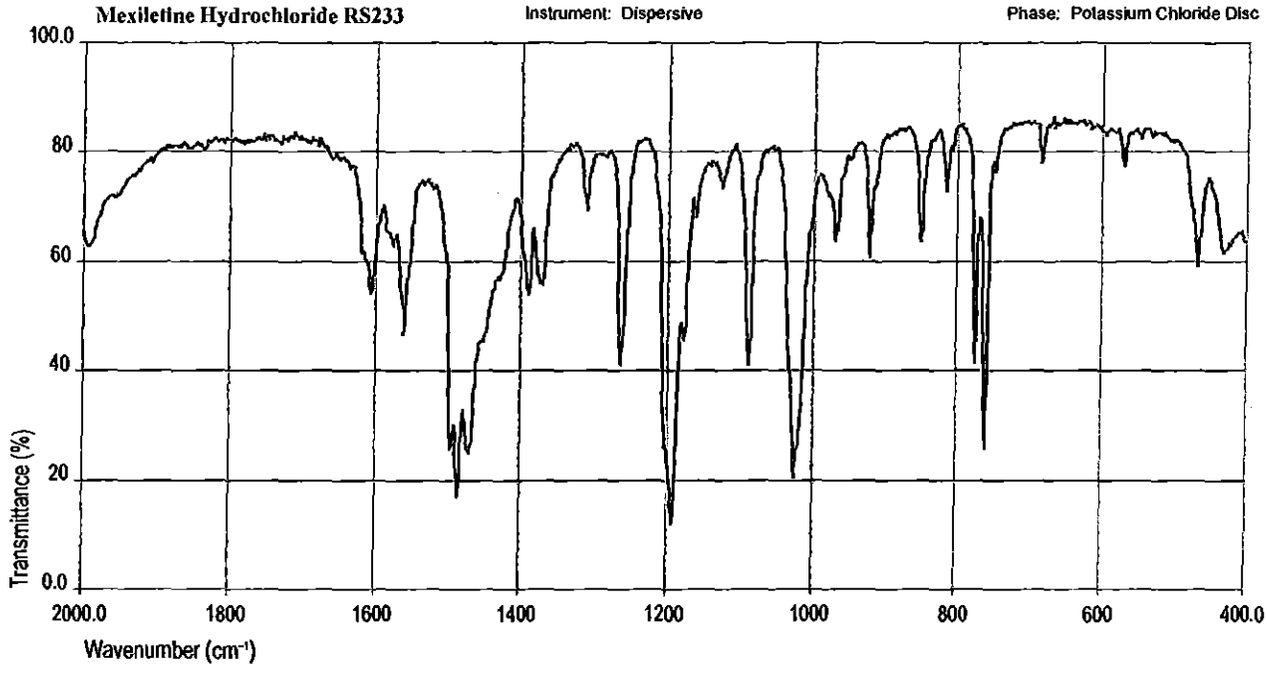


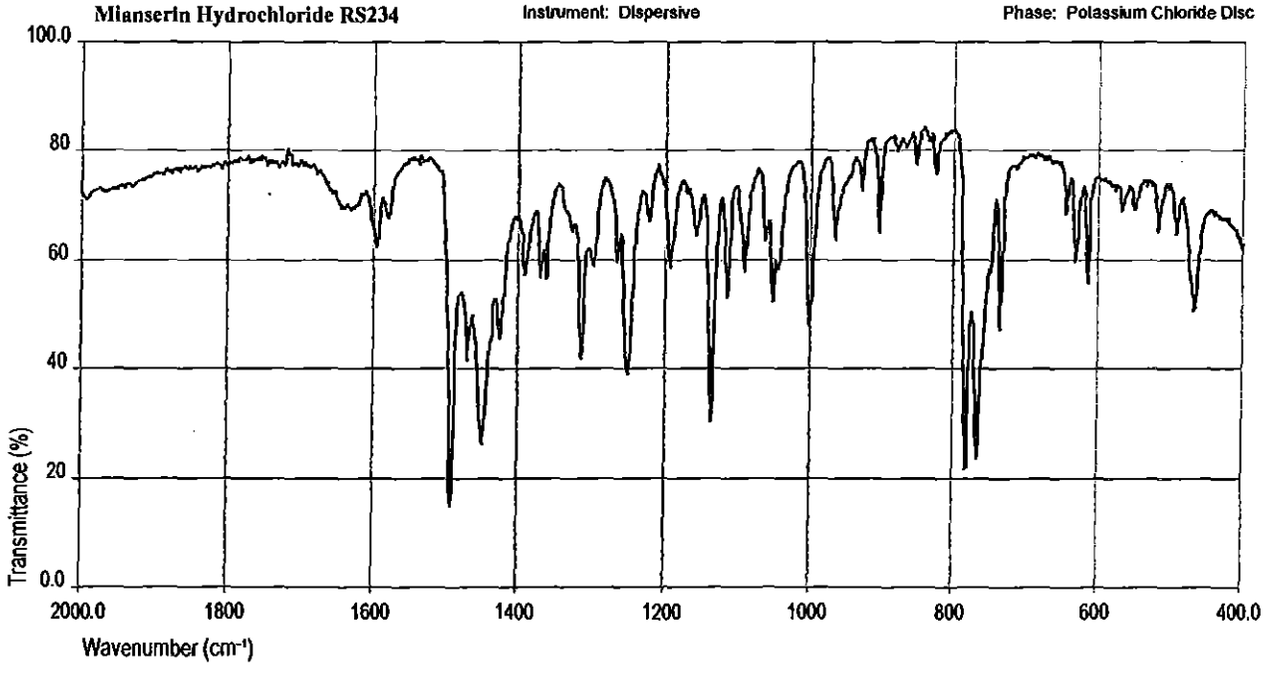


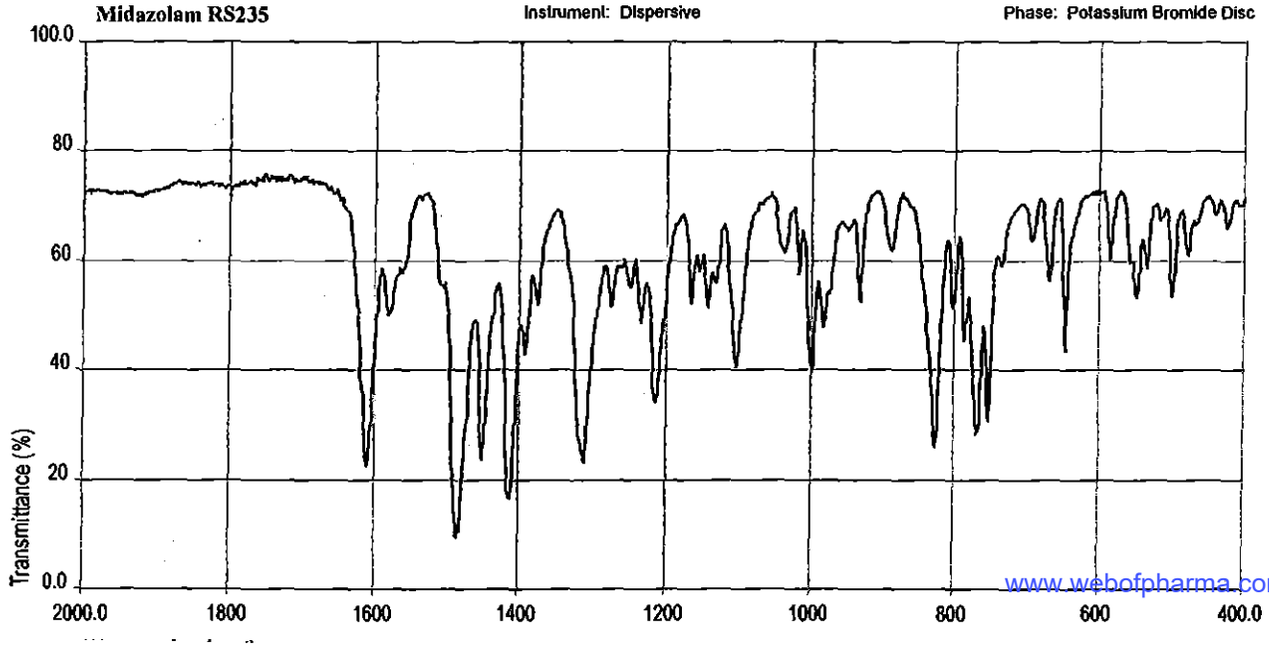


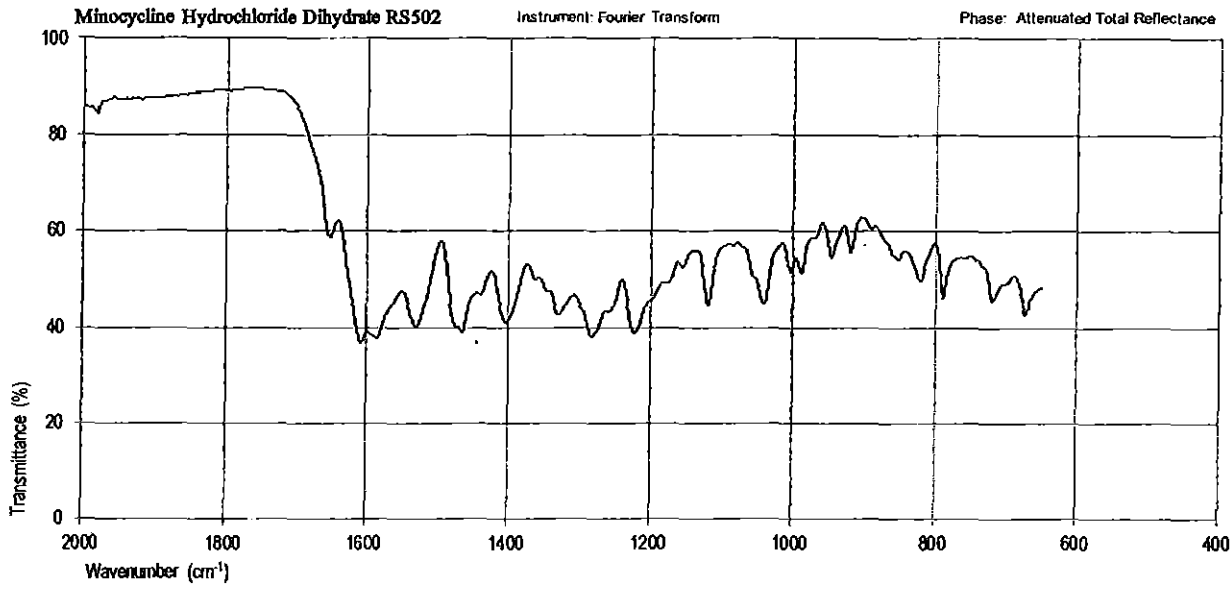


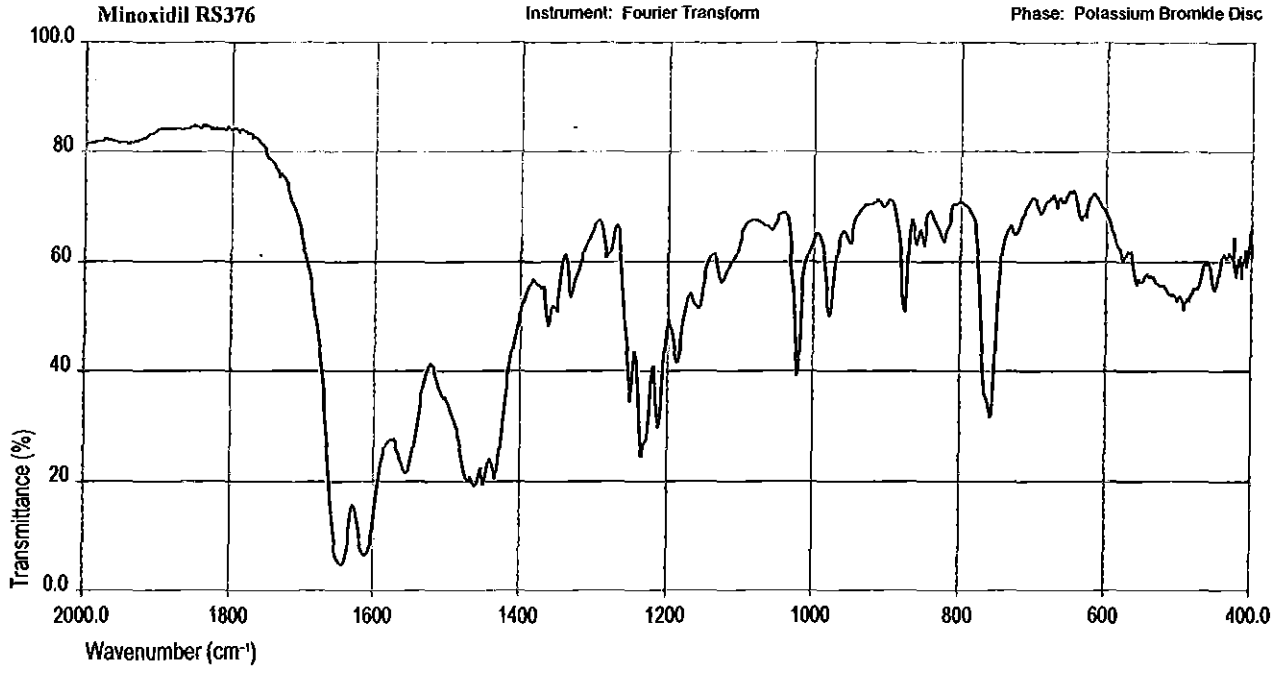


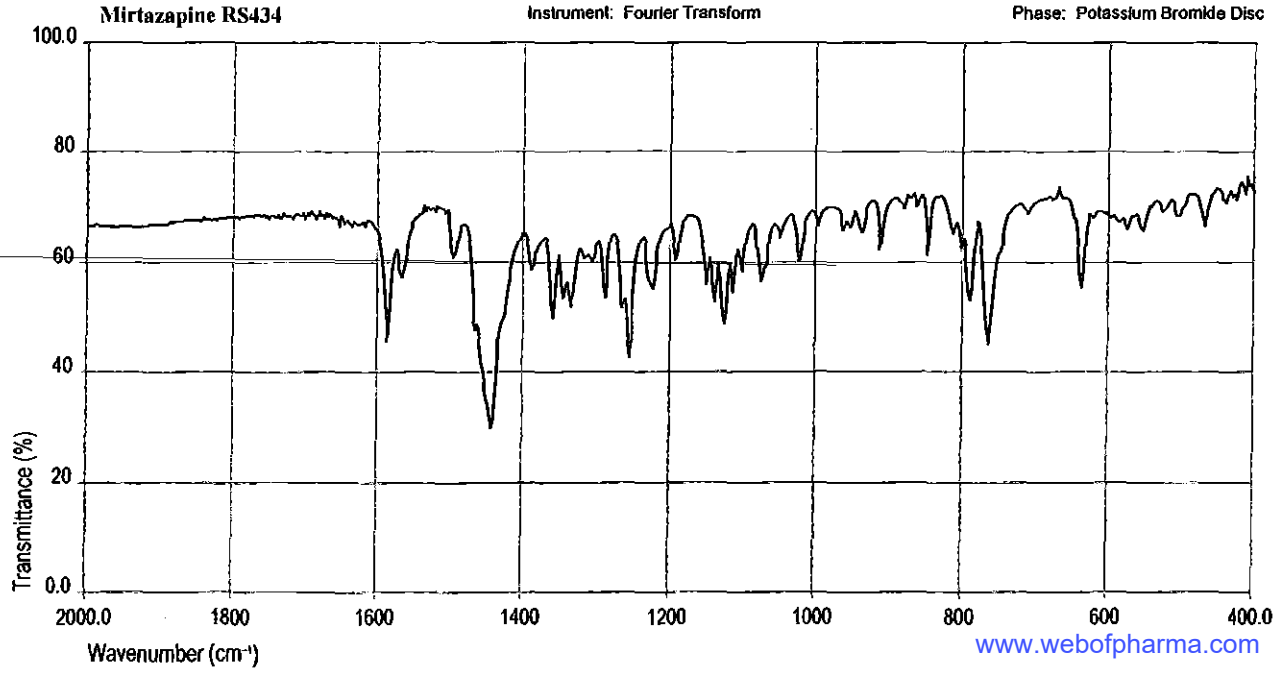


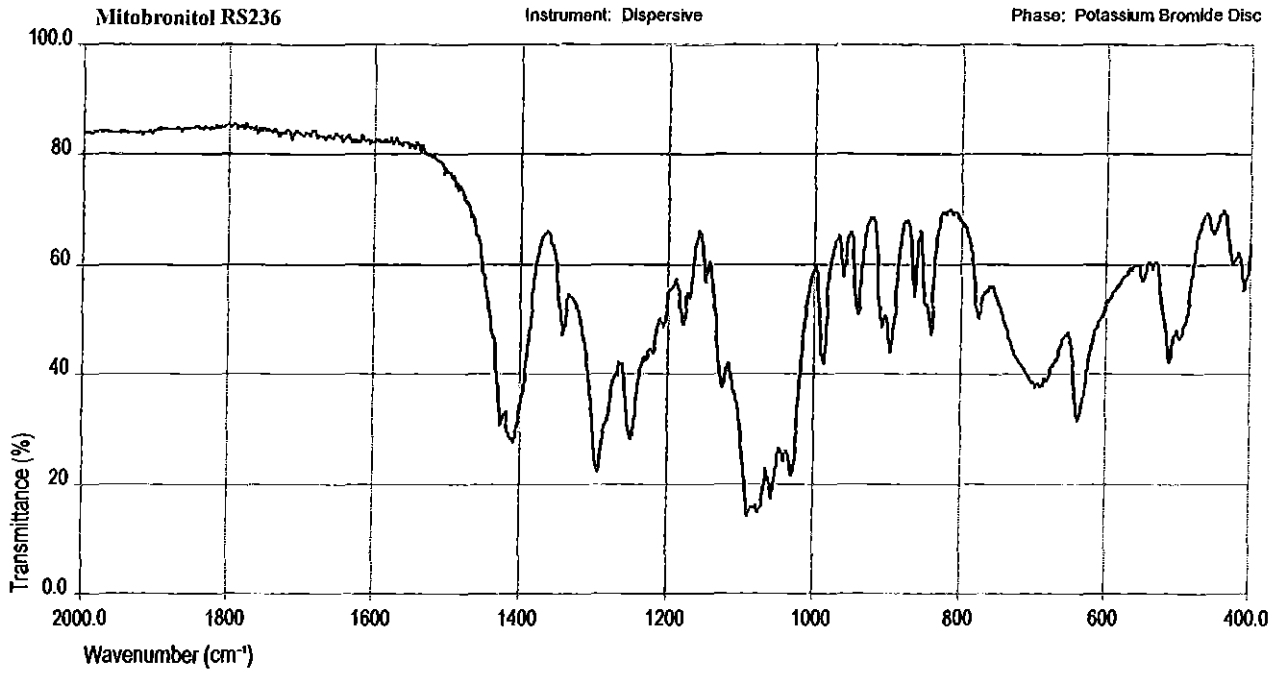


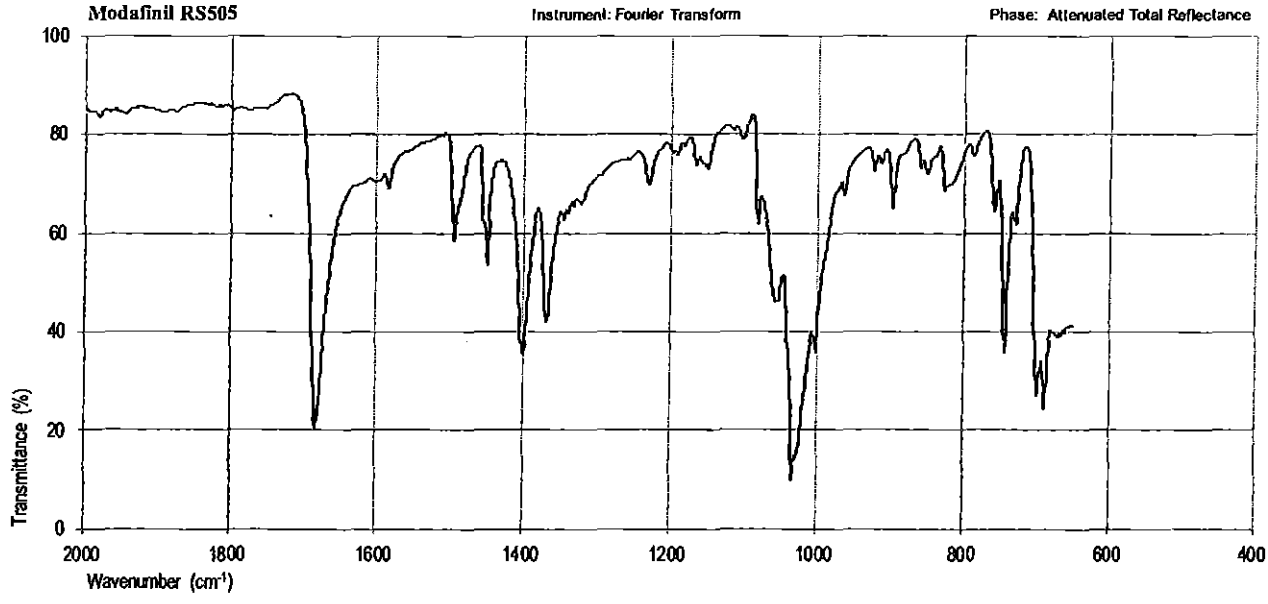


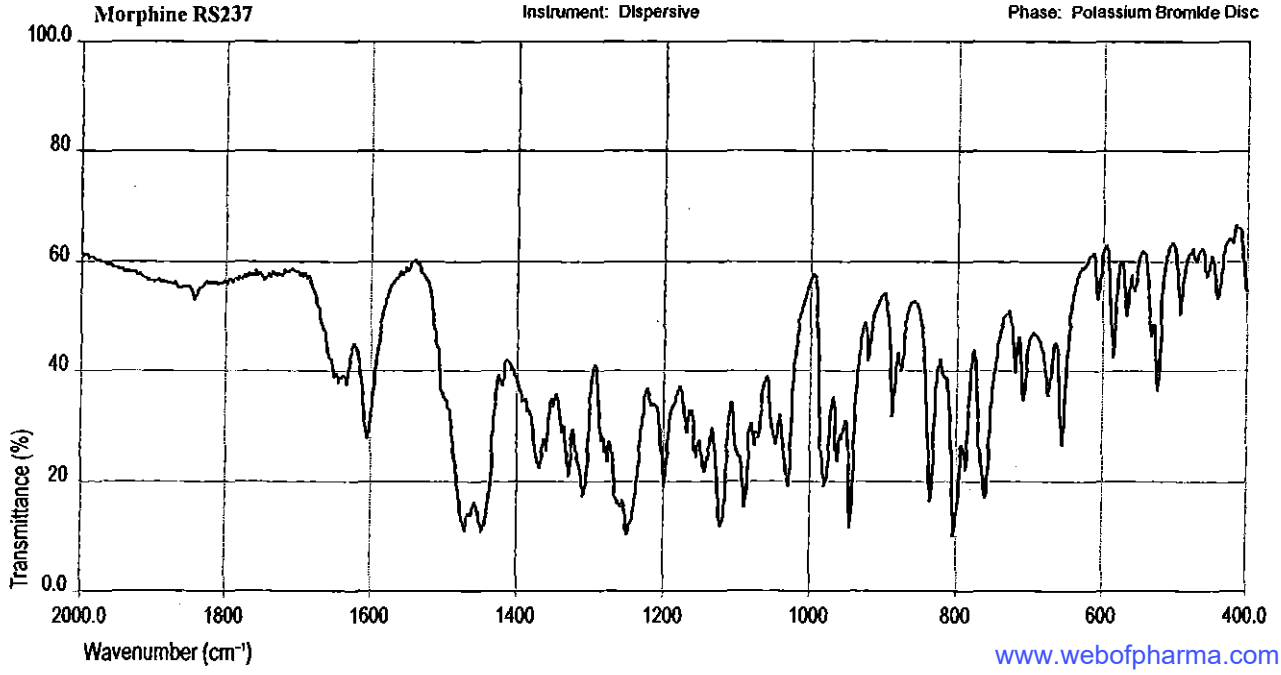








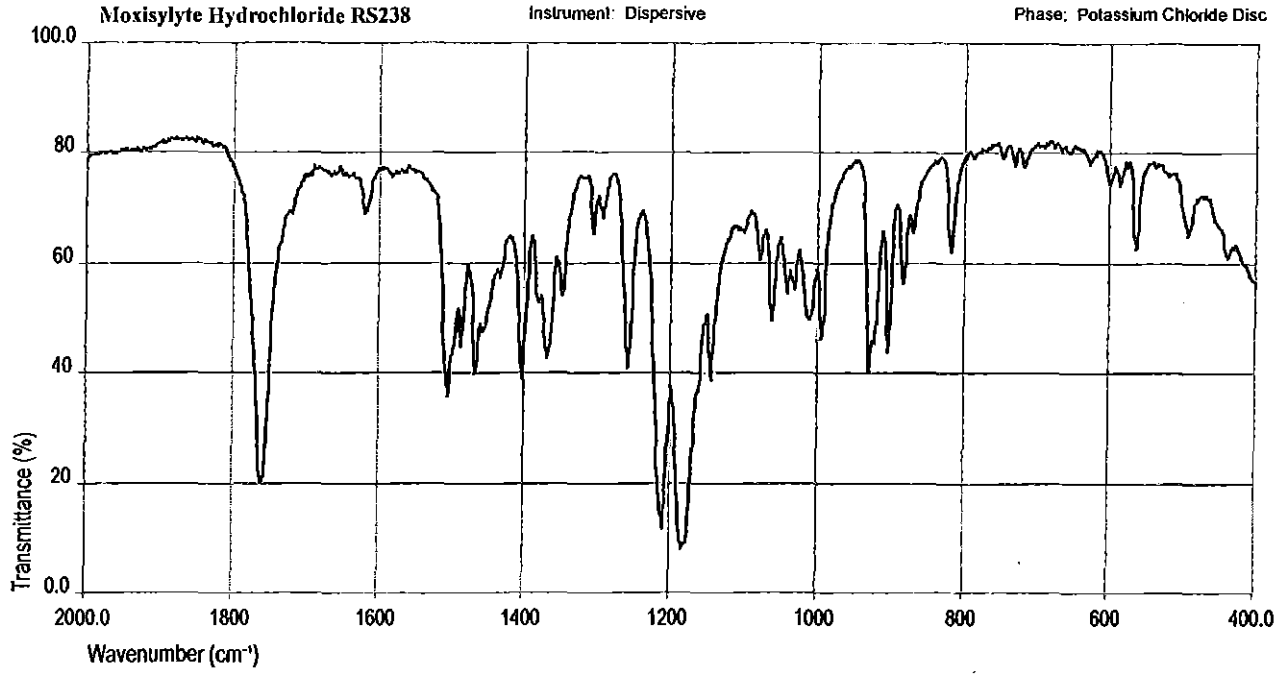




Moxisylyte Hydrochloride RS238

Instrument: Dispersive

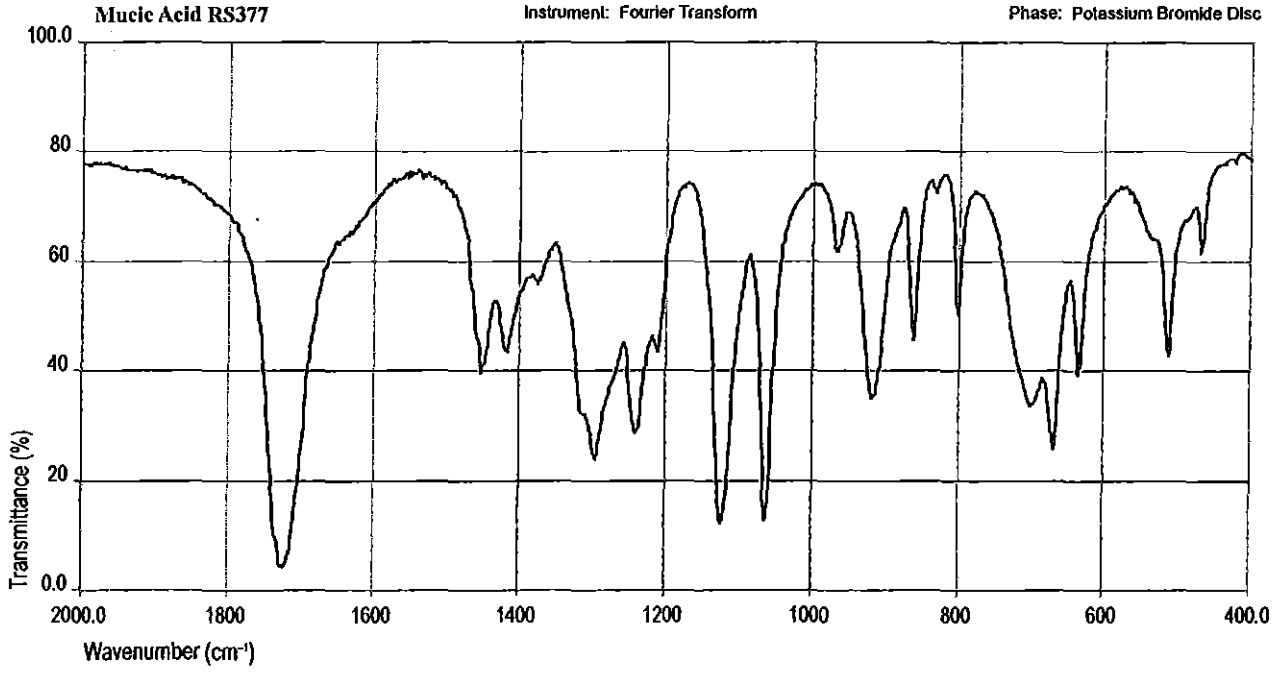
Phase: Potassium Chloride Disc



Mucic Acid RS377

Instrument: Fourier Transform

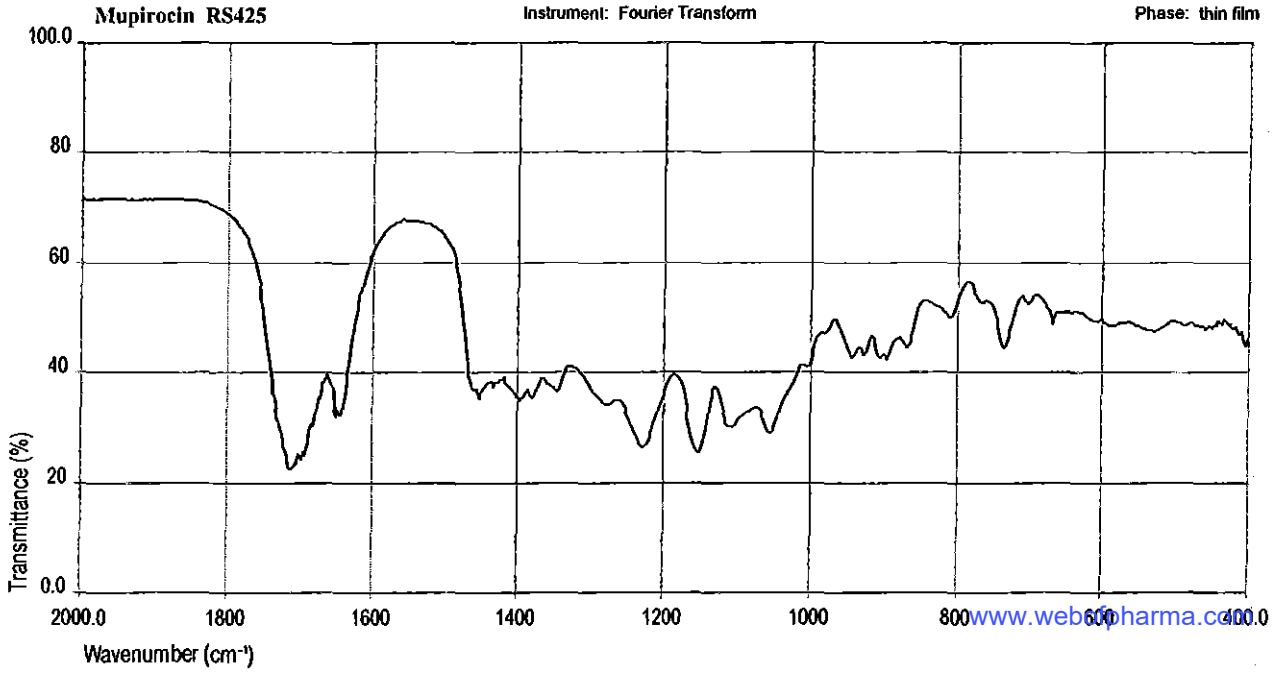
Phase: Potassium Bromide Disc

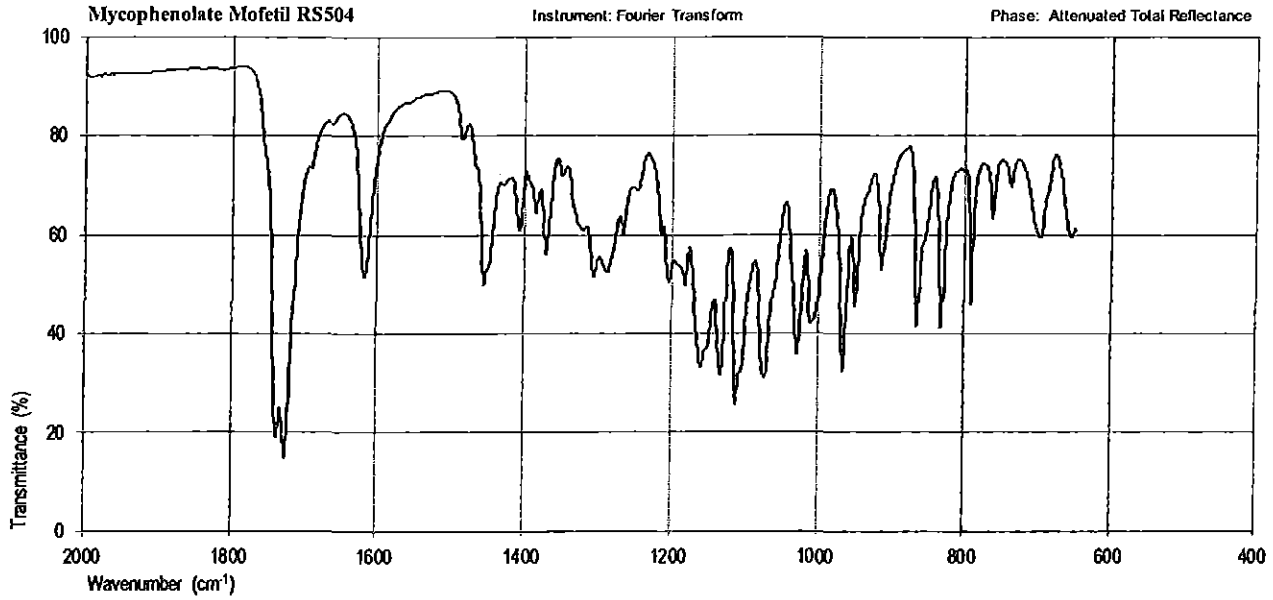


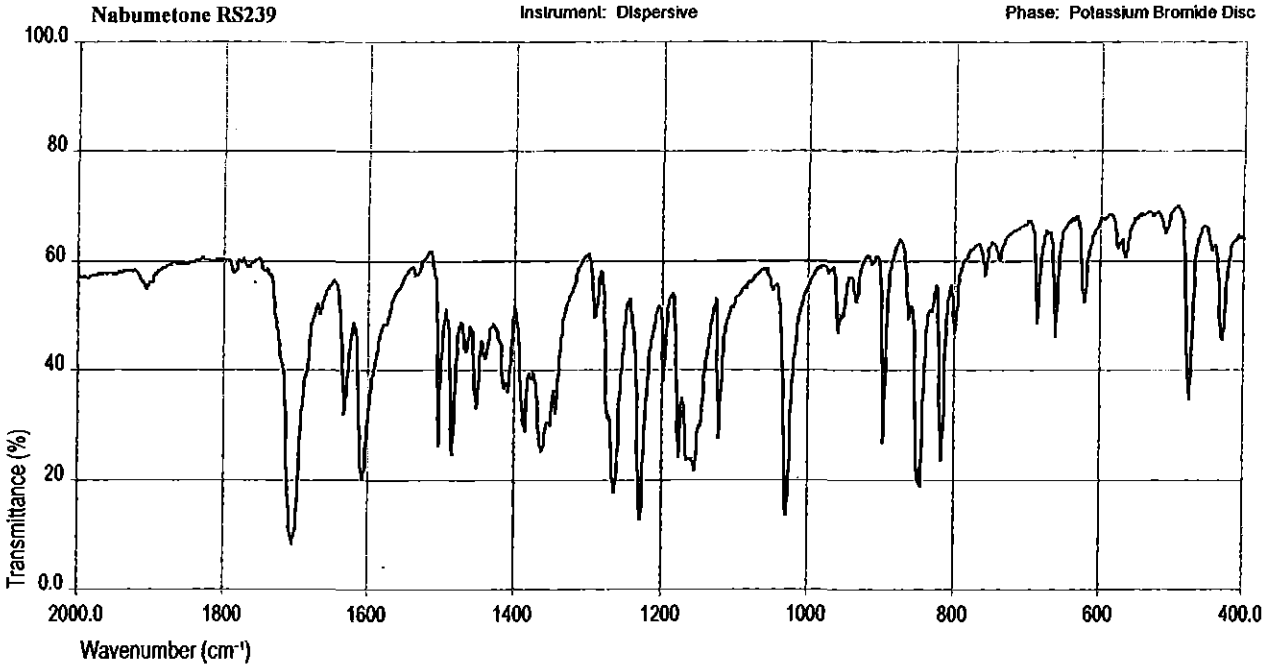
Mupirocin RS425

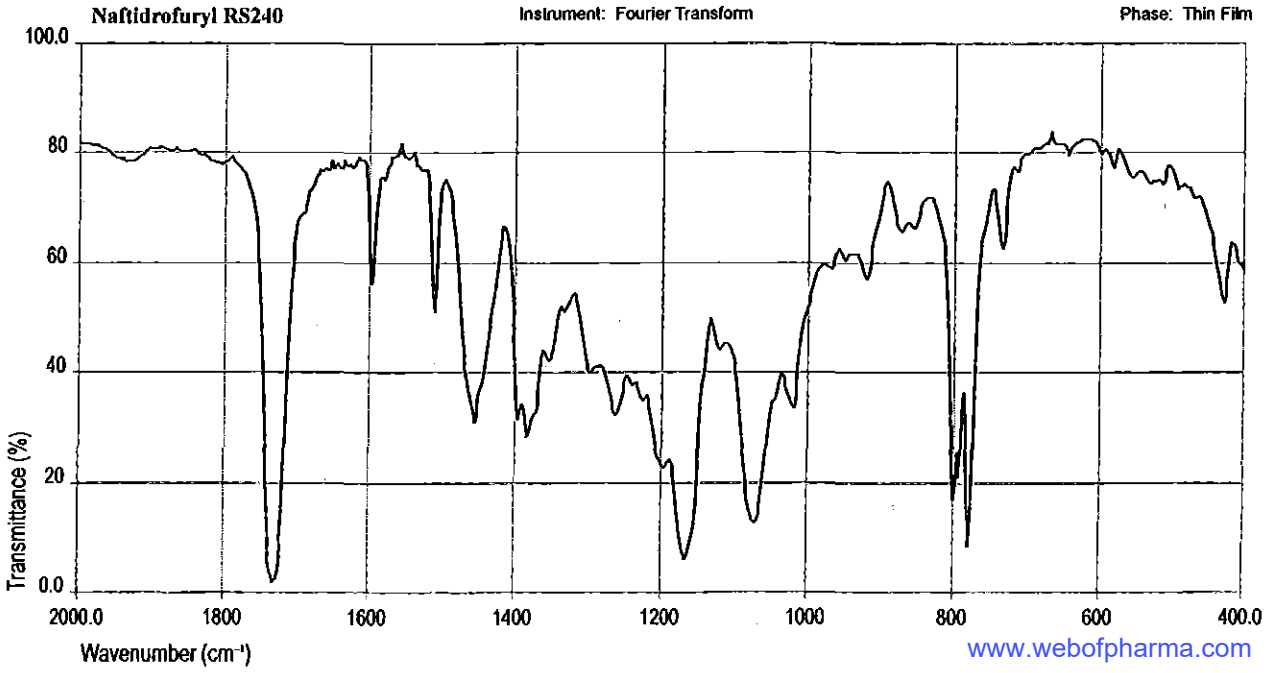
Instrument: Fourier Transform

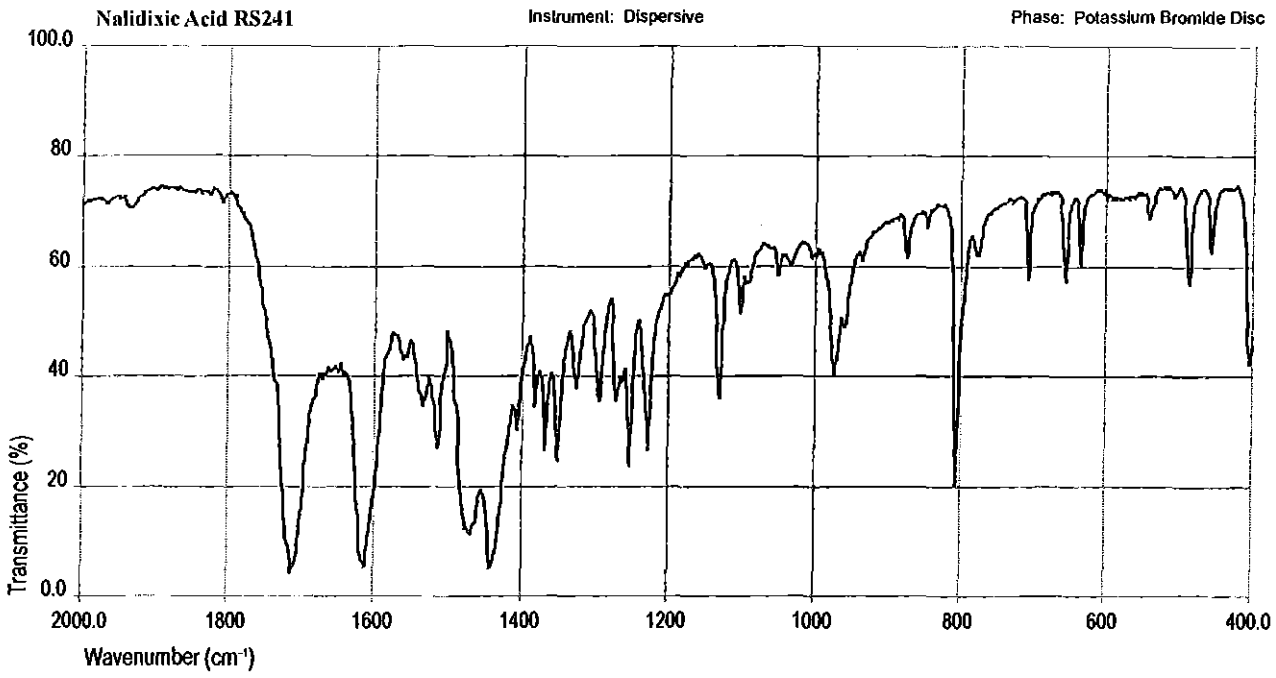
Phase: thin film

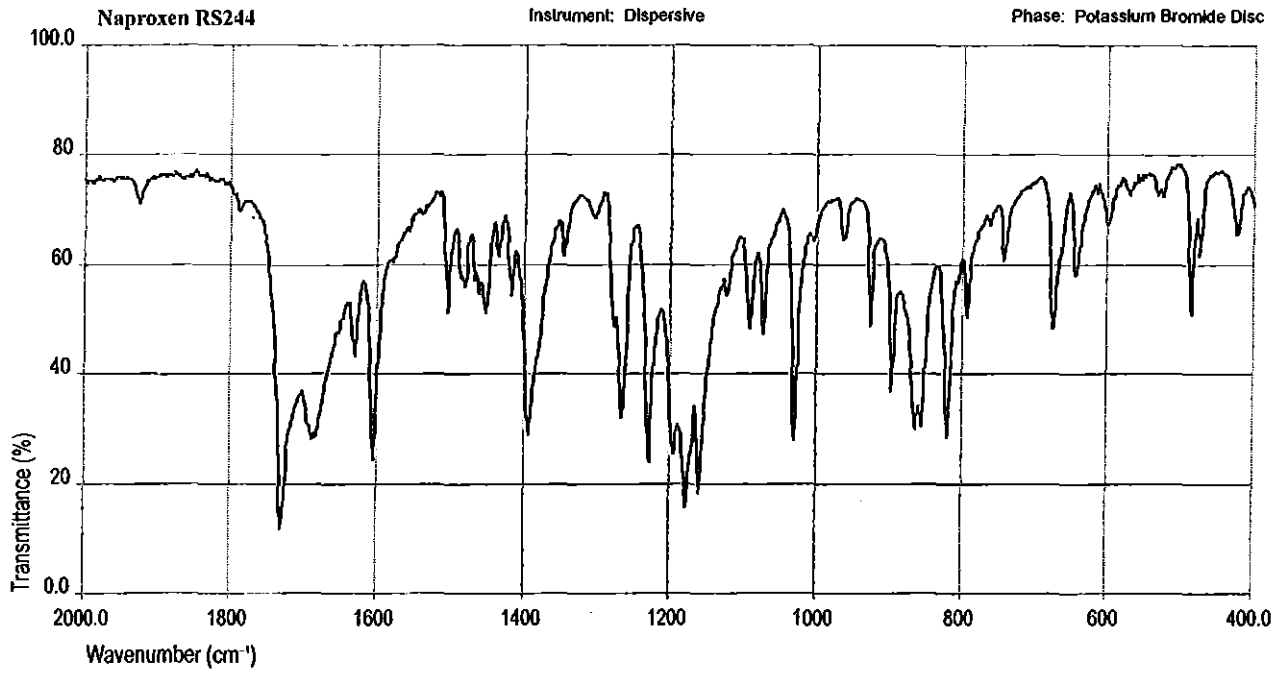


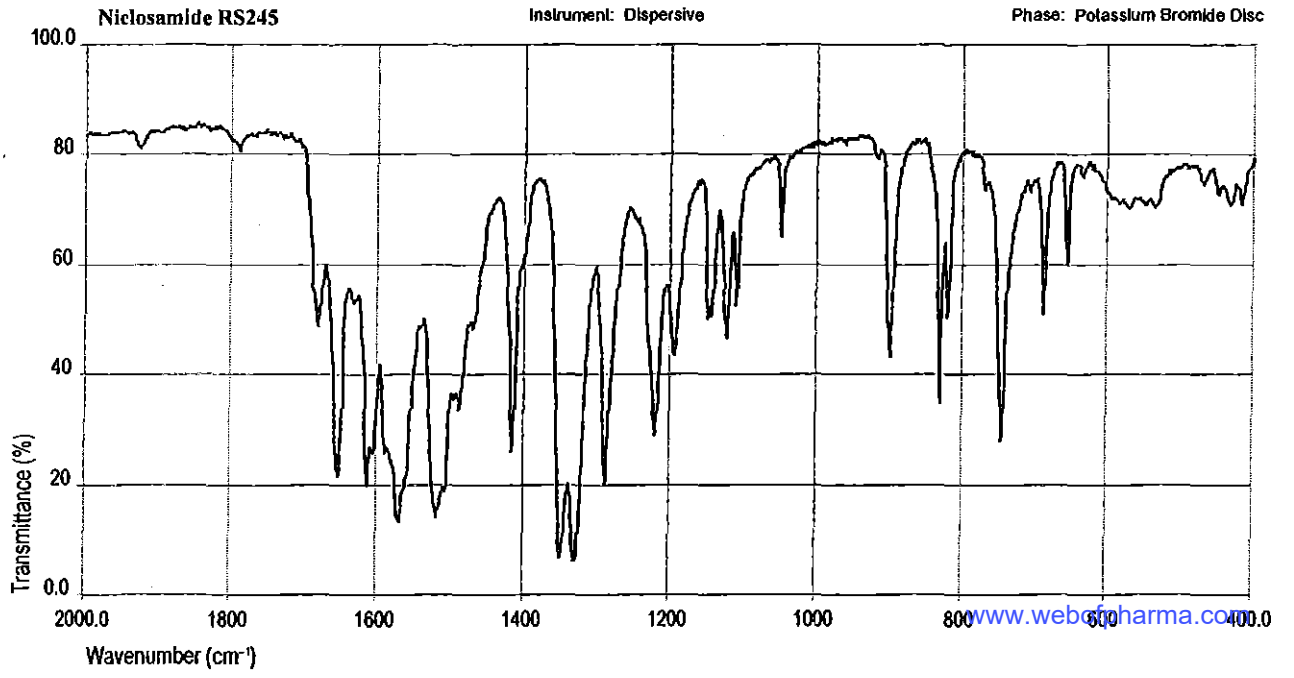


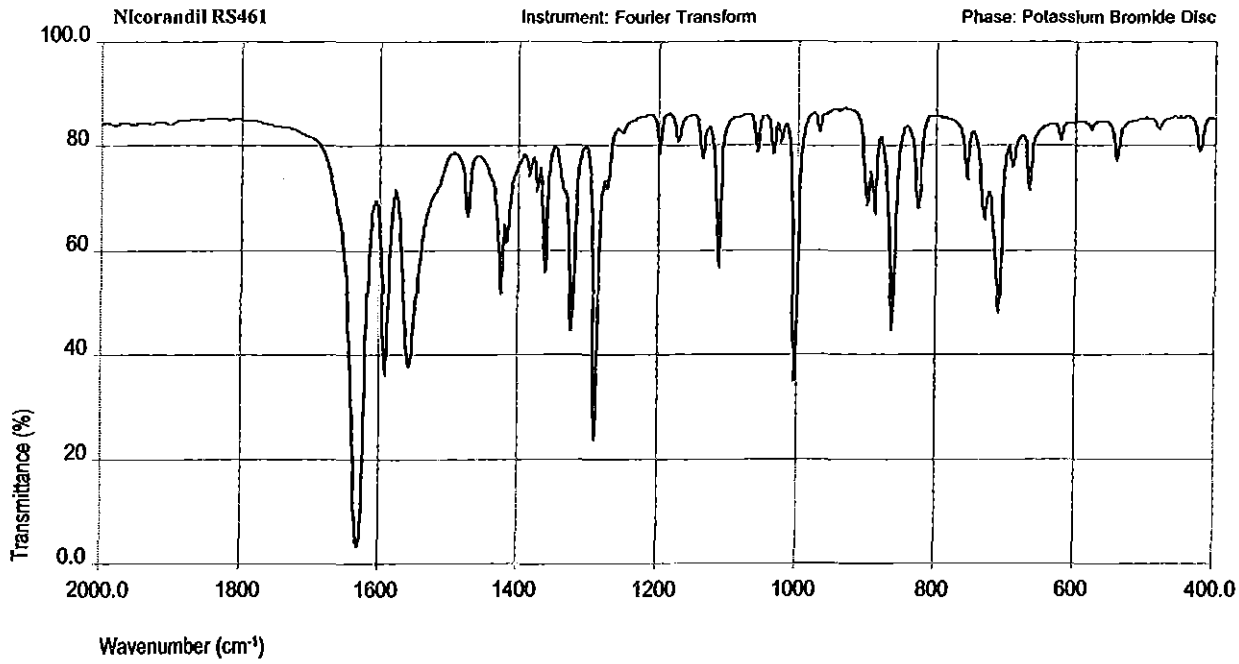


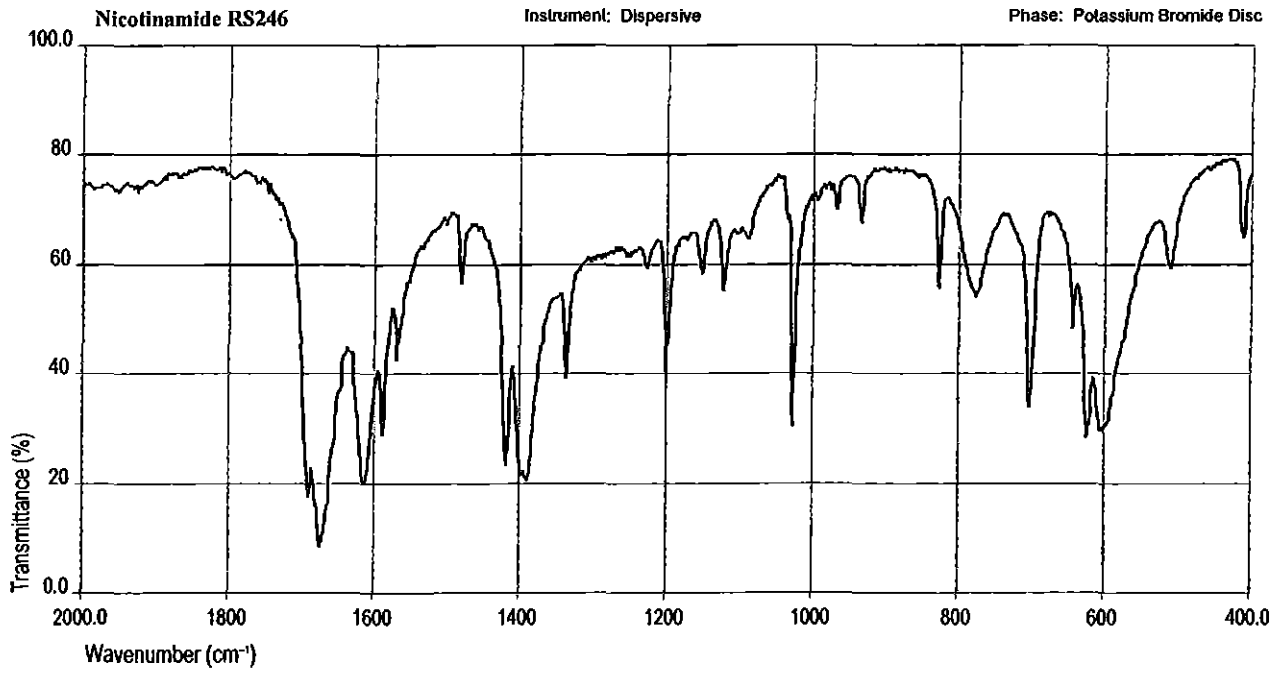


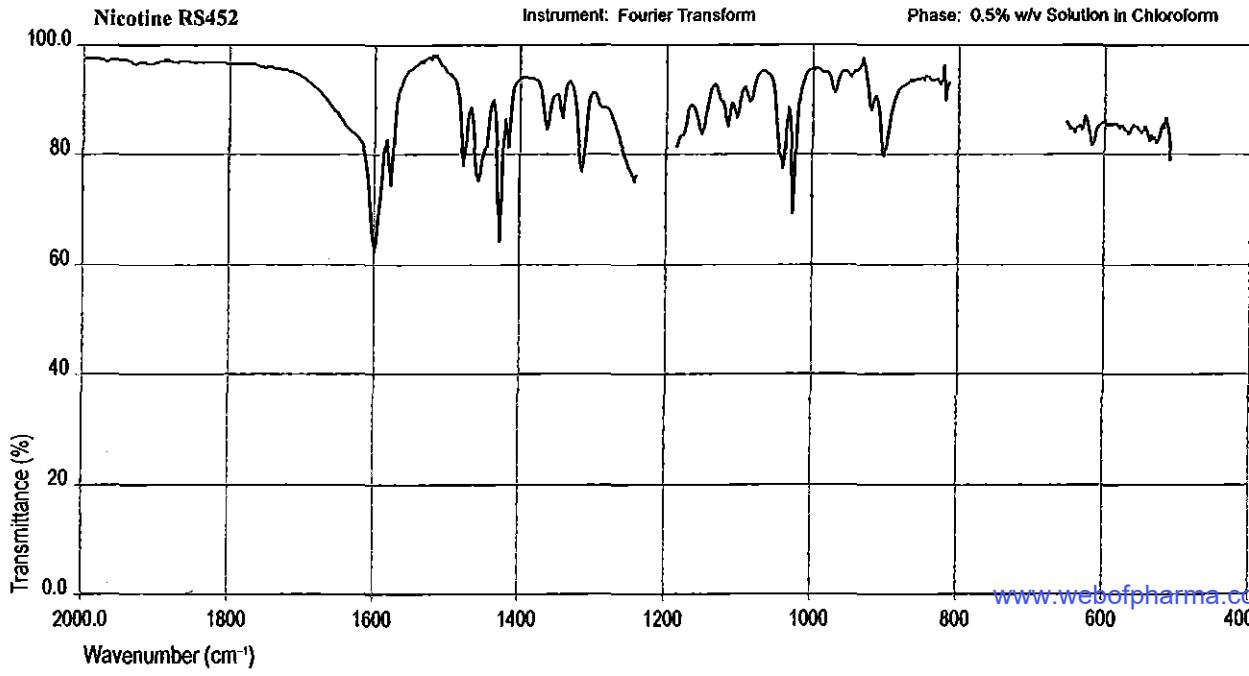


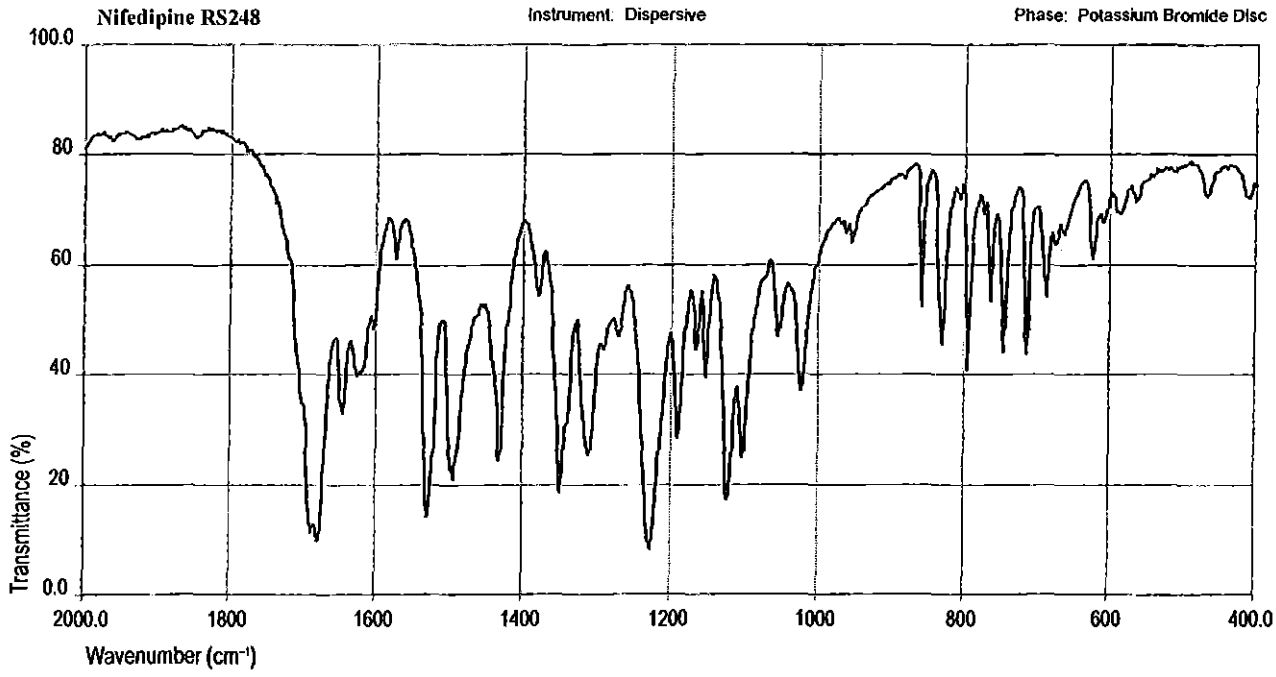


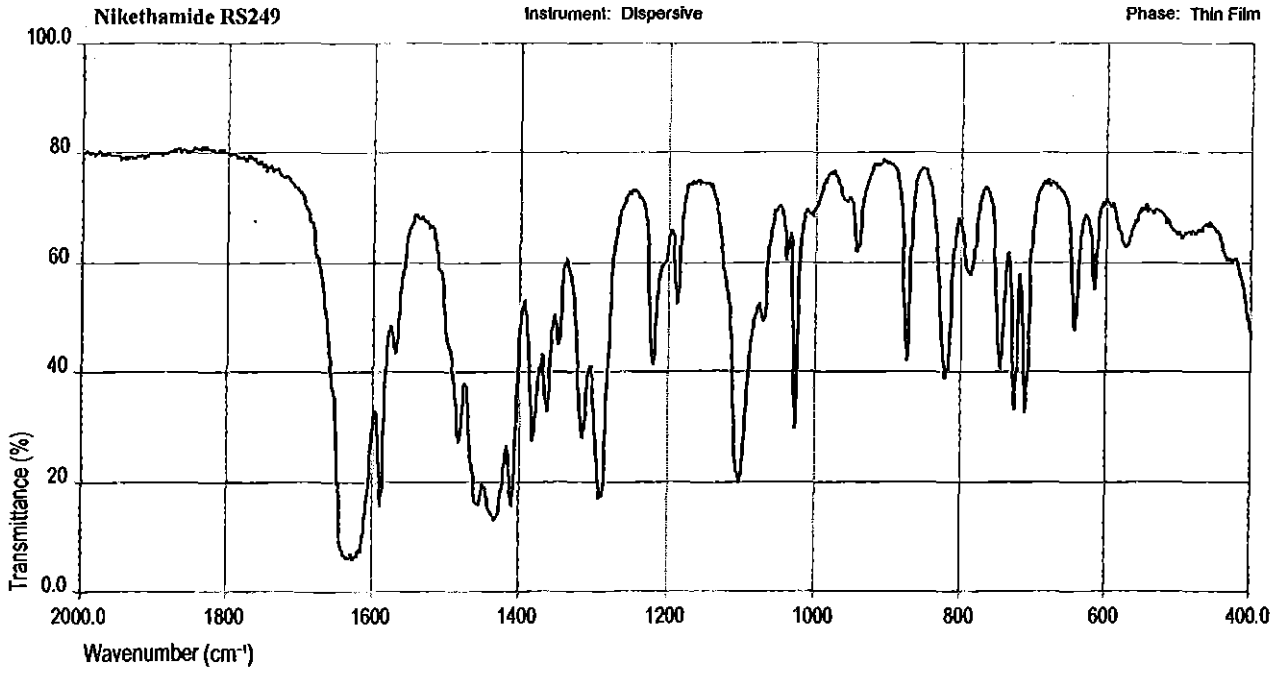


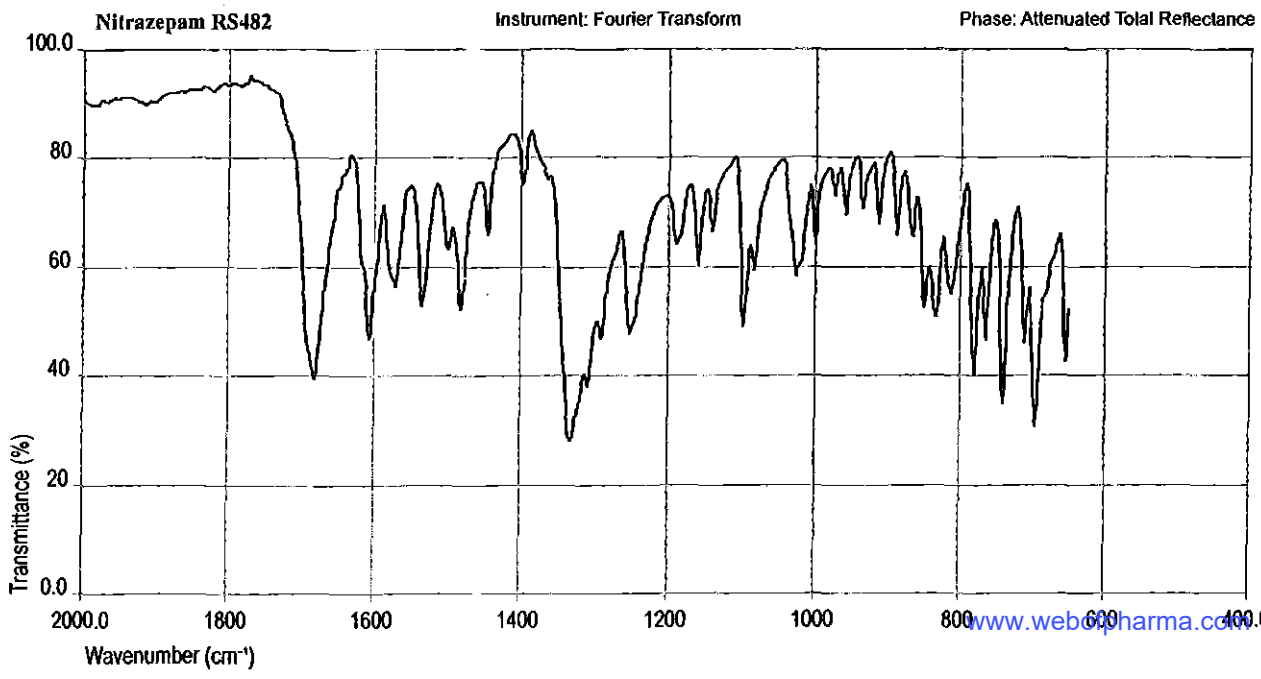


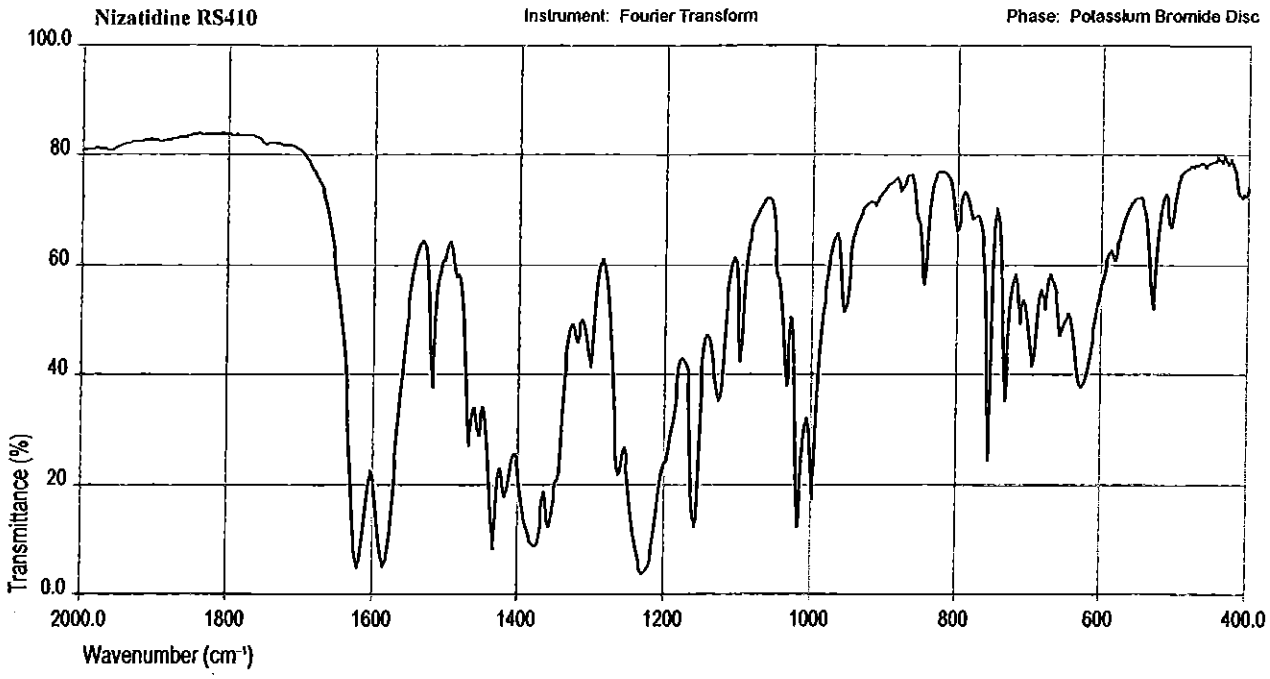


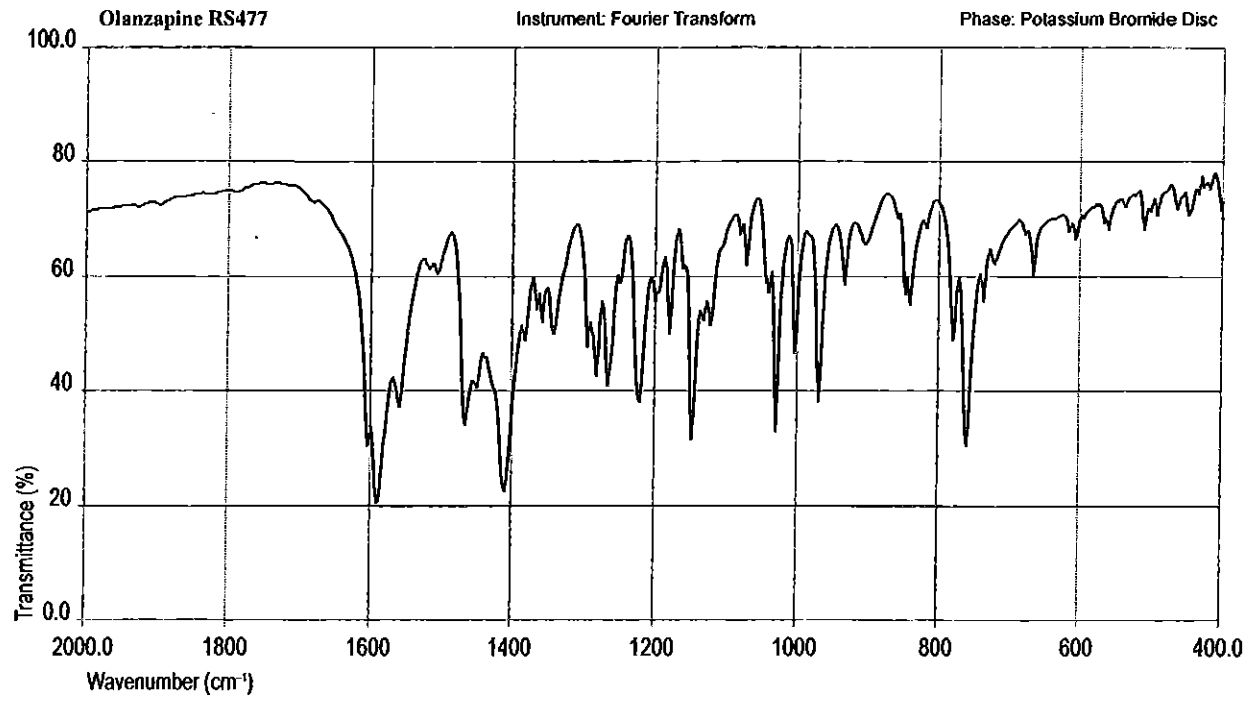


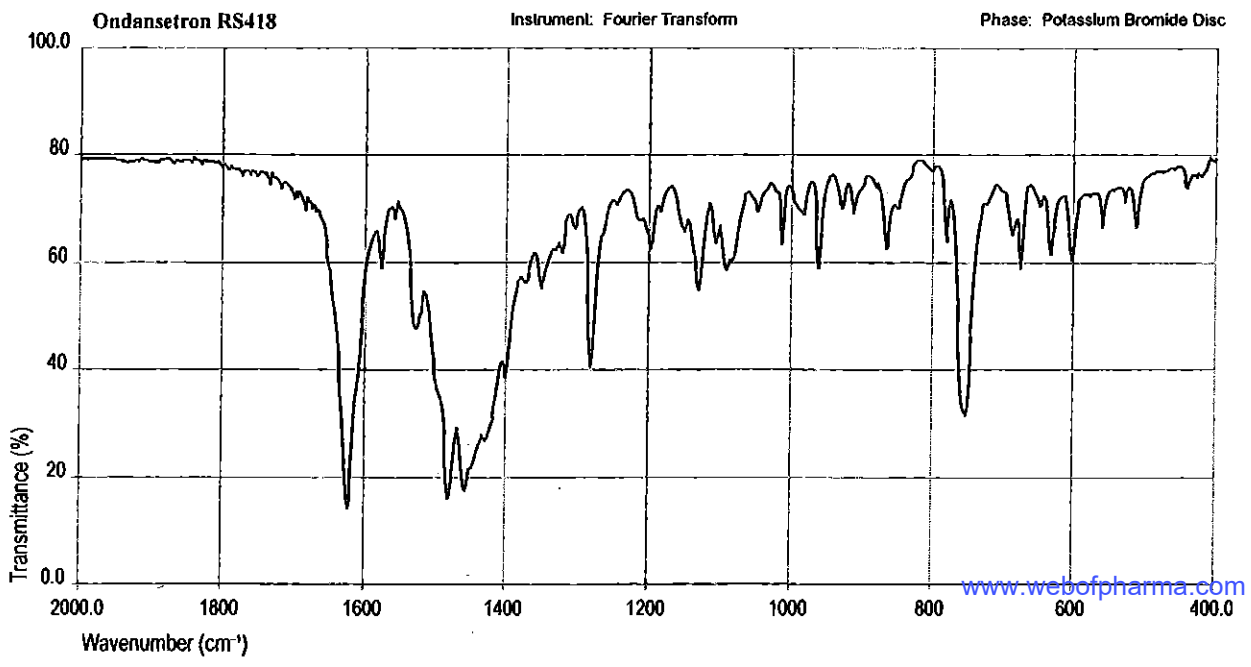




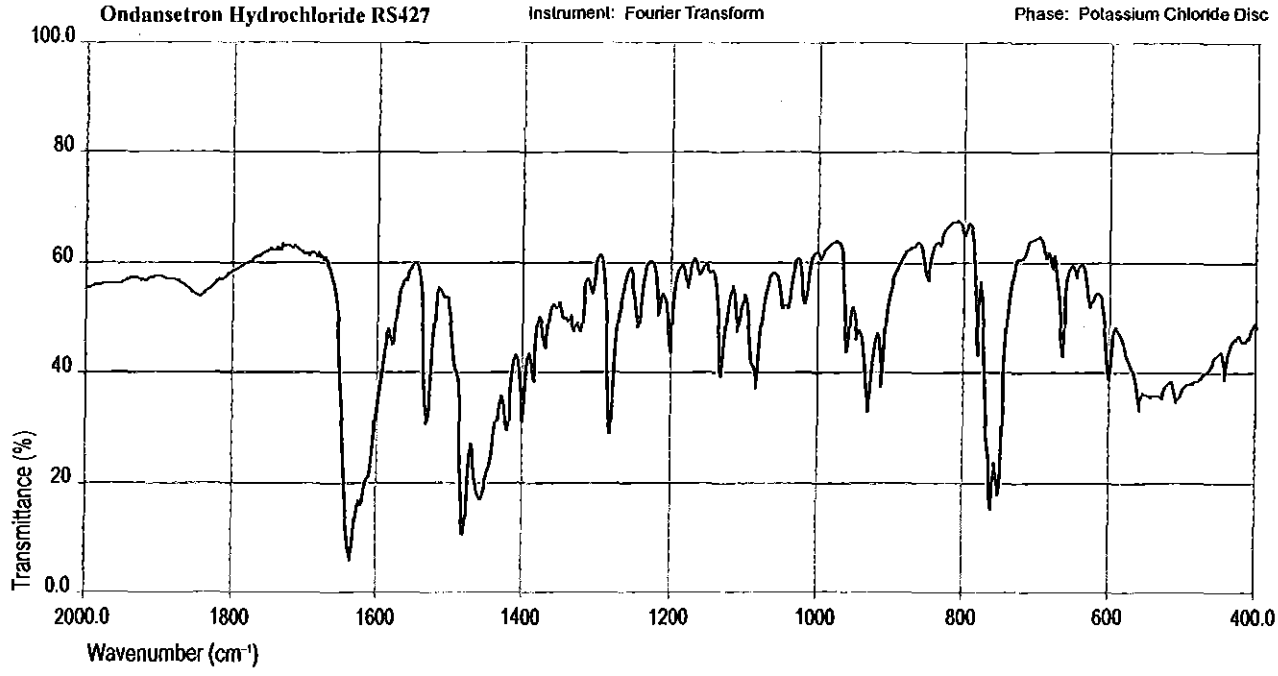




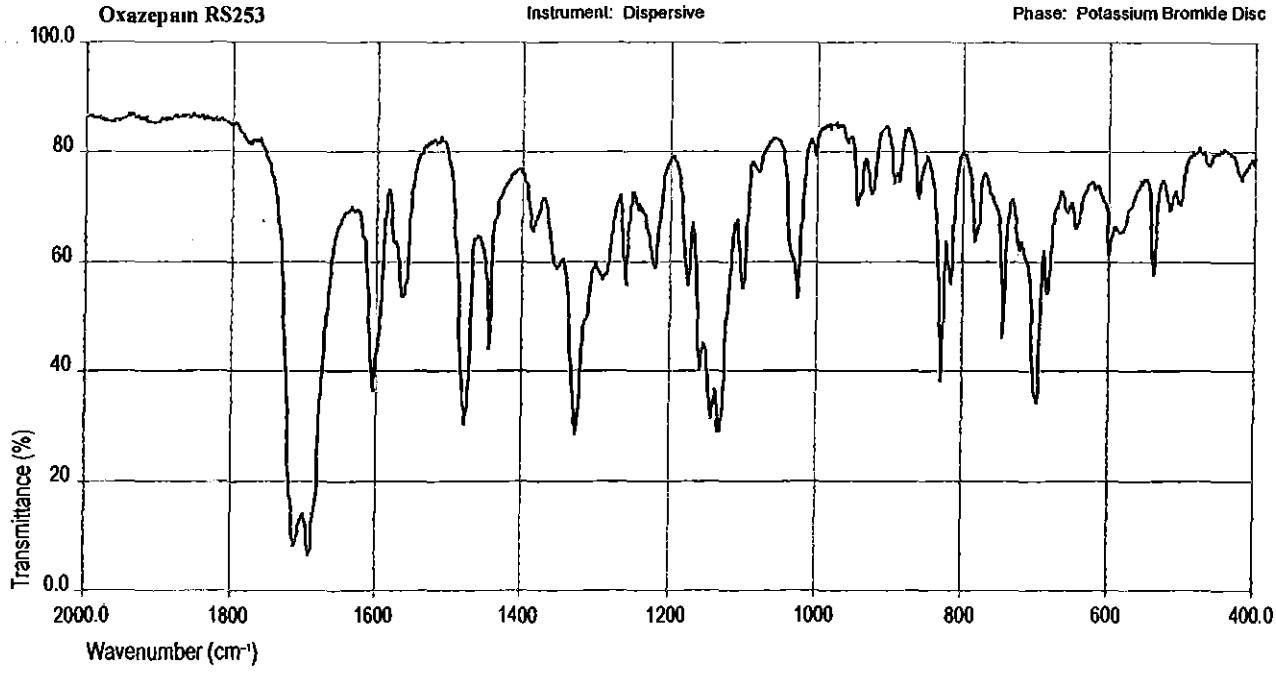




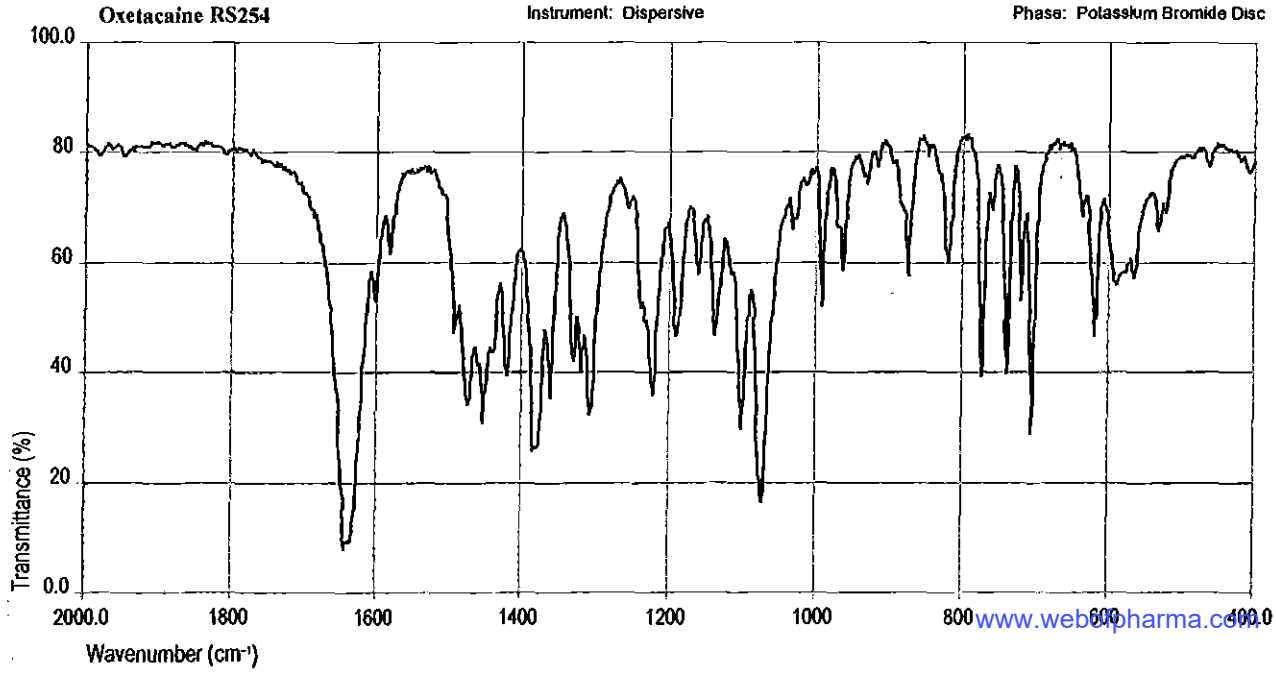
Ondansetron Hydrochloride RS427 Instrument: Fourier Transform Phase: Potassium Chloride Disc

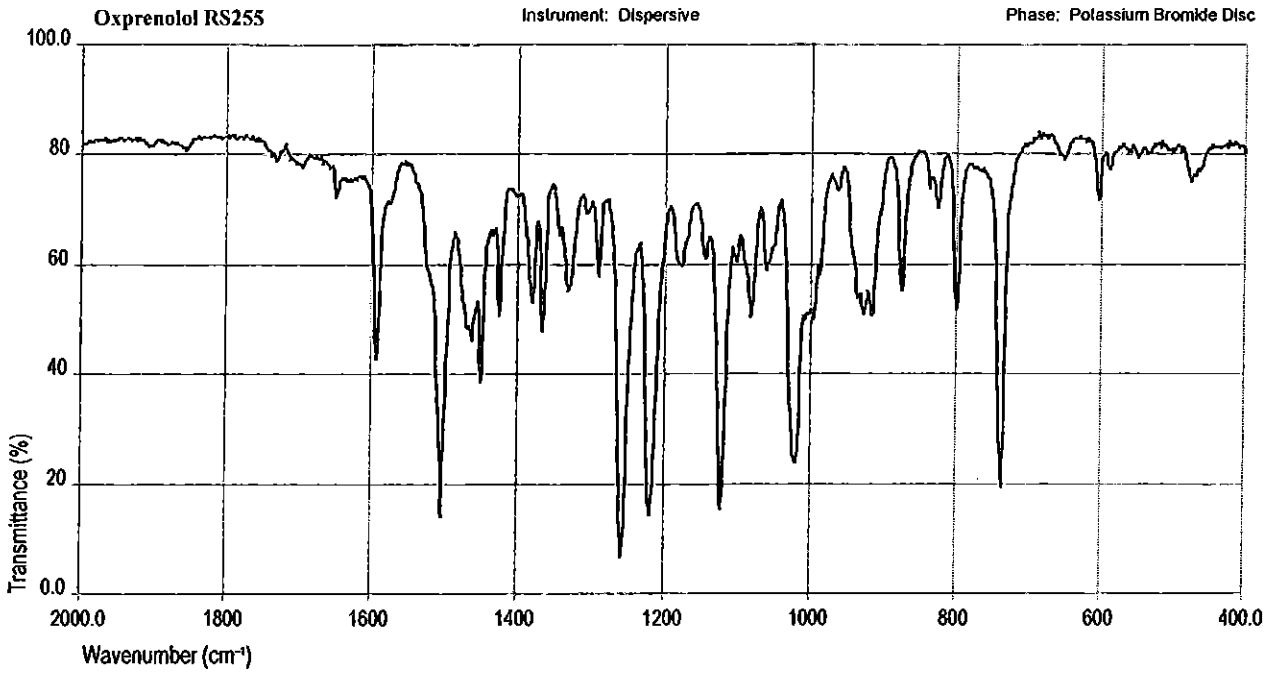


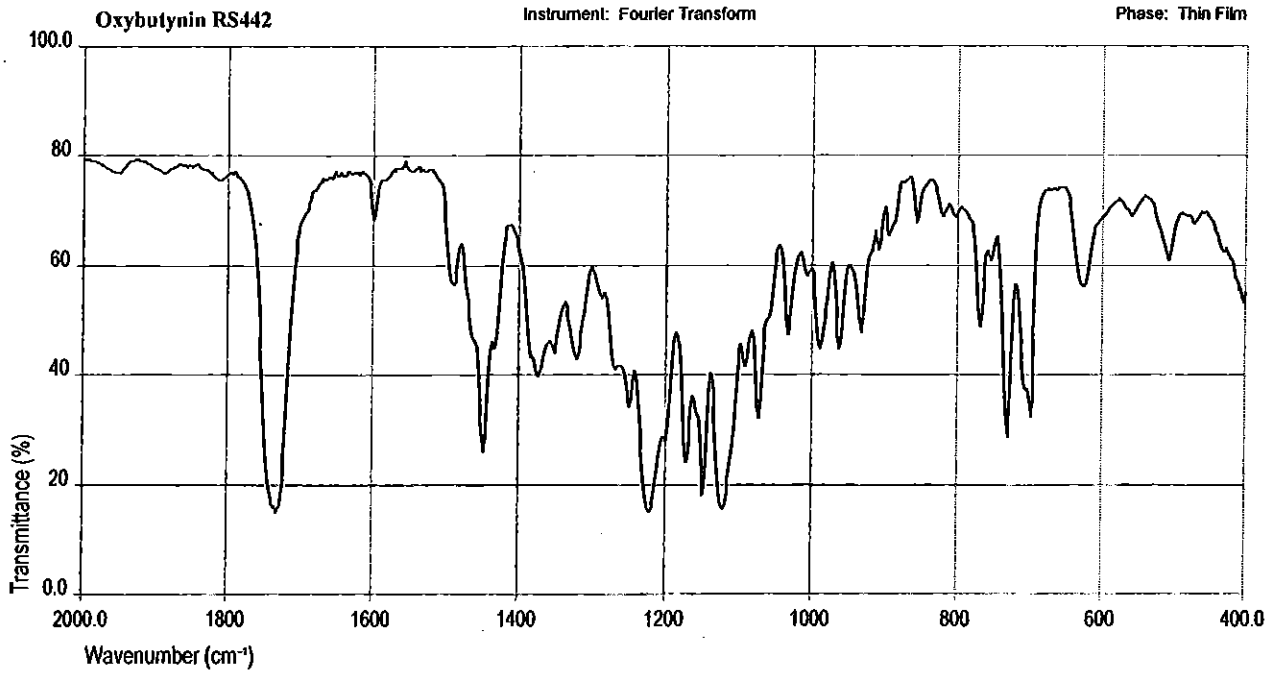
Oxazepam RS253 Instrument: Dispersive Phase: Potassium Bromide Disc

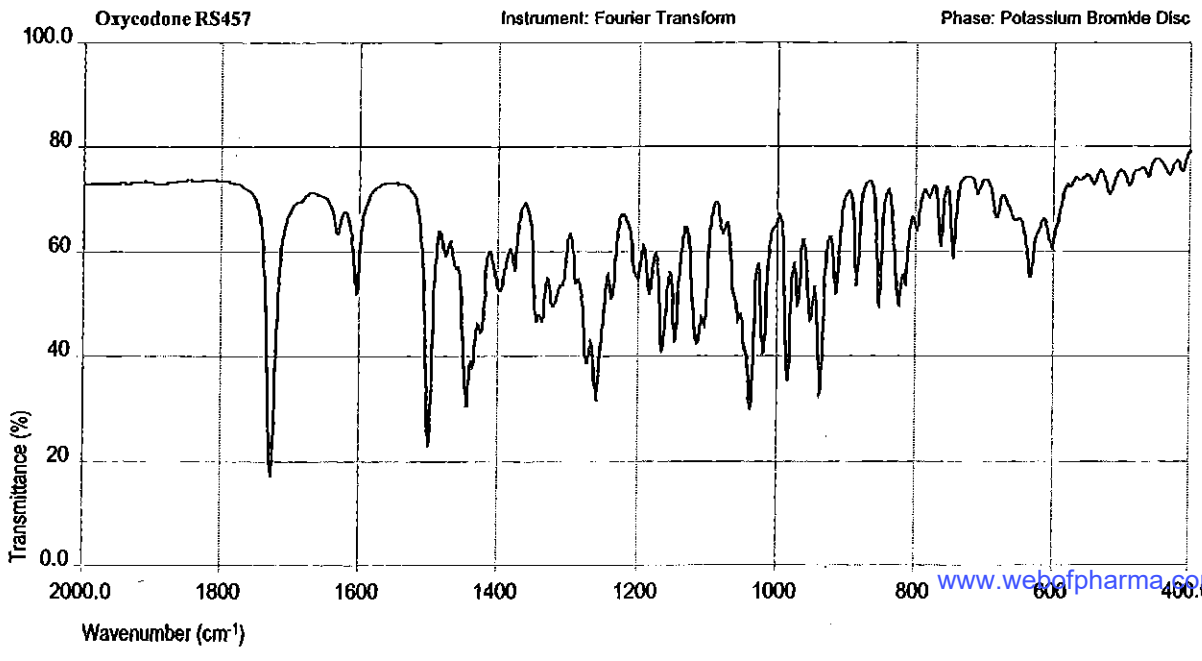


Oxetacaine RS254 Instrument: Dispersive Phase: Potassium Bromide Disc

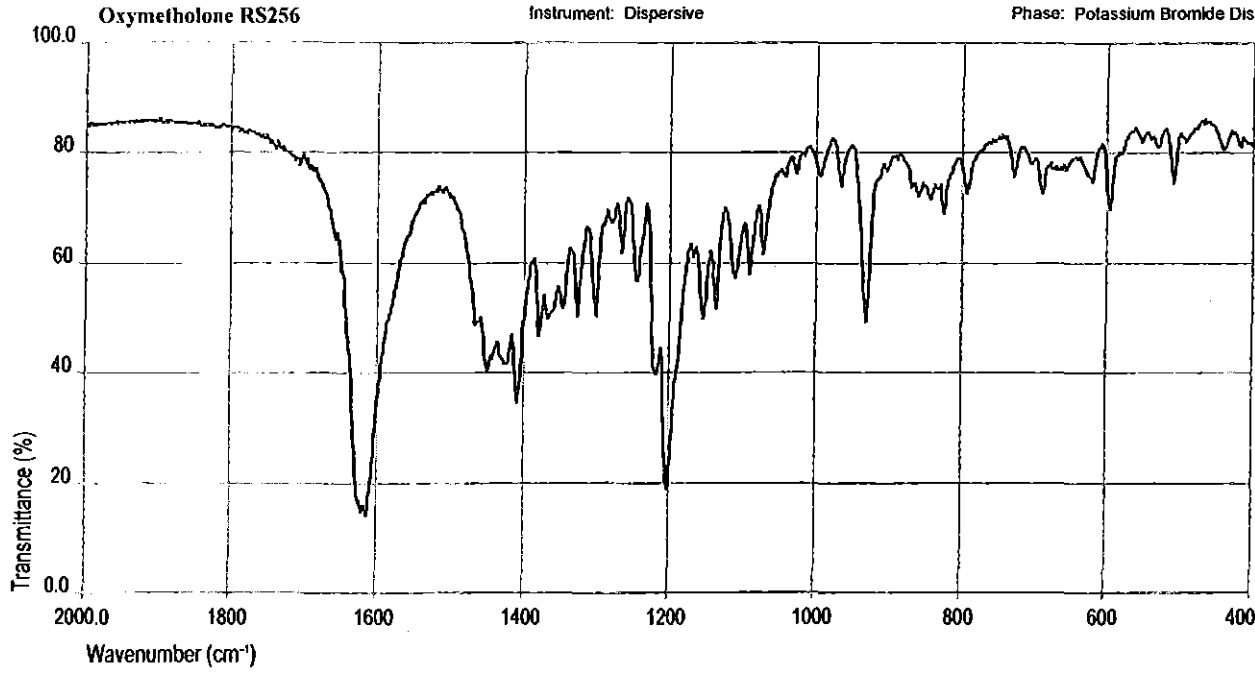




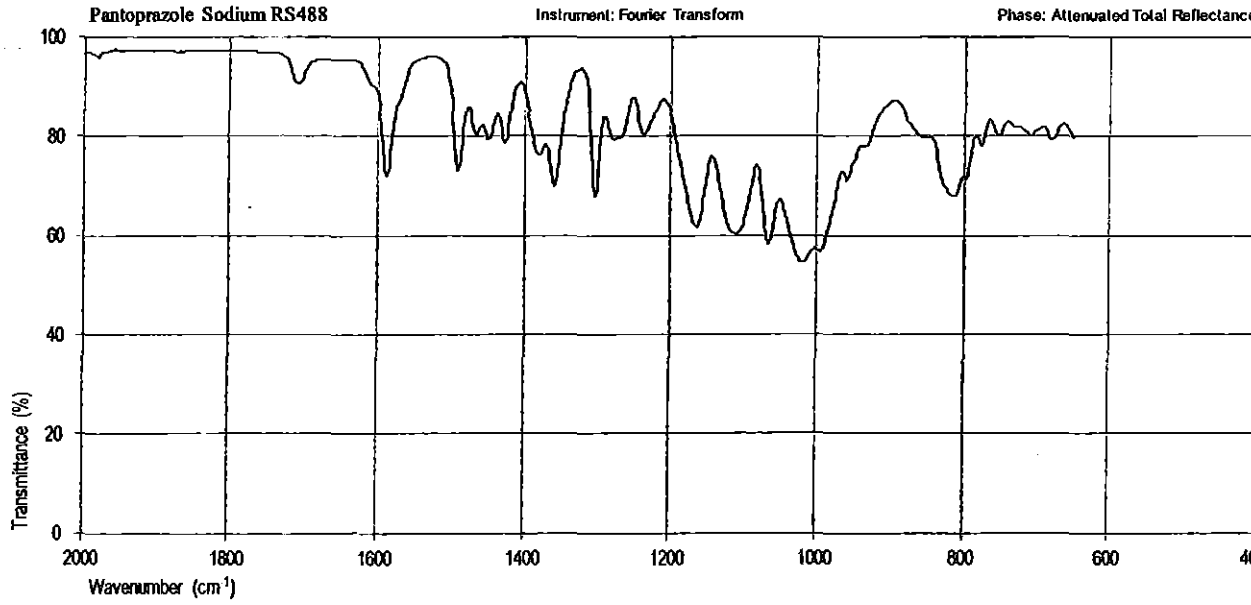




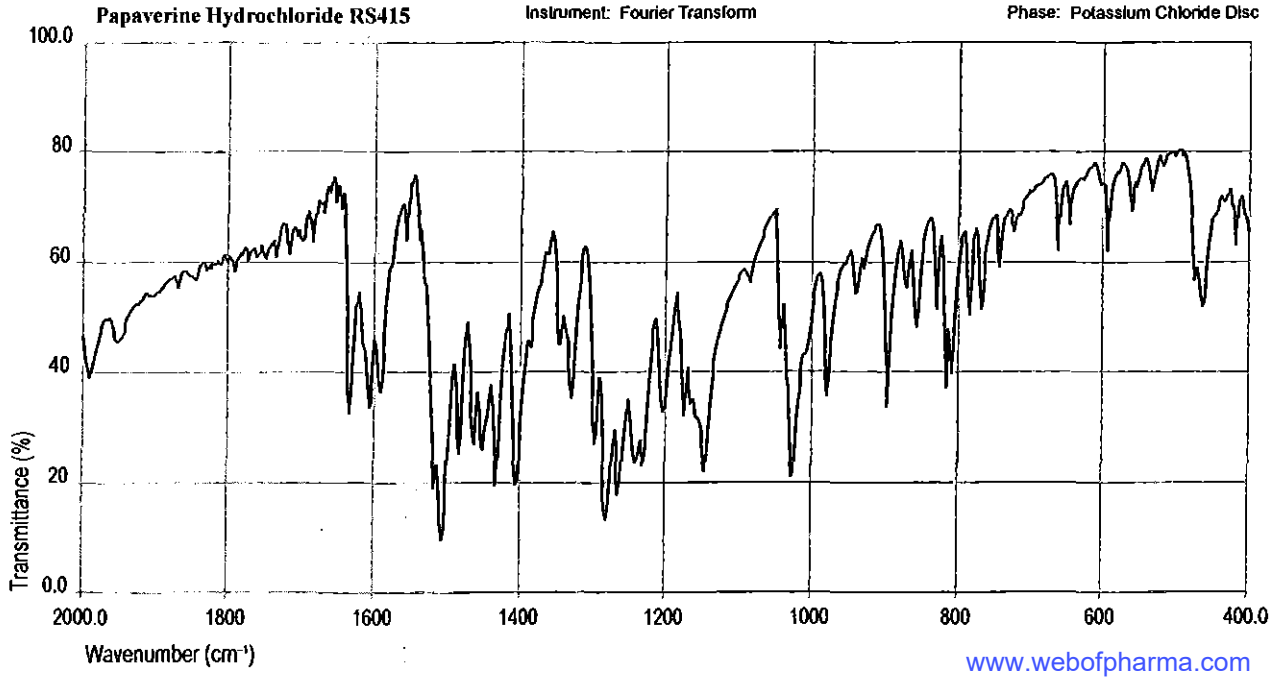
Oxymetholone RS256 Instrument: Dispersive Phase: Potassium Bromide Disc

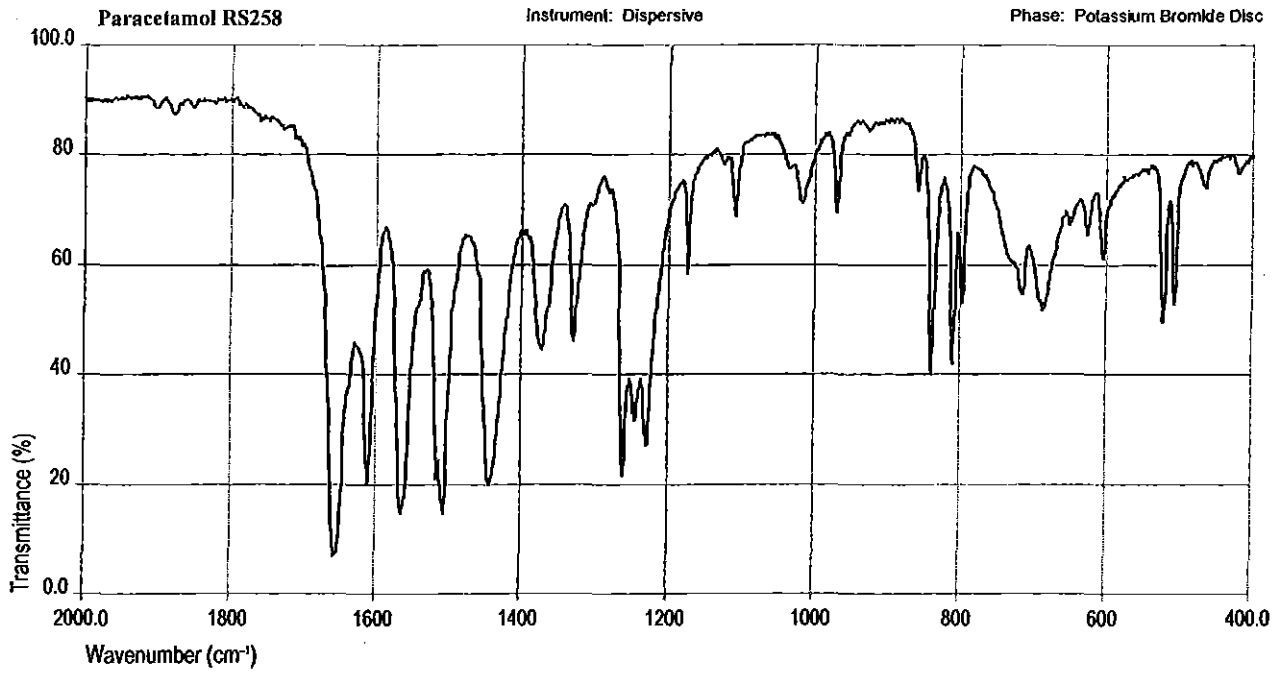


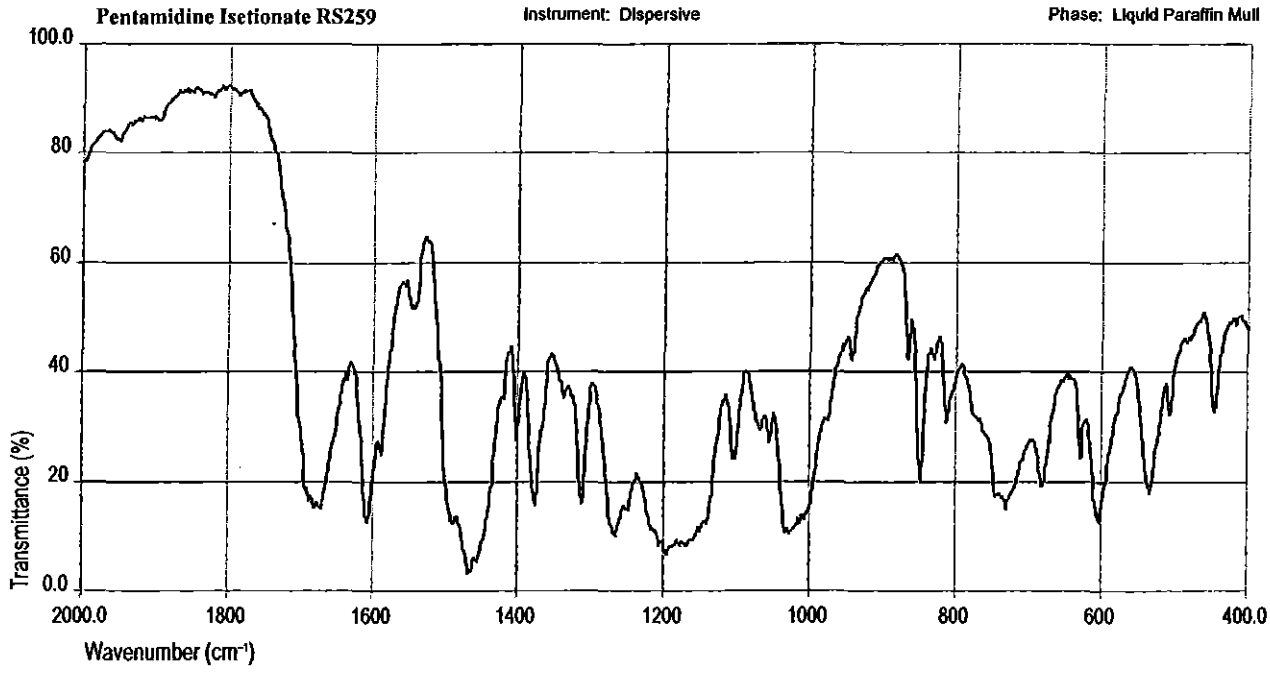
Pantoprazole Sodium RS488 Instrument: Fourier Transform Phase: Attenuated Total Reflectance

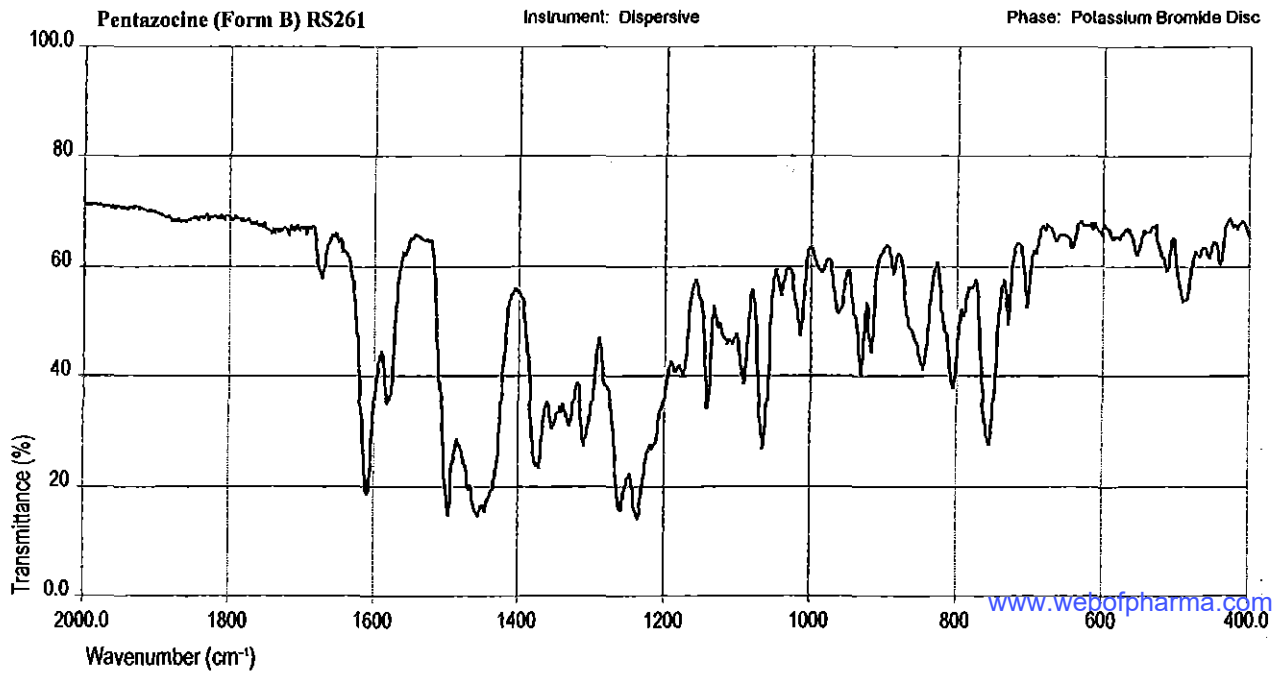


Papaverine Hydrochloride RS415 Instrument: Fourier Transform Phase: Potassium Chloride Disc





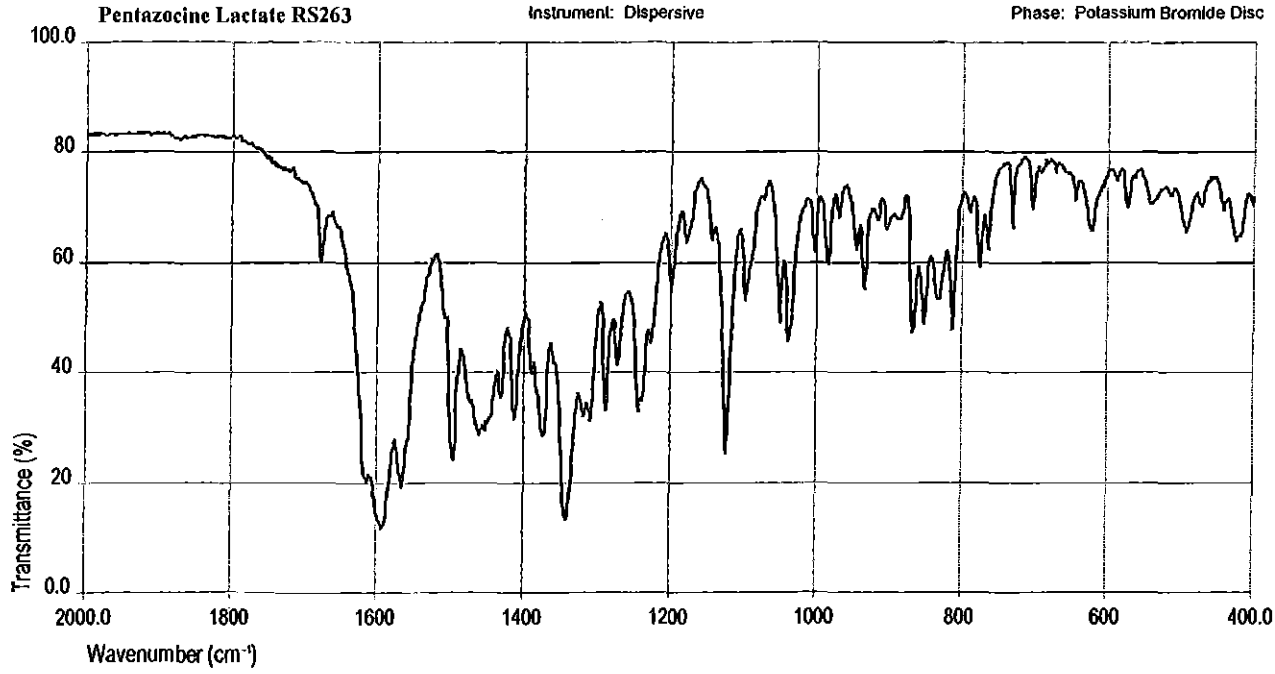




Pentazocine Lactate RS263

Instrument: Dispersive

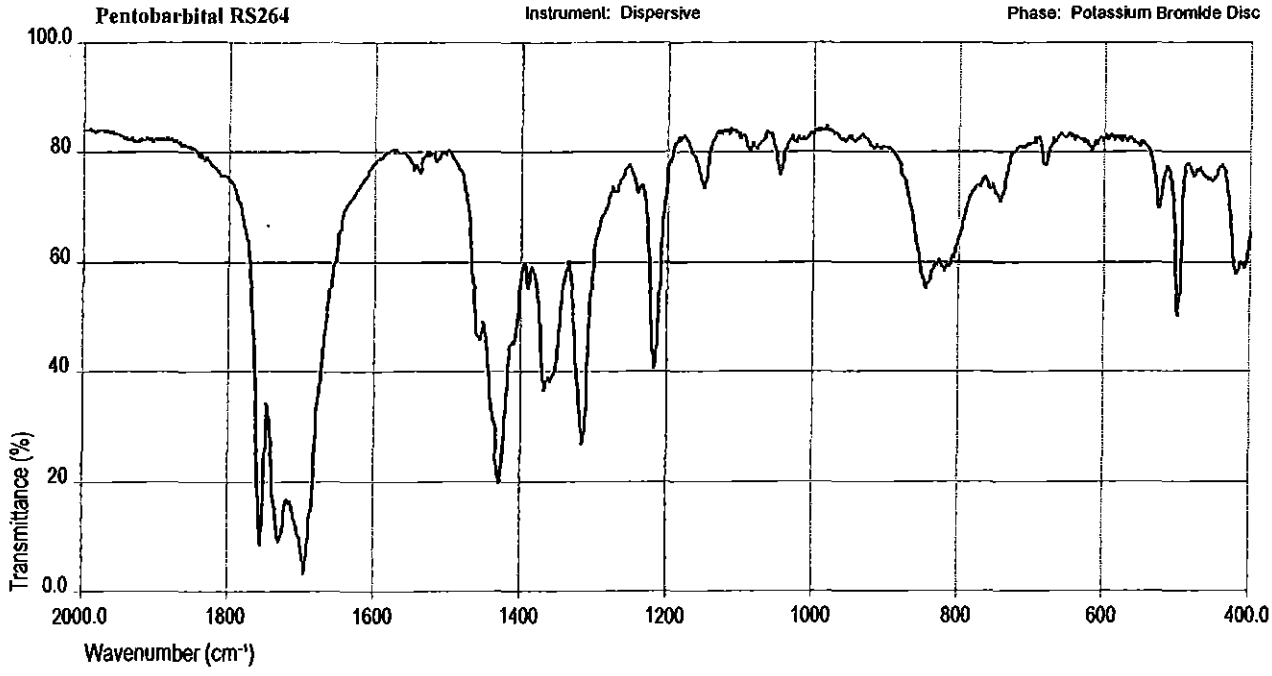
Phase: Potassium Bromide Disc



Pentobarbital RS264

Instrument: Dispersive

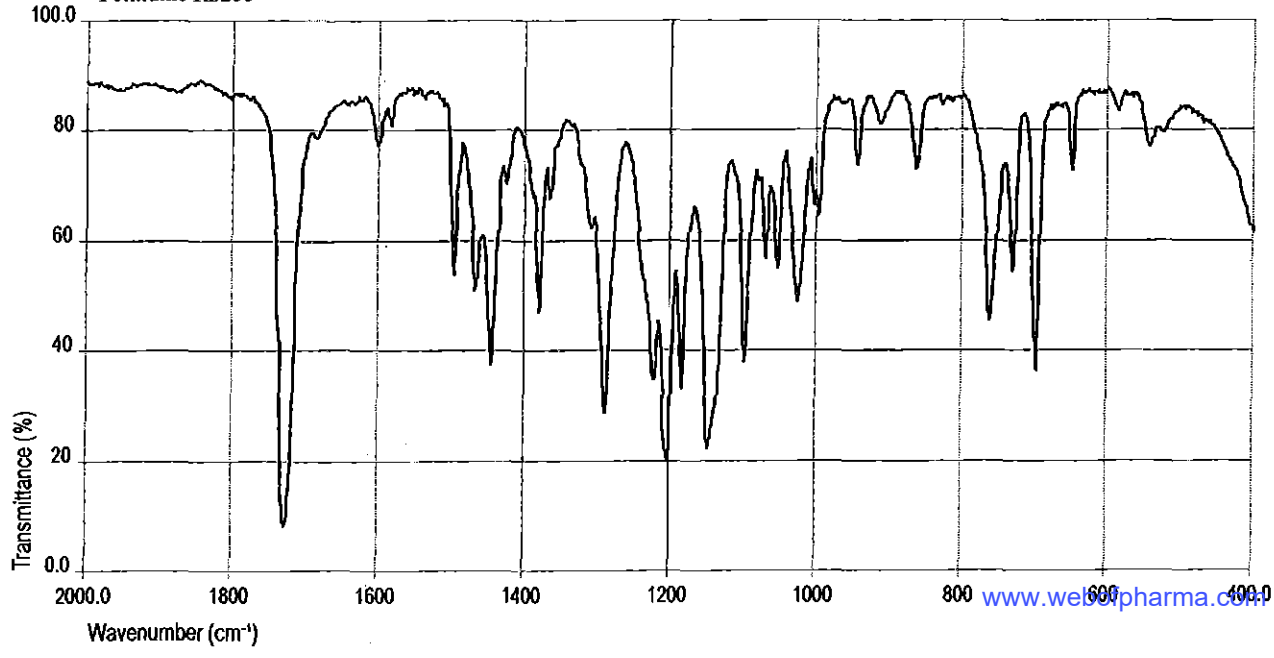
Phase: Potassium Bromide Disc



Pethidine RS266

Instrument: Dispersive

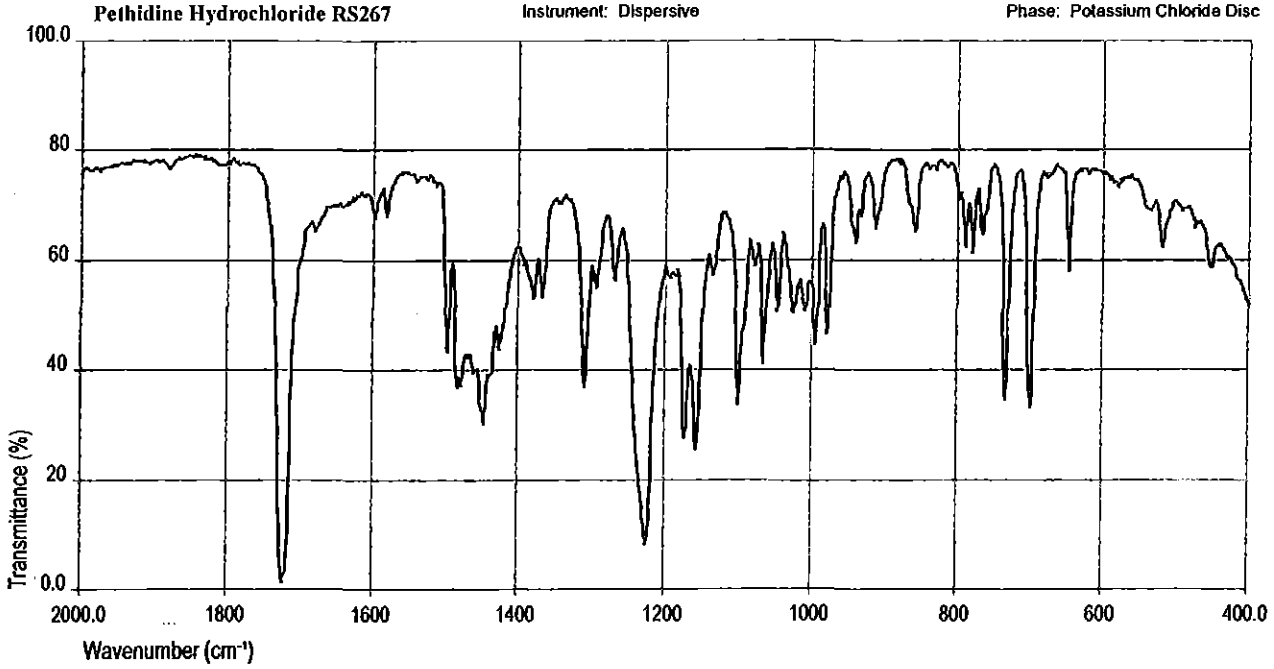
Phase: Thin Film



Pethidine Hydrochloride RS267

Instrument: Dispersive

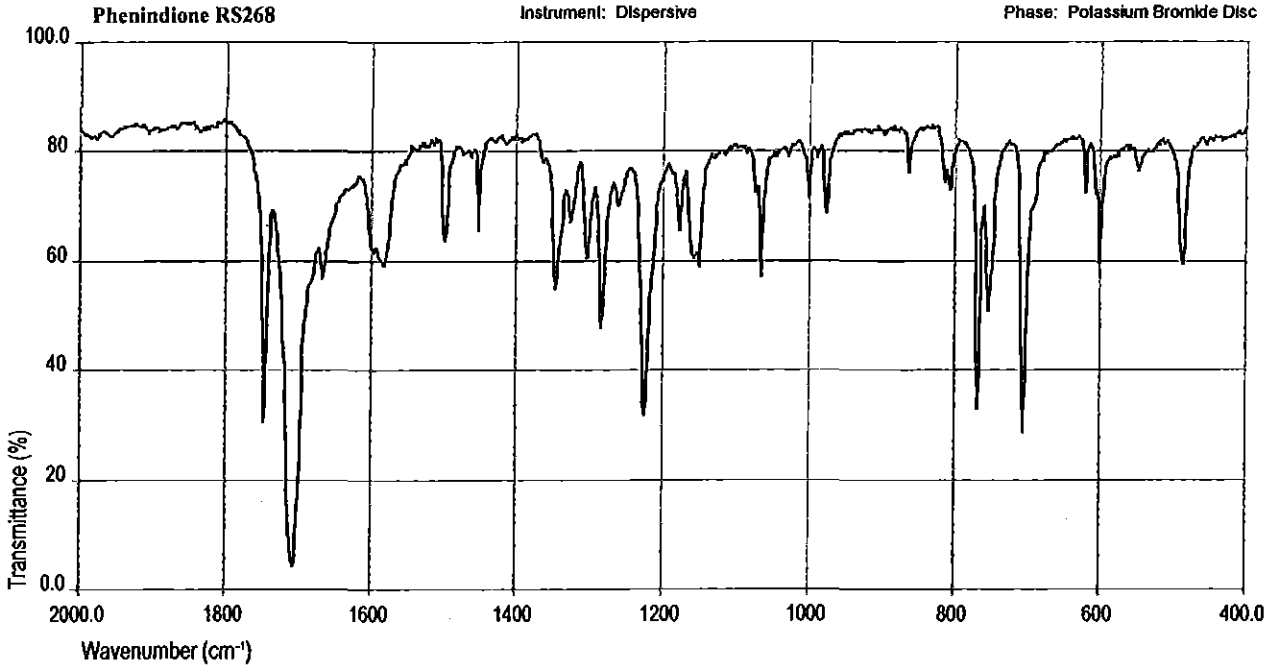
Phase: Potassium Chloride Disc



Phenindione RS268

Instrument: Dispersive

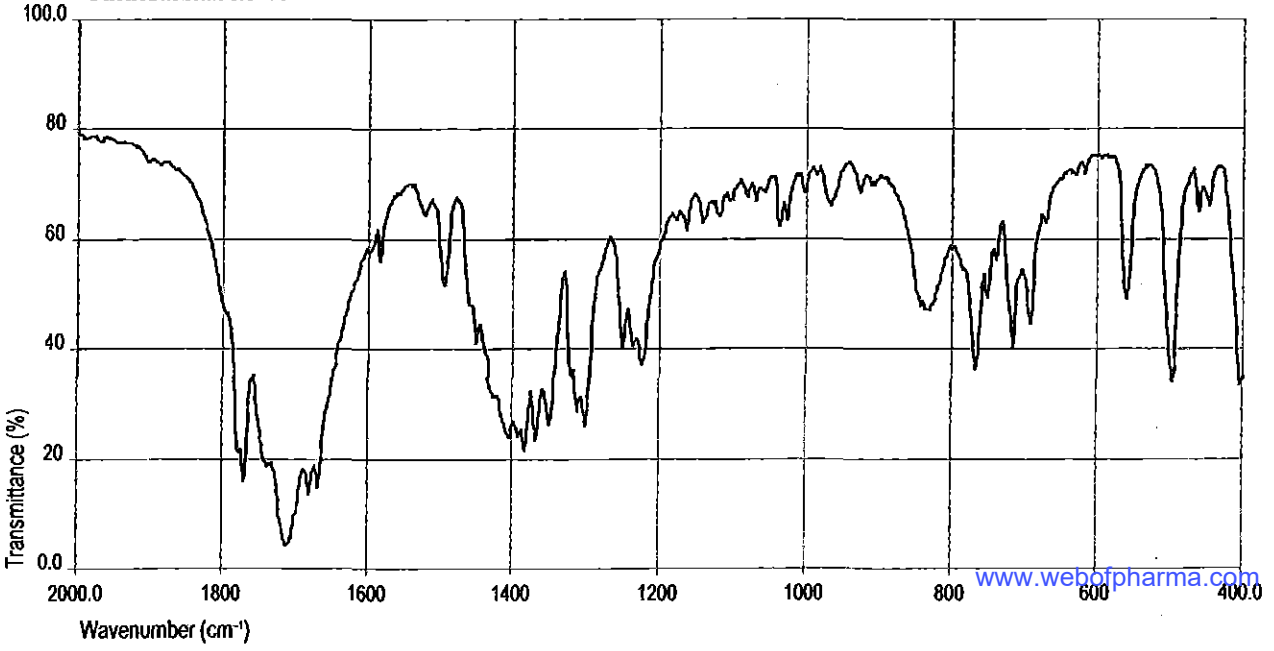
Phase: Potassium Bromide Disc



Phenobarbital RS270

Instrument: Dispersive

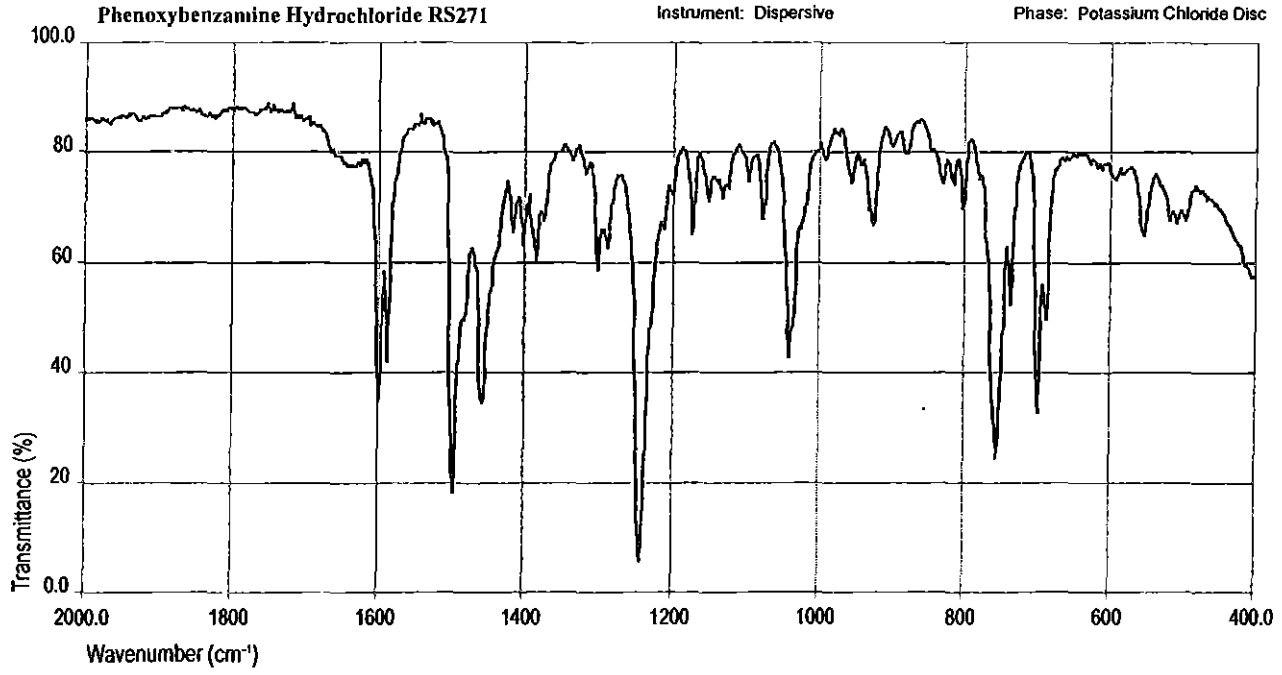
Phase: Potassium Bromide Disc



Phenoxybenzamine Hydrochloride RS271

Instrument: Dispersive

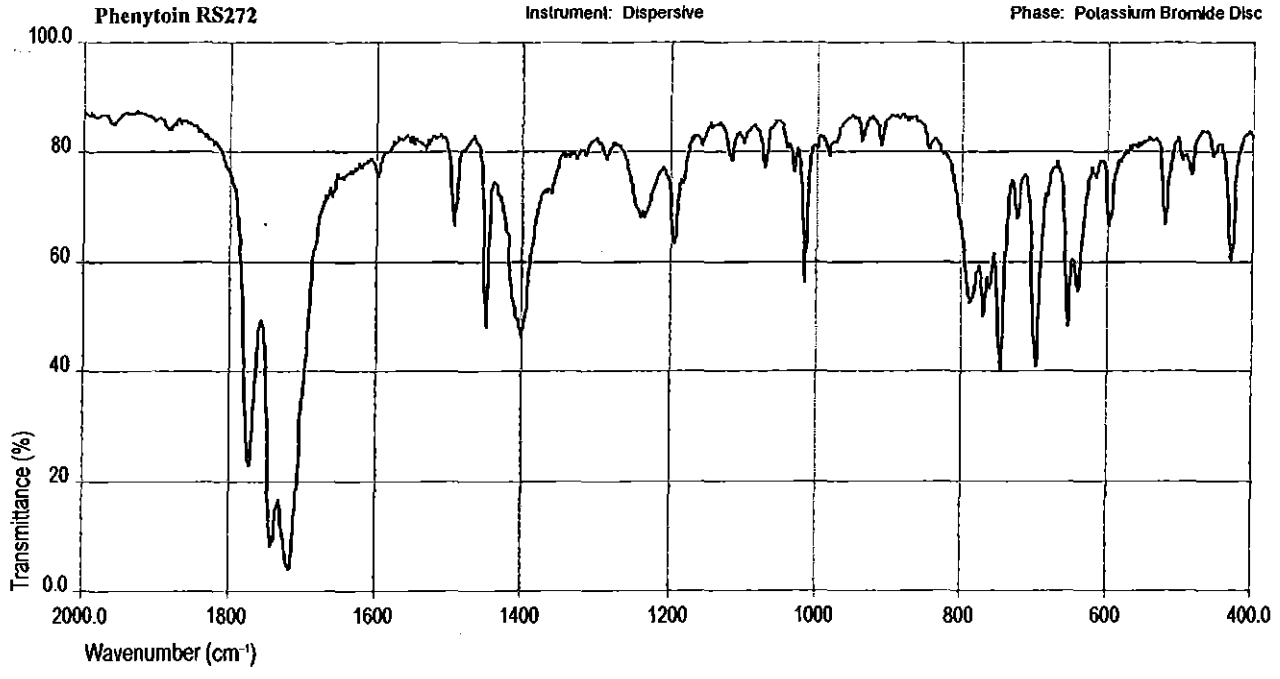
Phase: Potassium Chloride Disc



Phenytoin RS272

Instrument: Dispersive

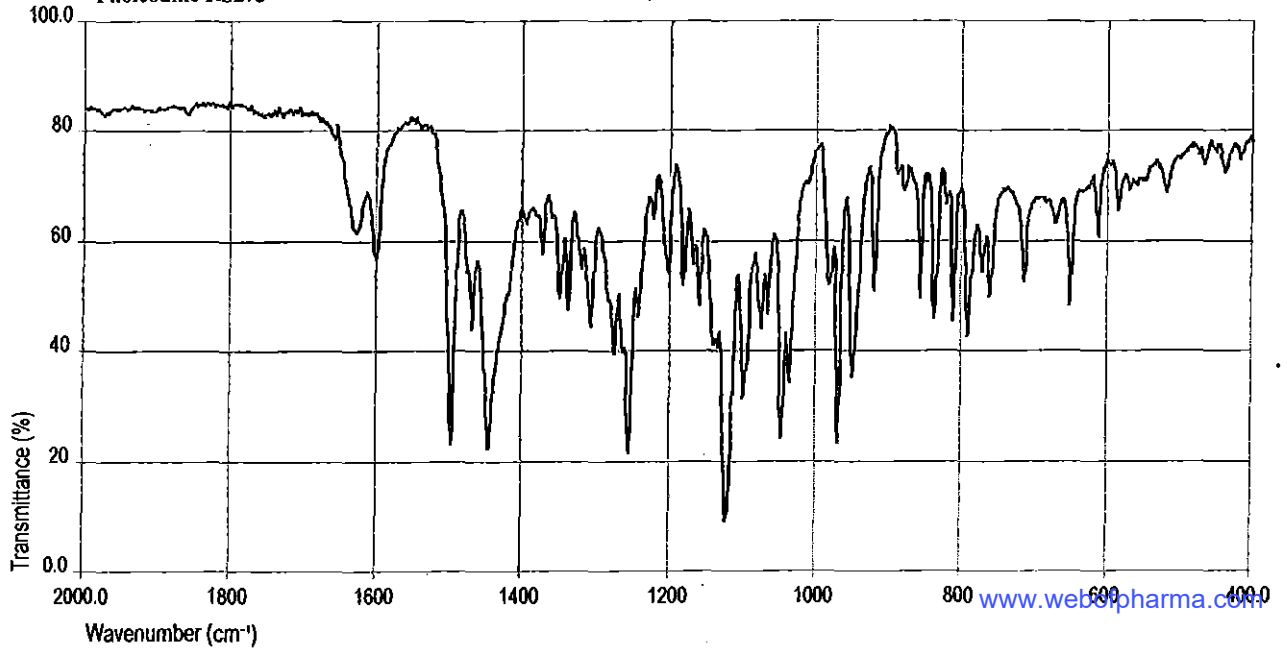
Phase: Potassium Bromide Disc

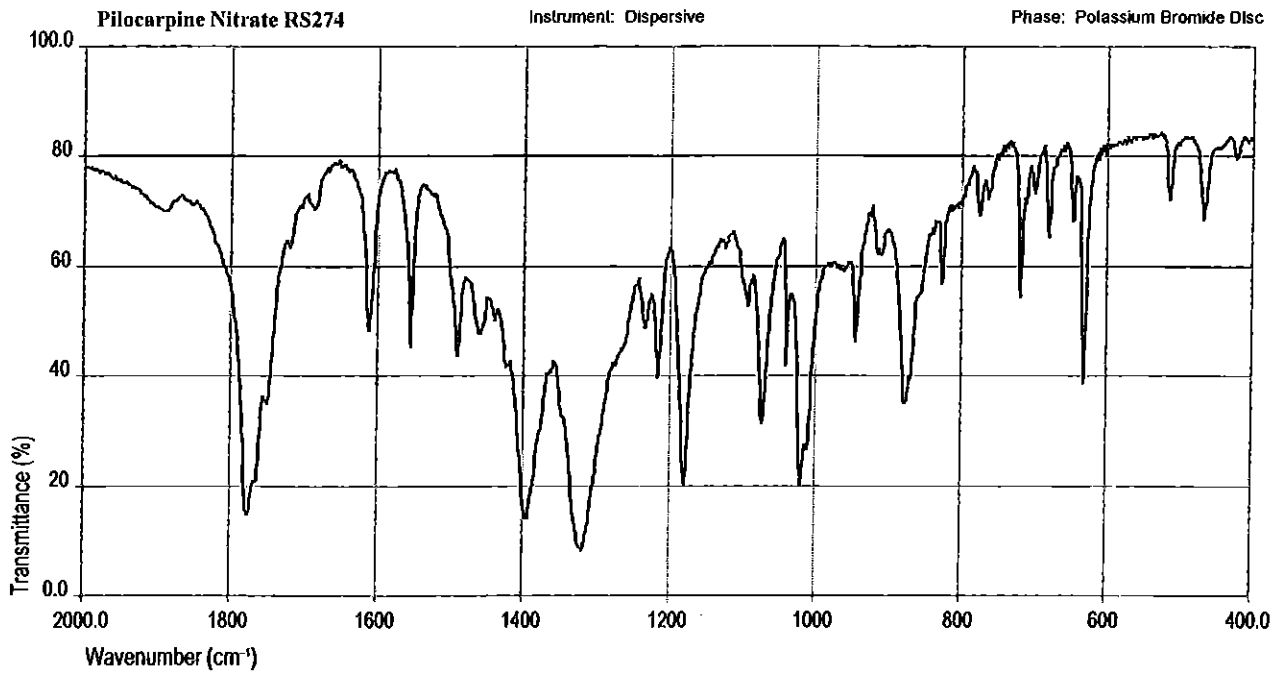


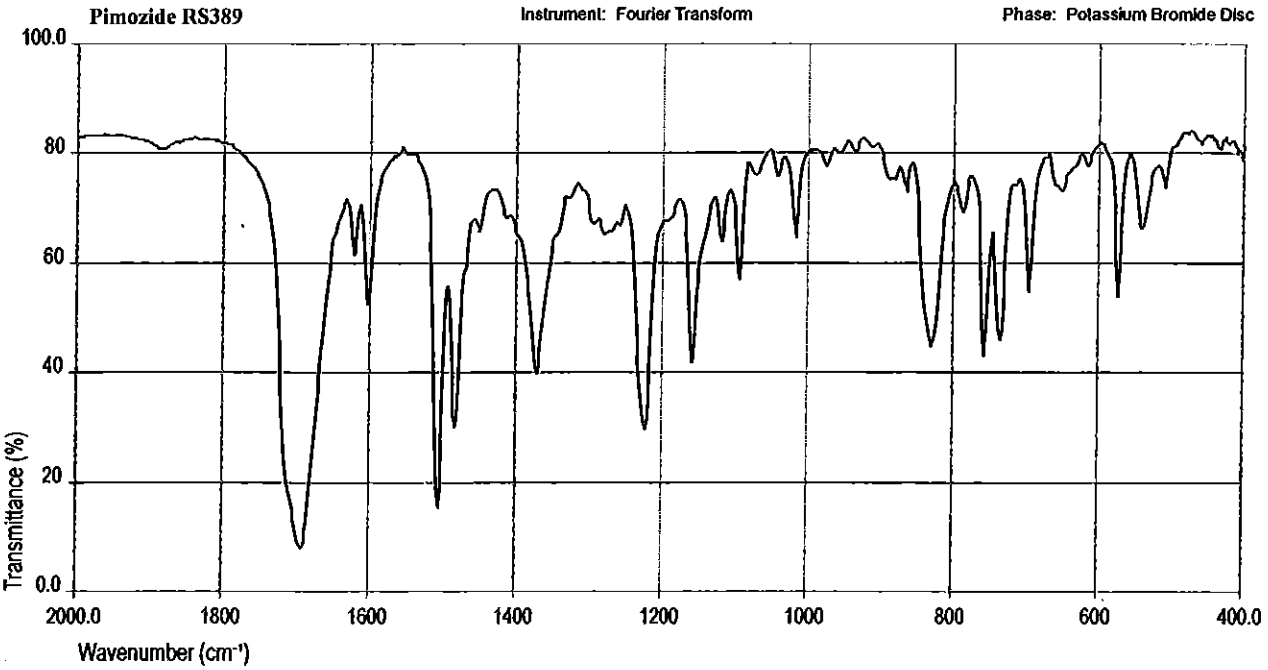
Pholcodine RS273

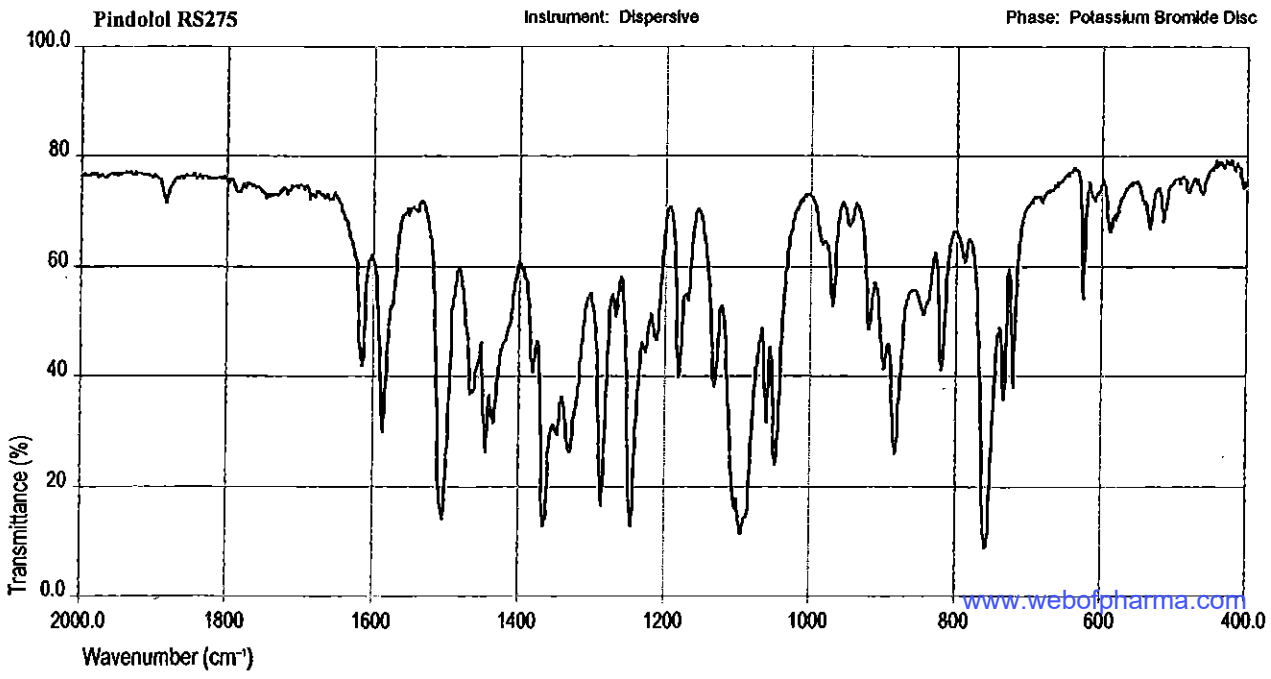
Instrument: Dispersive

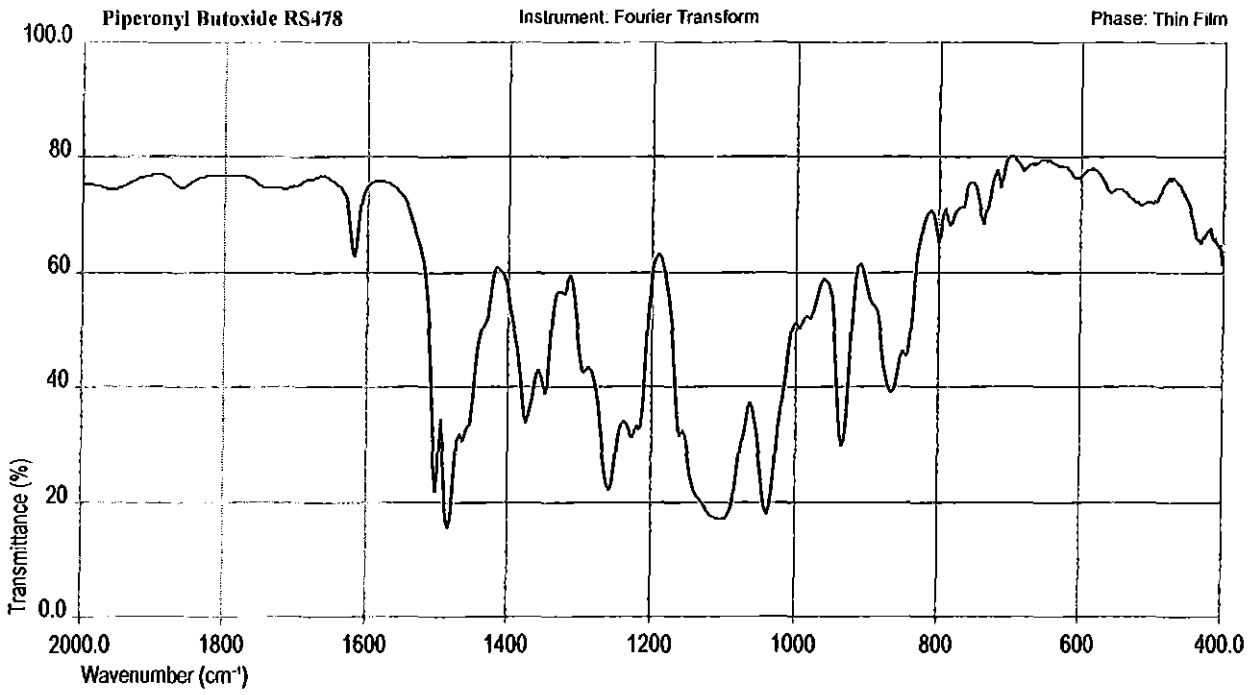
Phase: Potassium Bromide Disc

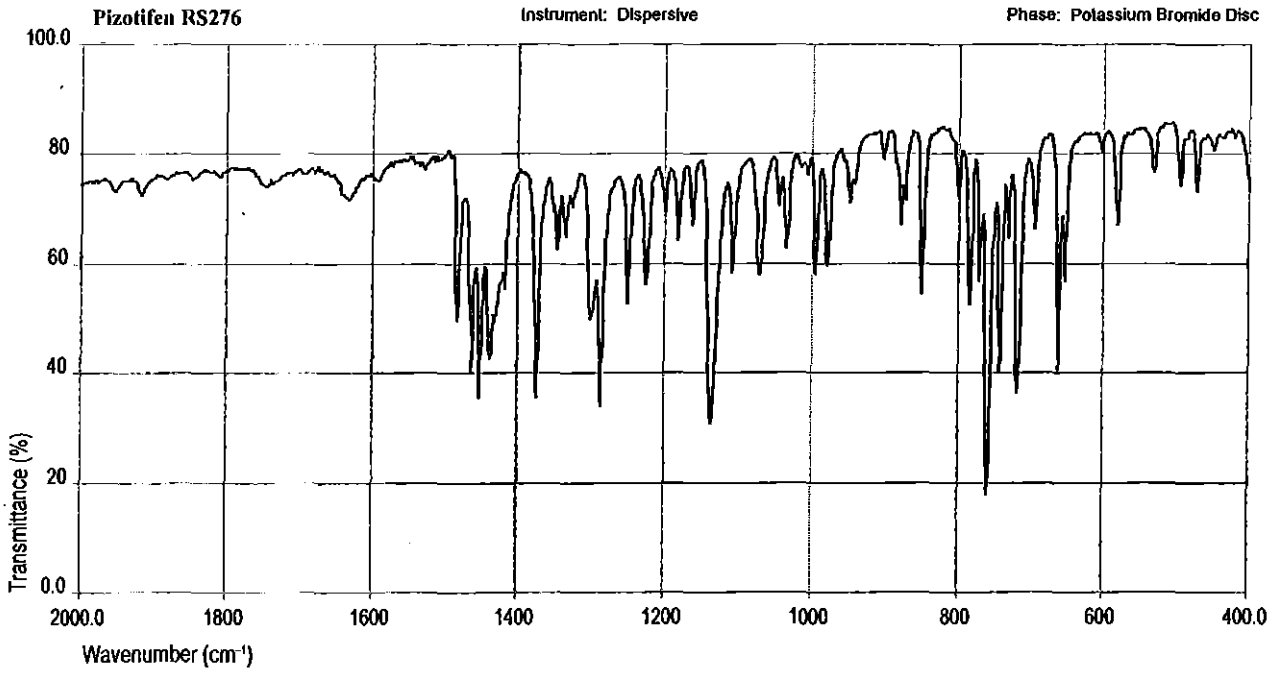


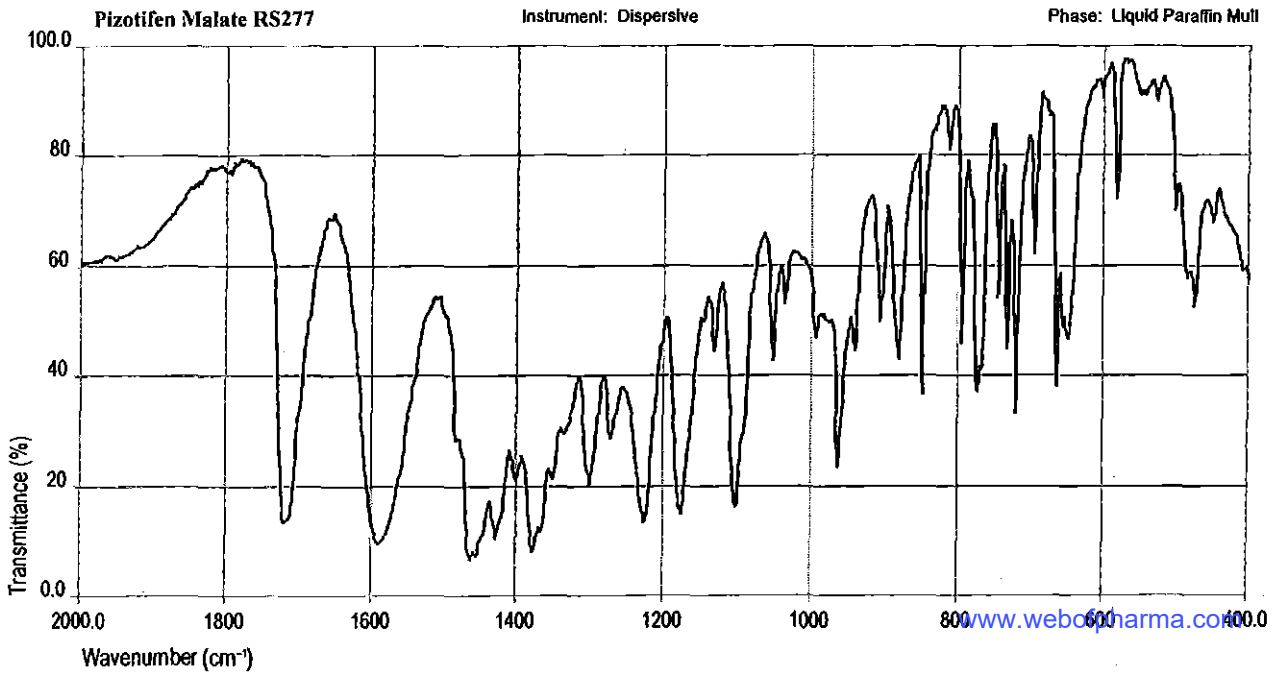








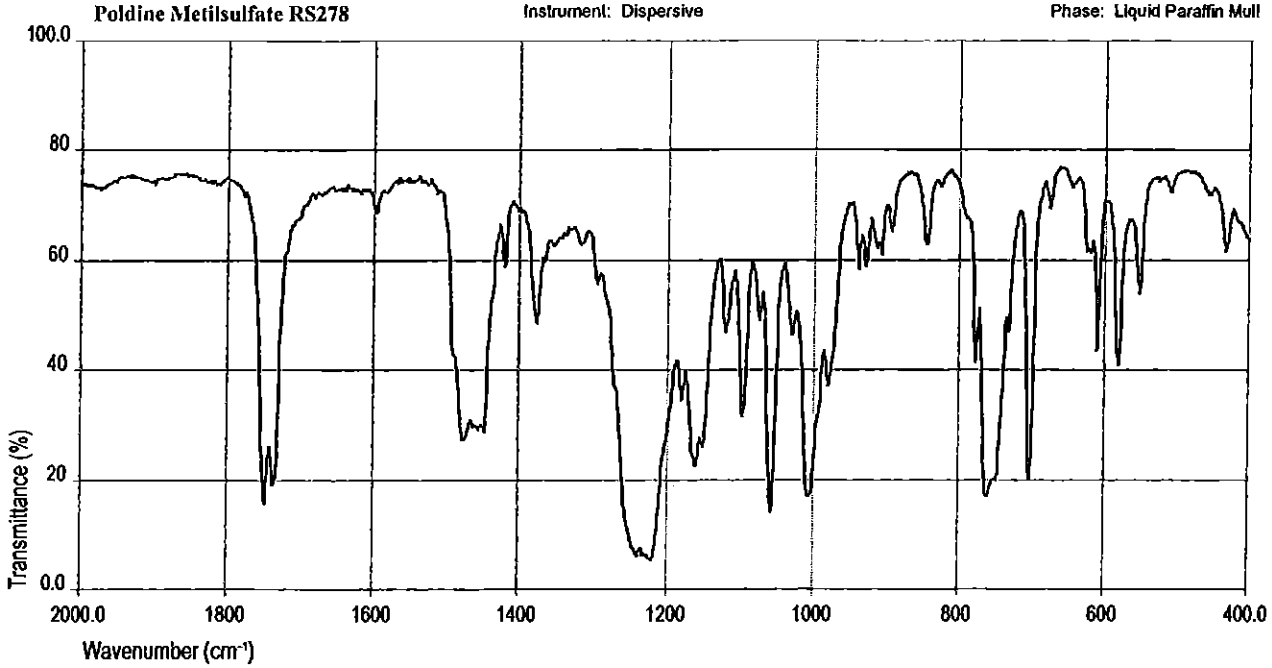




Poldine Metilsulfate RS278

Instrument: Dispersive

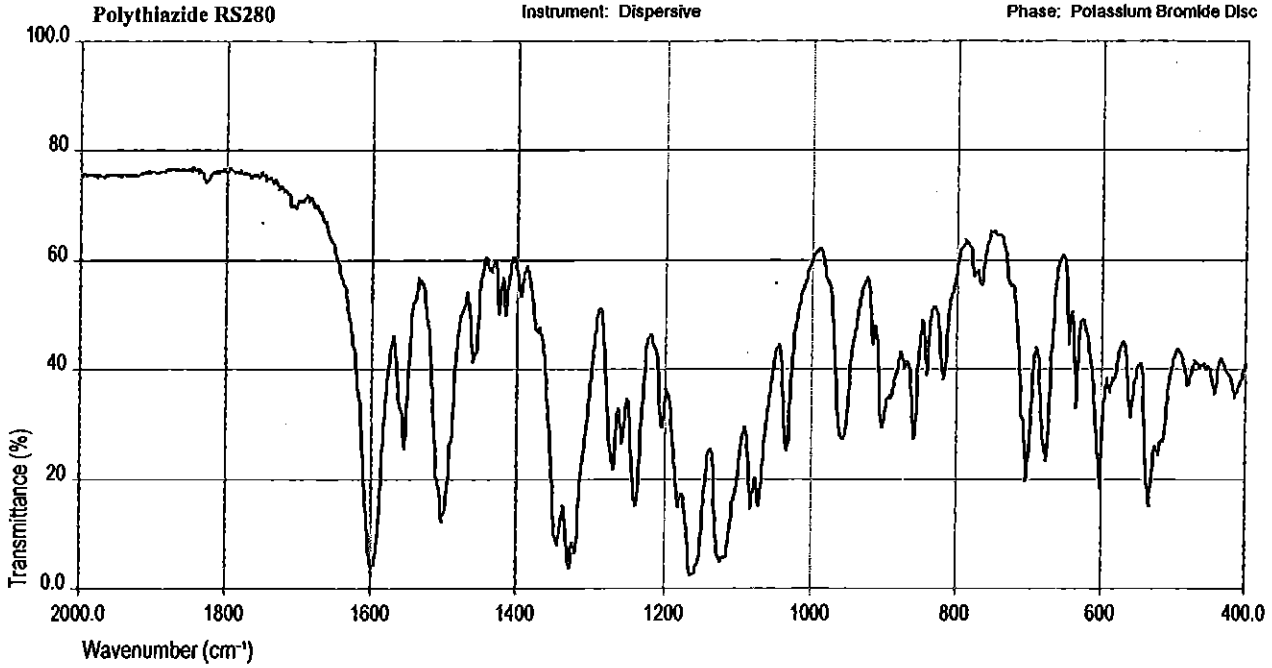
Phase: Liquid Paraffin Mull



Polythiazide RS280

Instrument: Dispersive

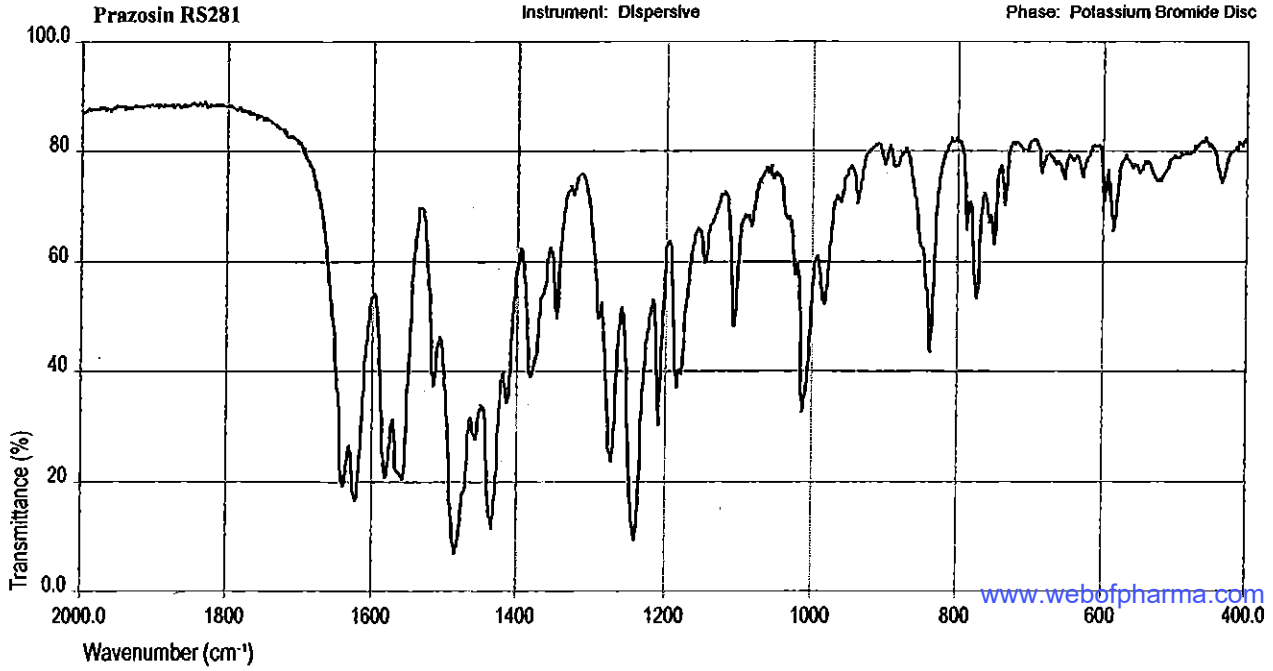
Phase: Potassium Bromide Disc

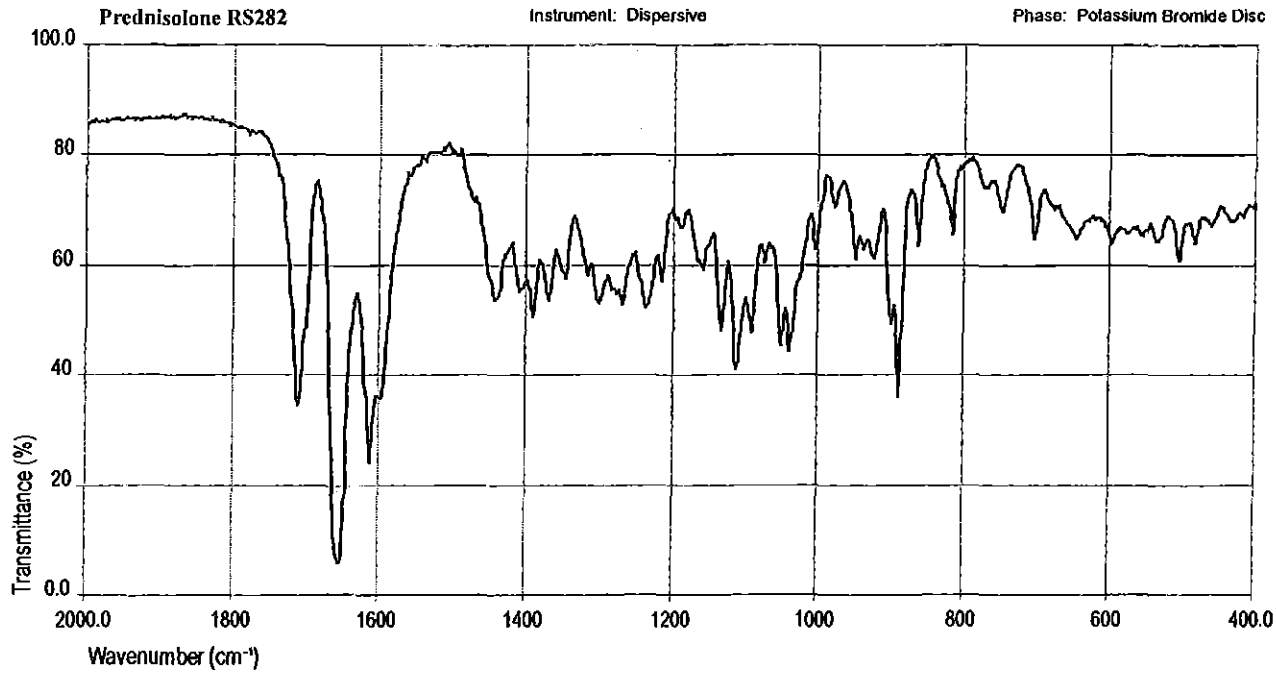


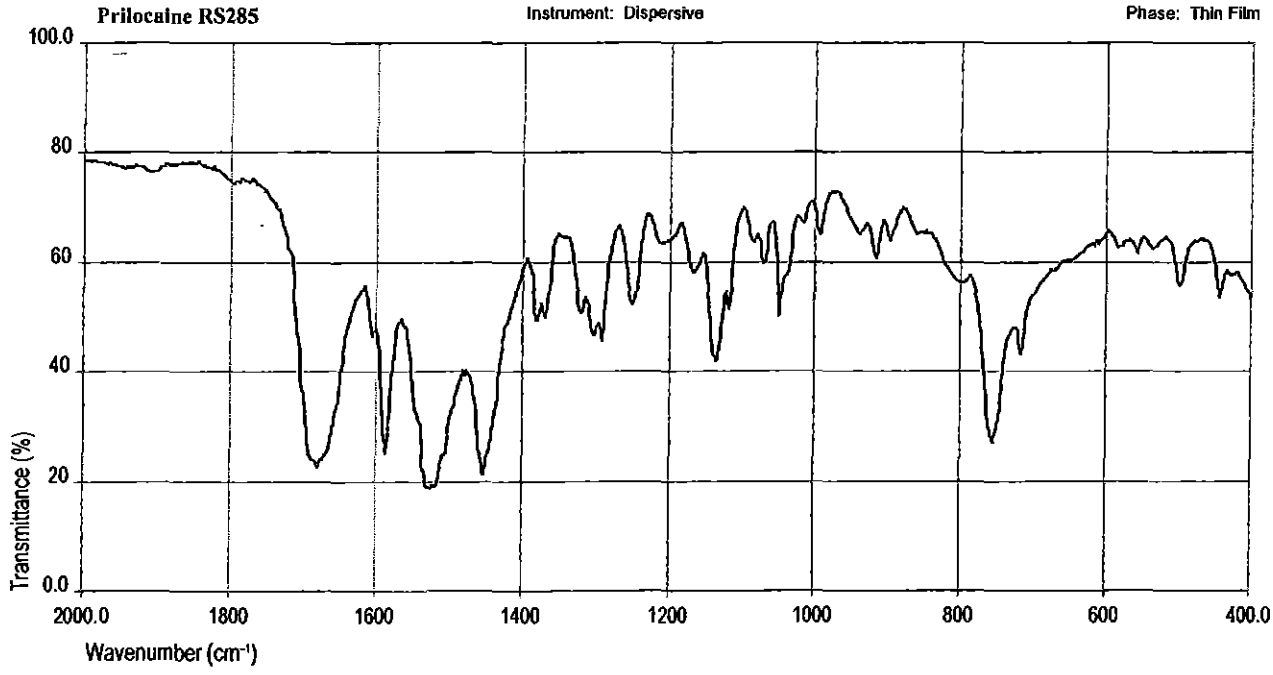
Prazosin RS281

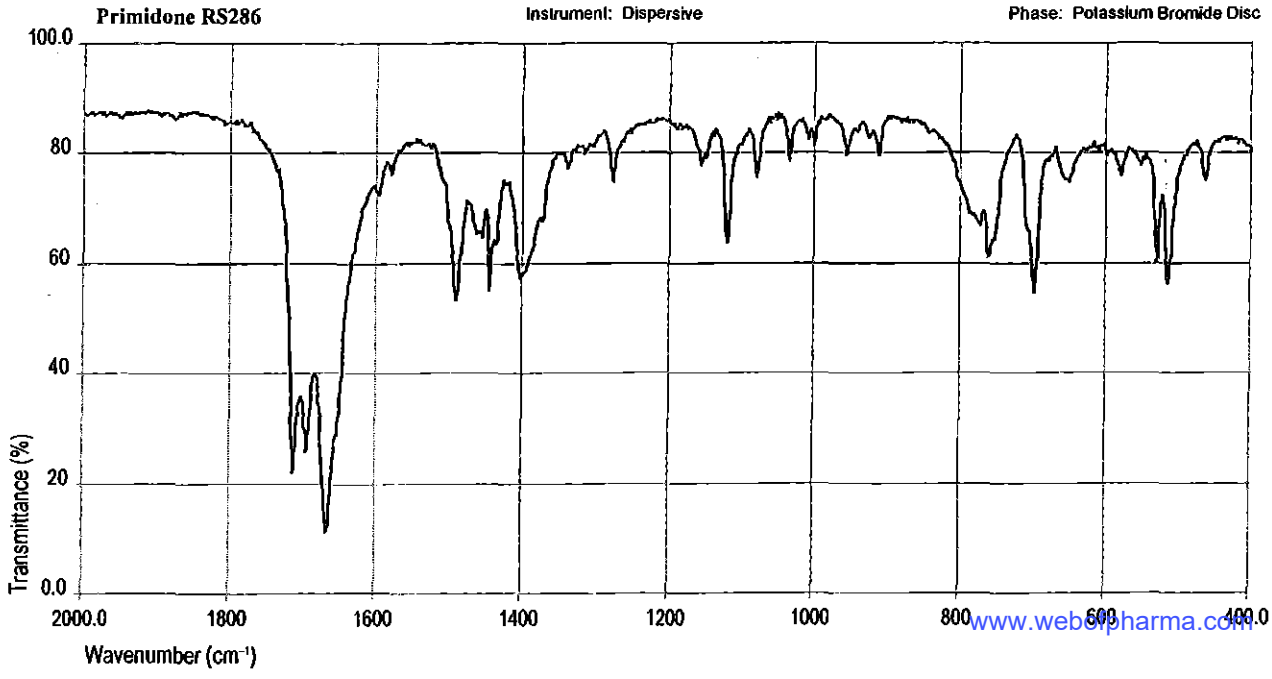
Instrument: Dispersive

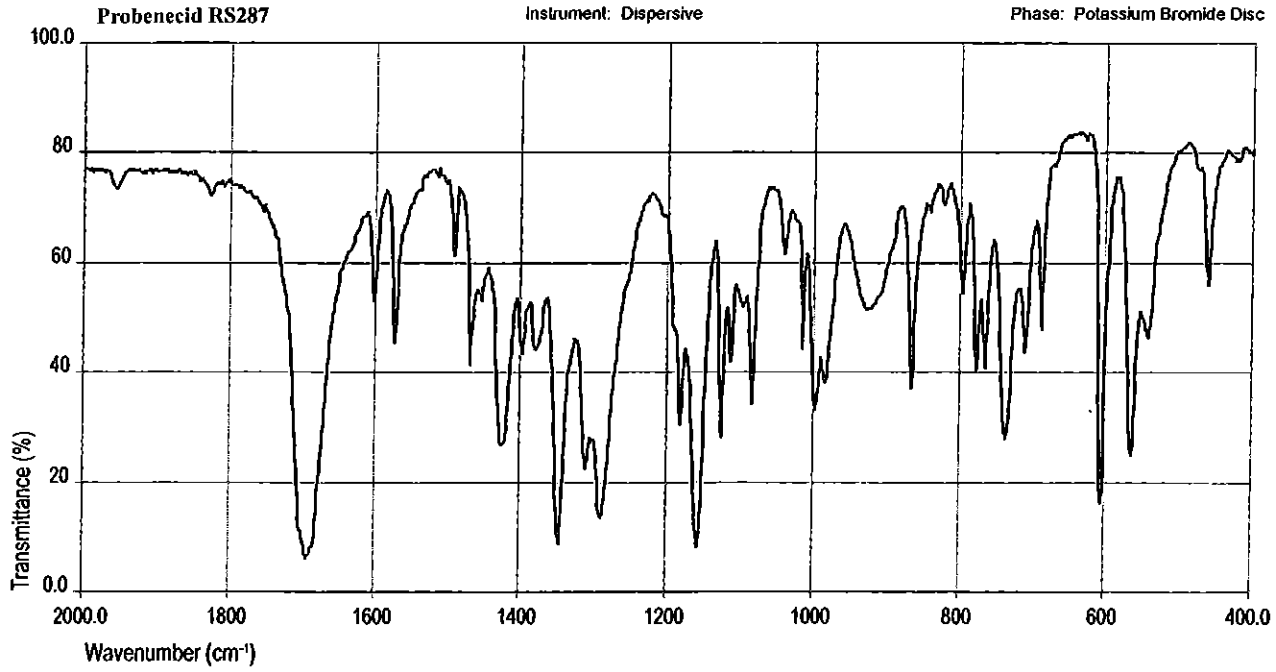
Phase: Potassium Bromide Disc

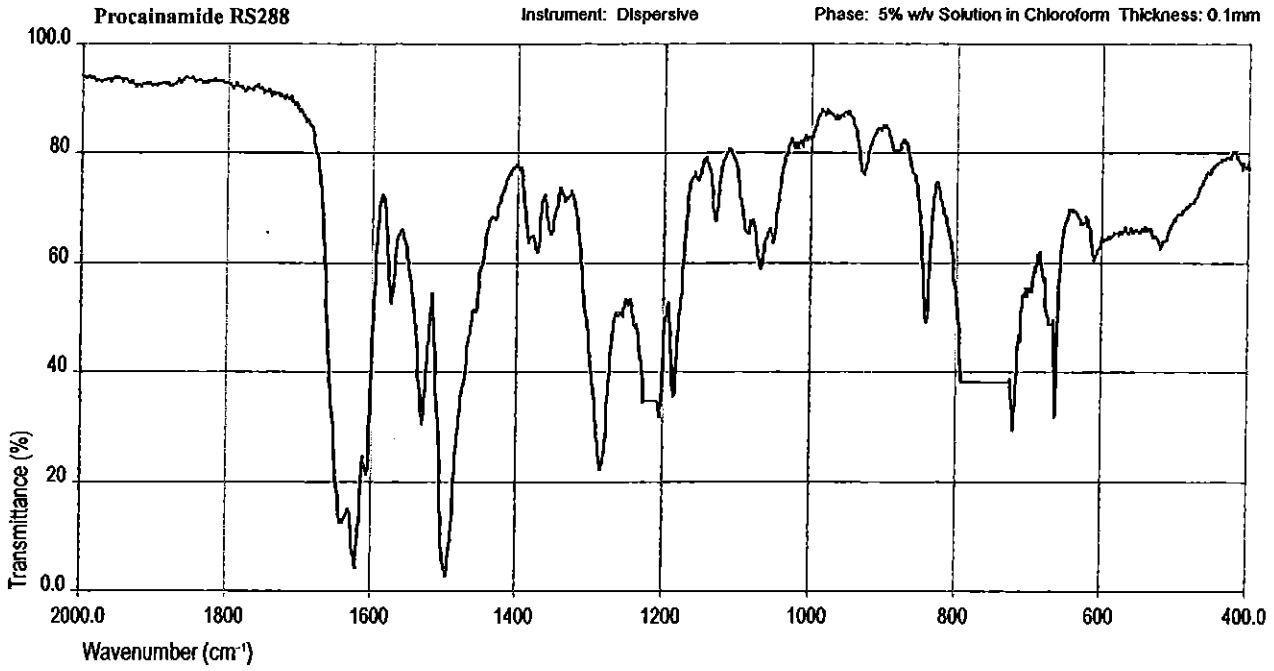


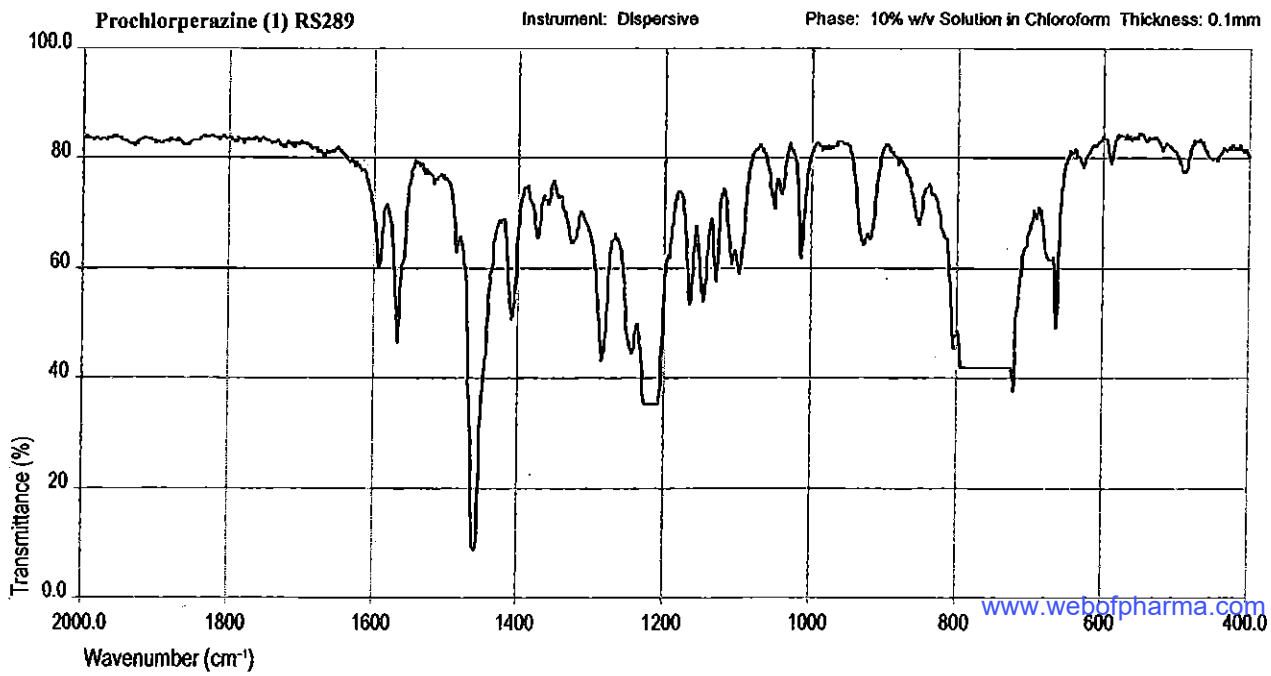




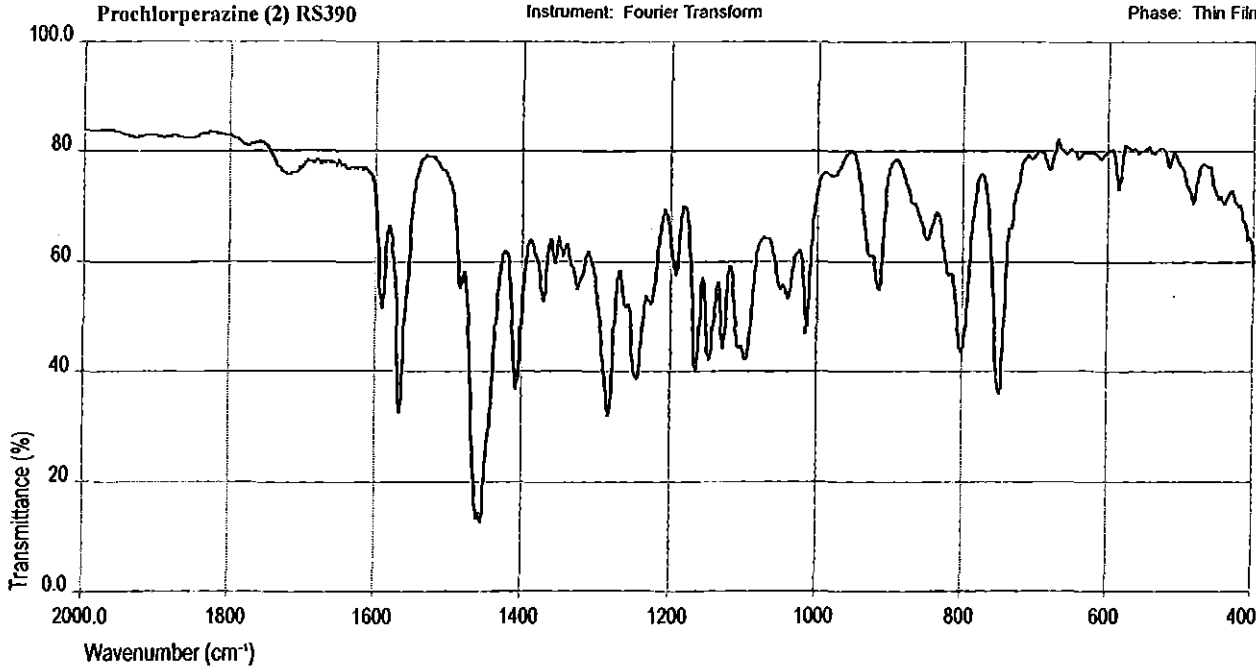




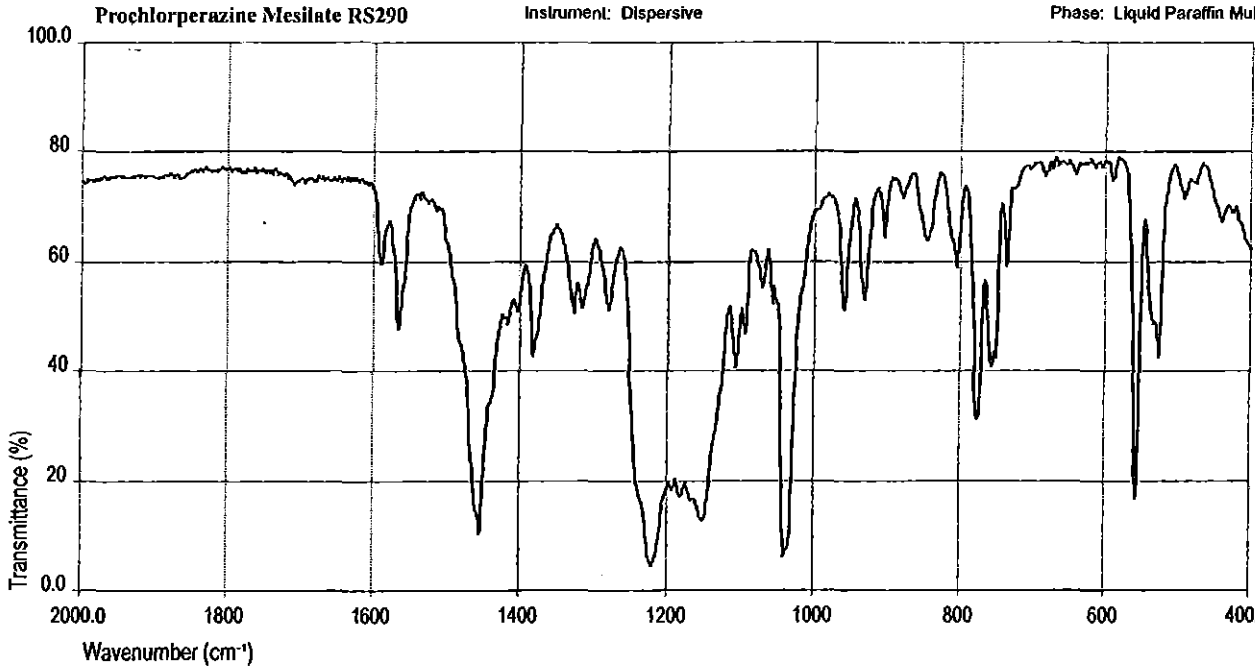




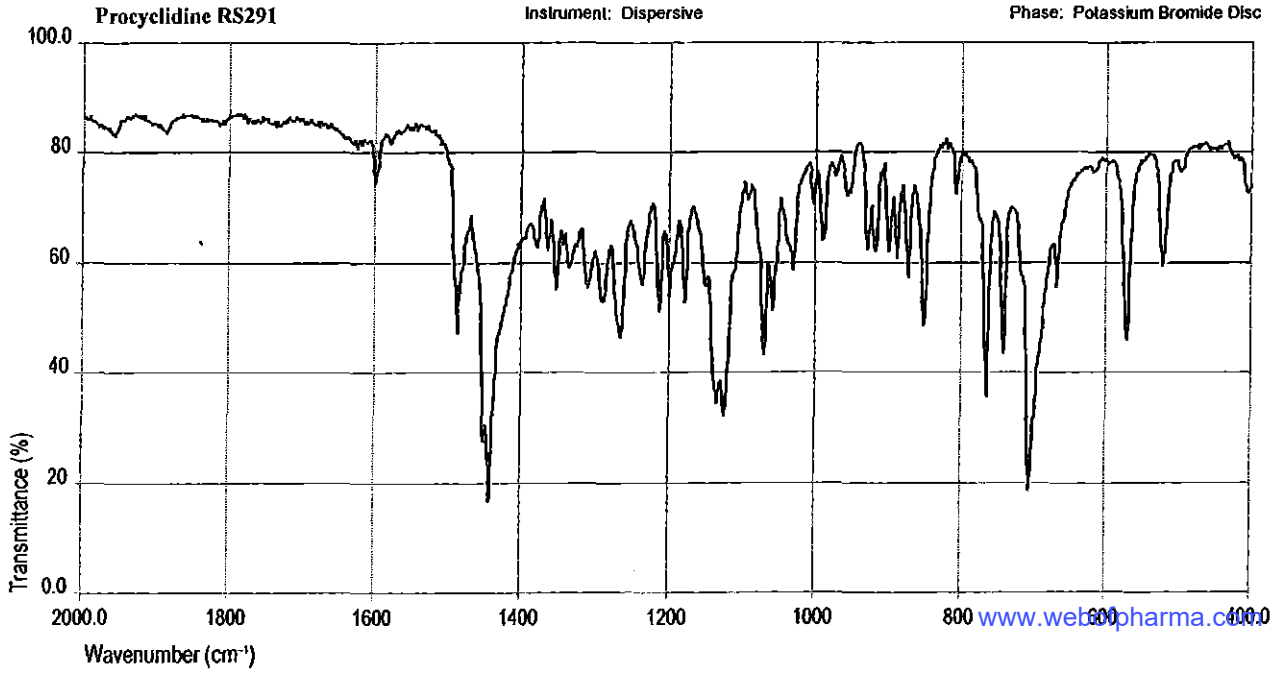
Prochlorperazine (2) RS390 Instrument: Fourier Transform Phase: Thin Film



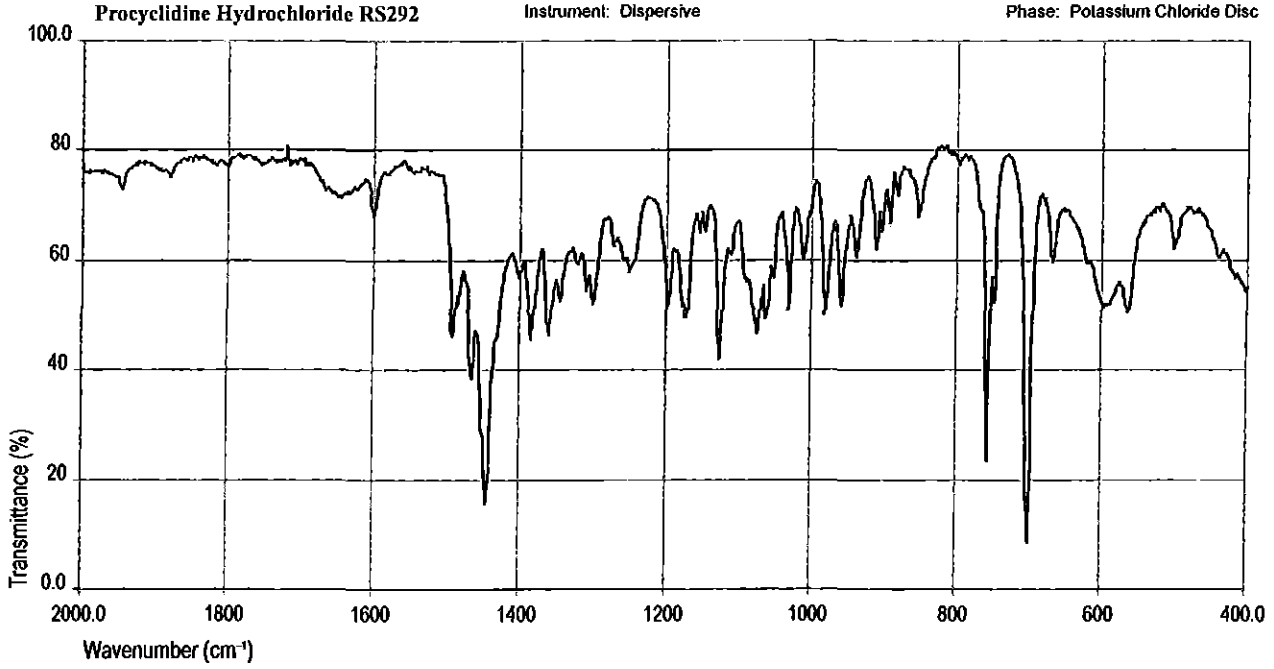
Prochlorperazine Mesilate RS290 Instrument: Dispersive Phase: Liquid Paraffin Mull



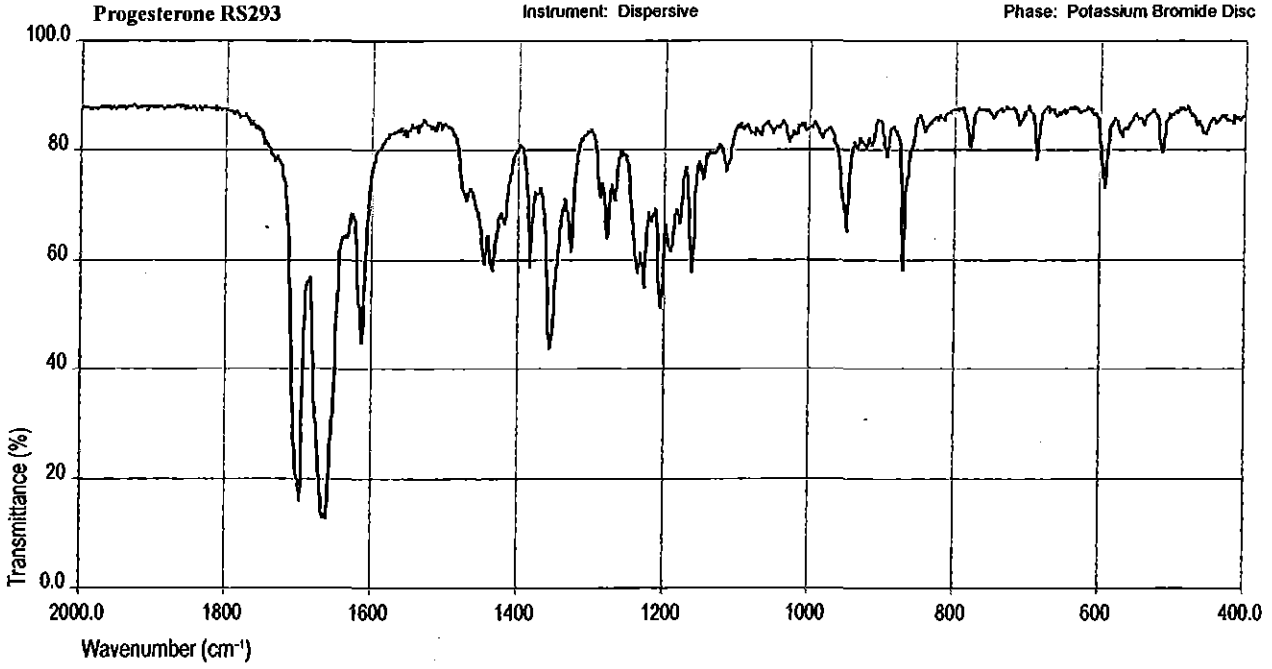
Procyclidine RS291 Instrument: Dispersive Phase: Potassium Bromide Disc



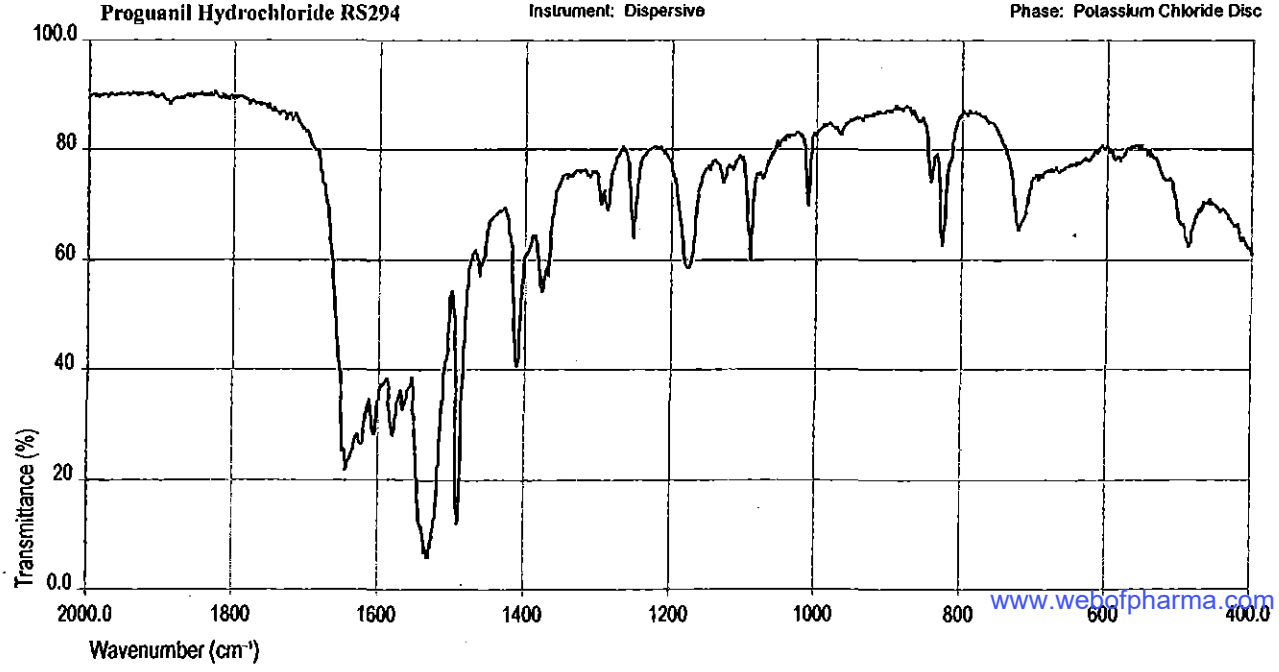
Procyclidine Hydrochloride RS292 Instrument: Dispersive Phase: Potassium Chloride Disc

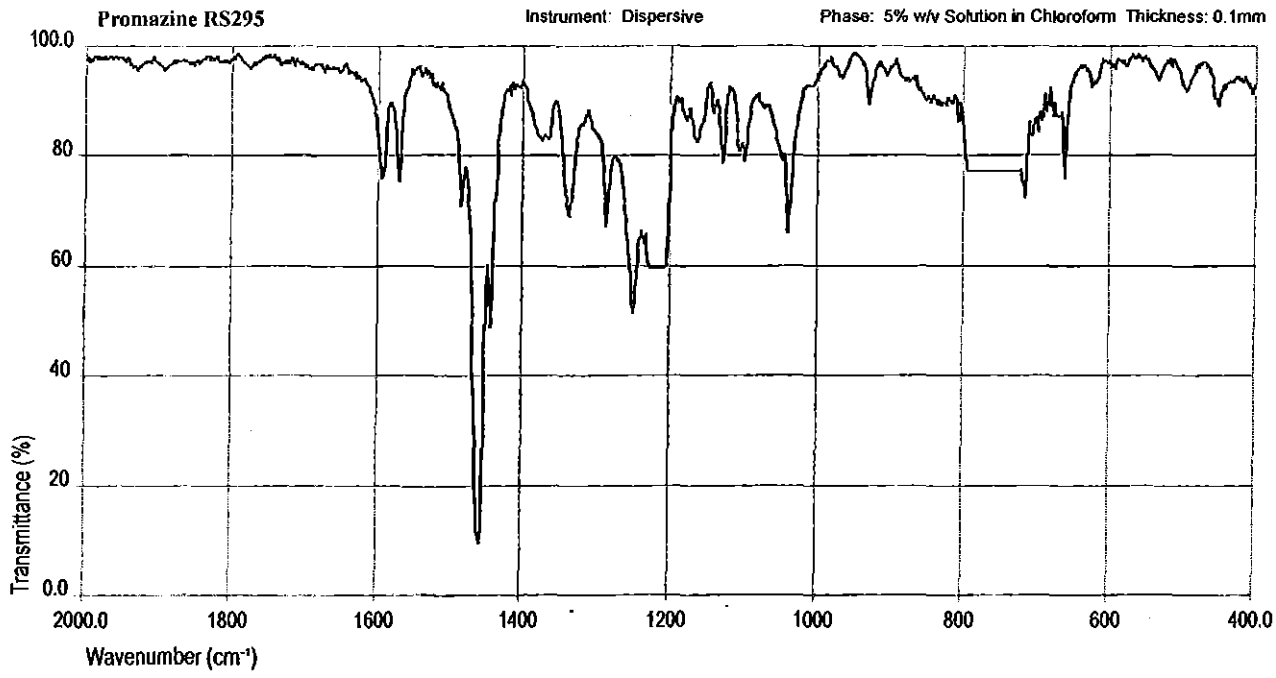


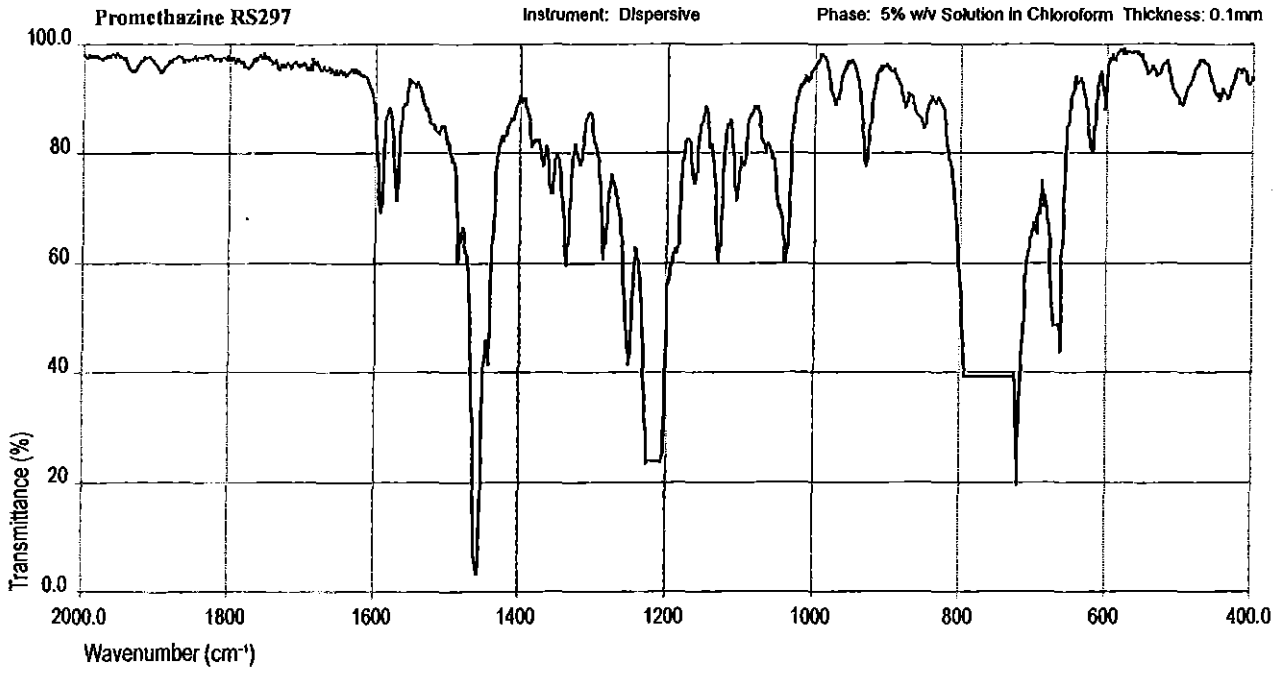
Progesterone RS293 Instrument: Dispersive Phase: Potassium Bromide Disc

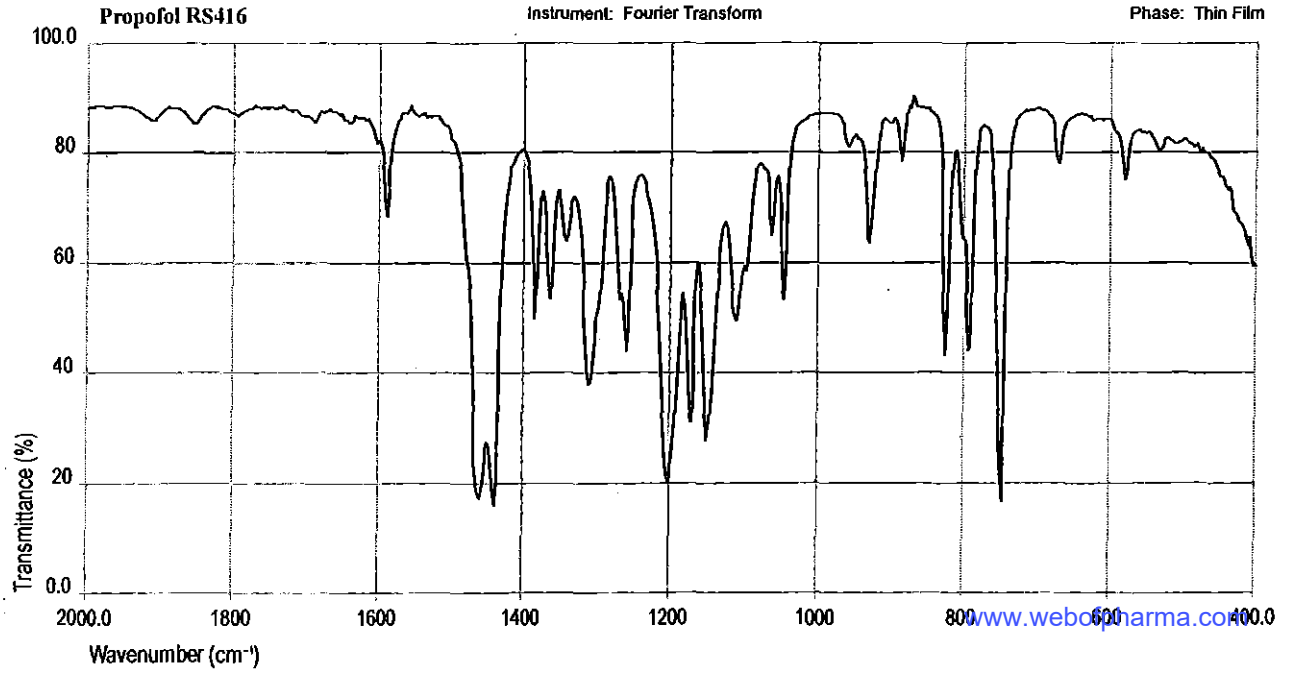


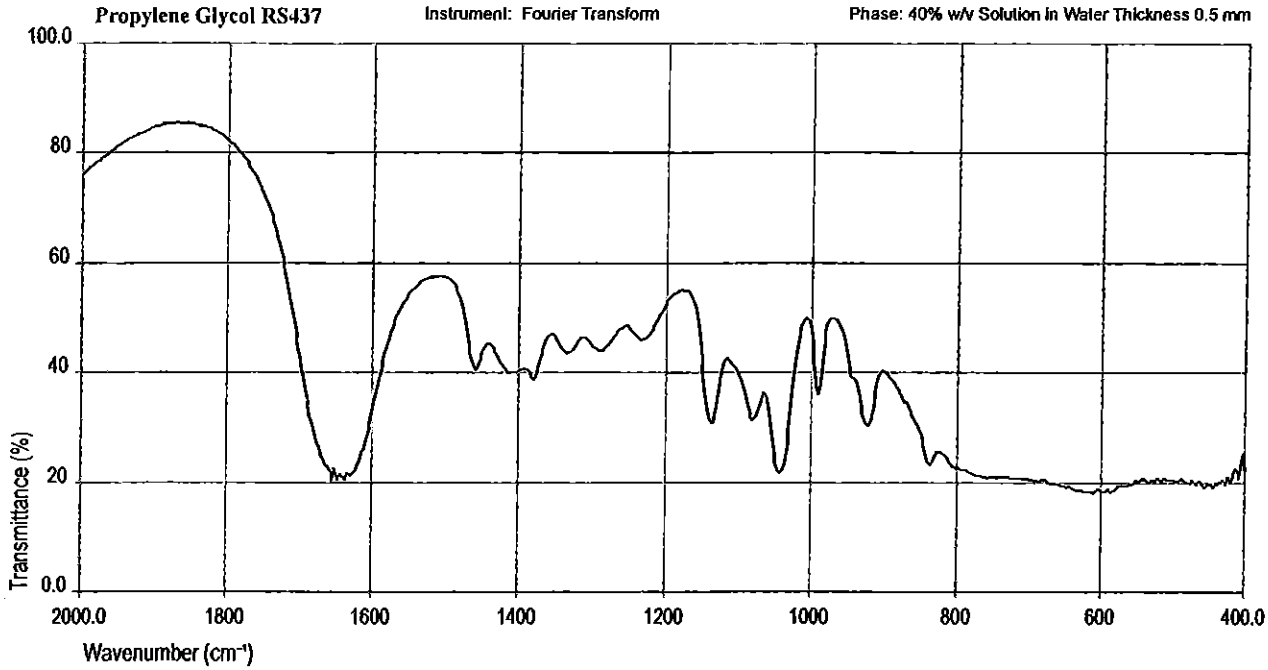
Proguanil Hydrochloride RS294 Instrument: Dispersive Phase: Potassium Chloride Disc

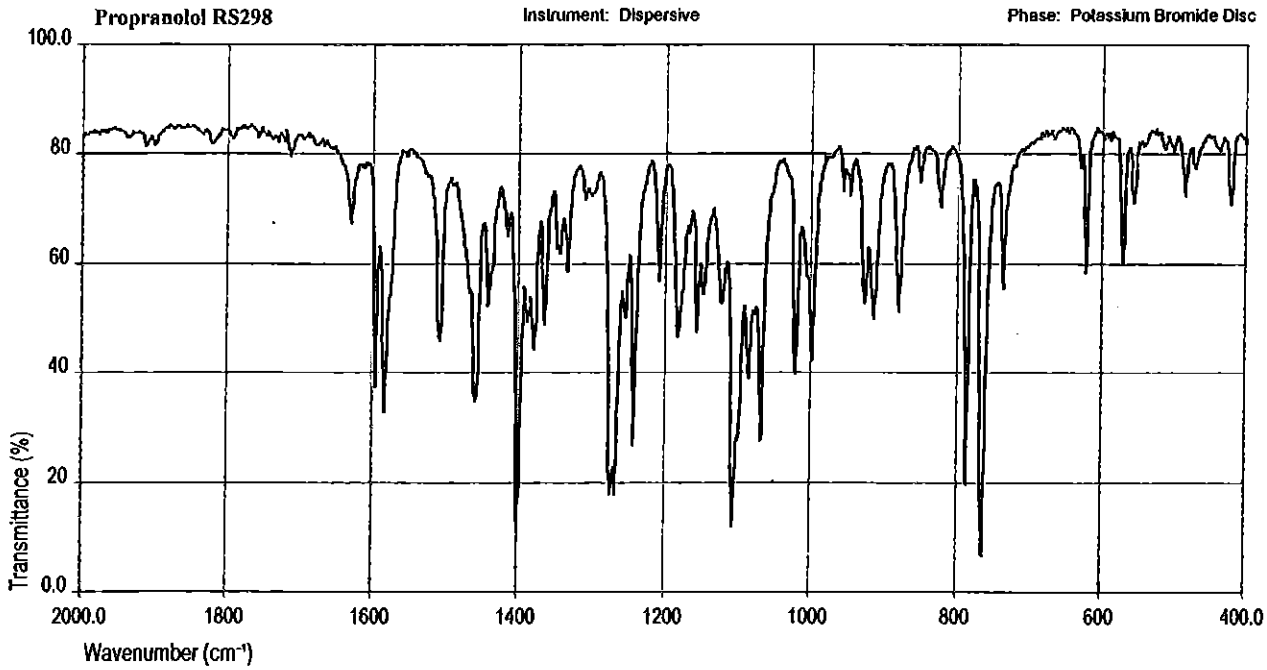


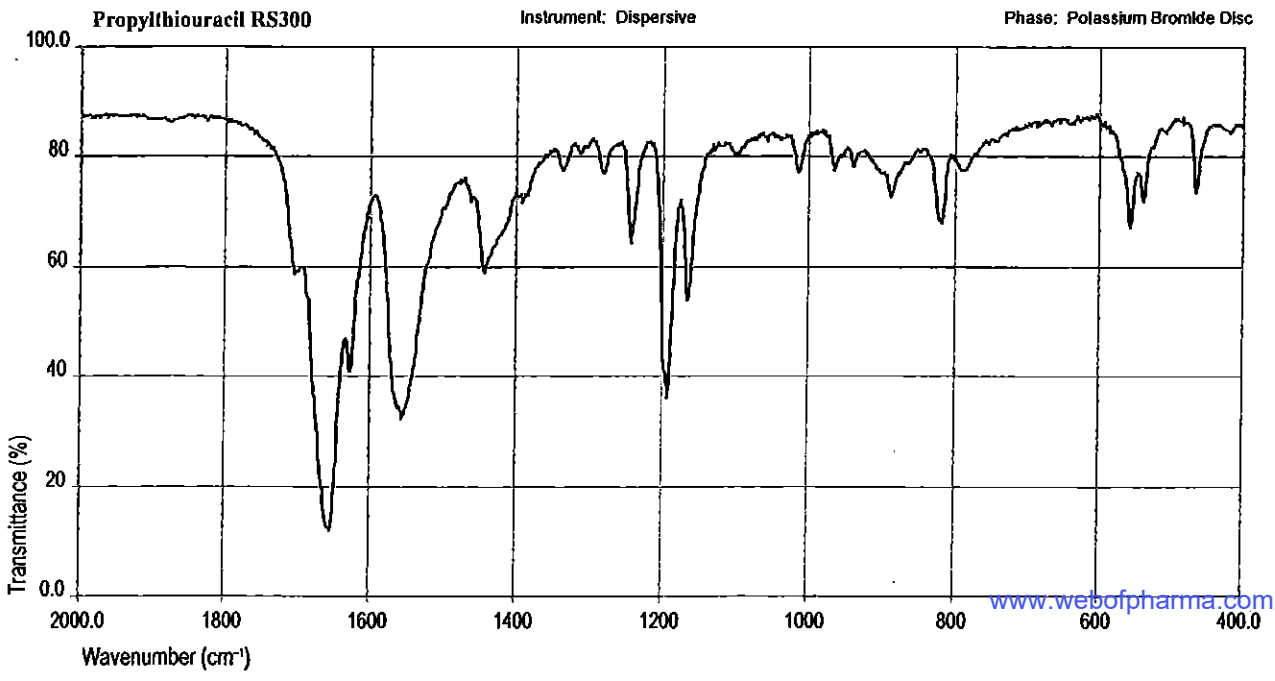


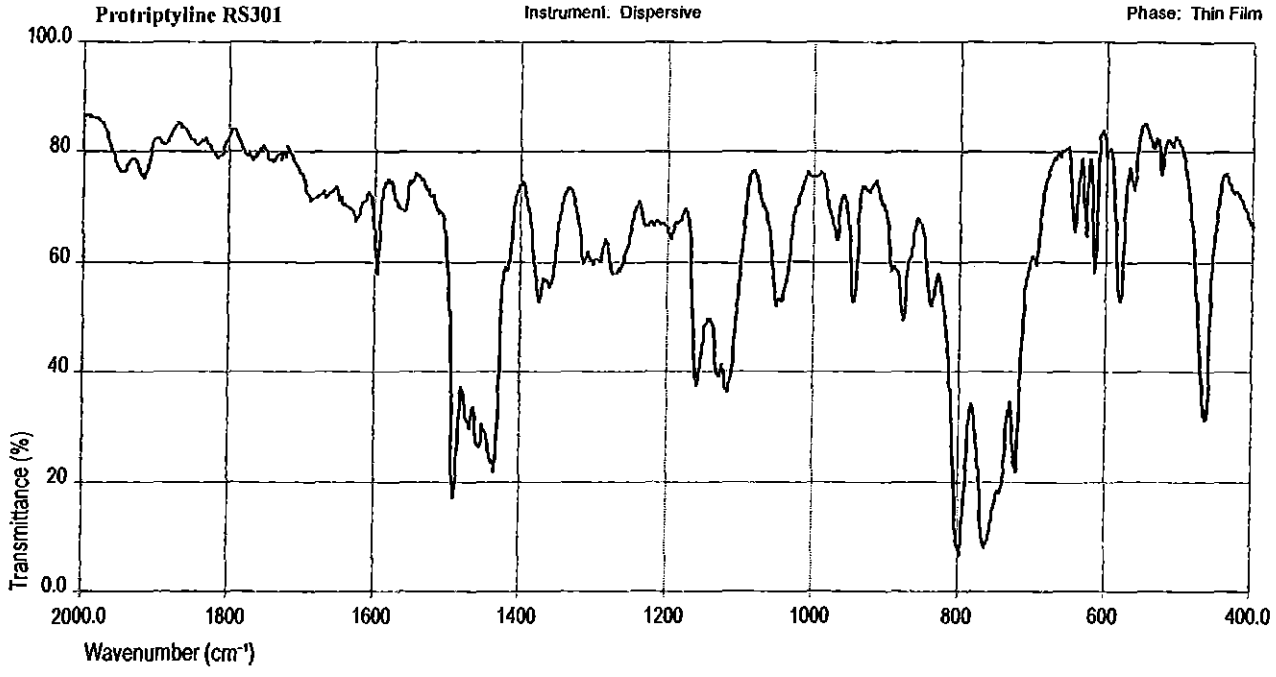


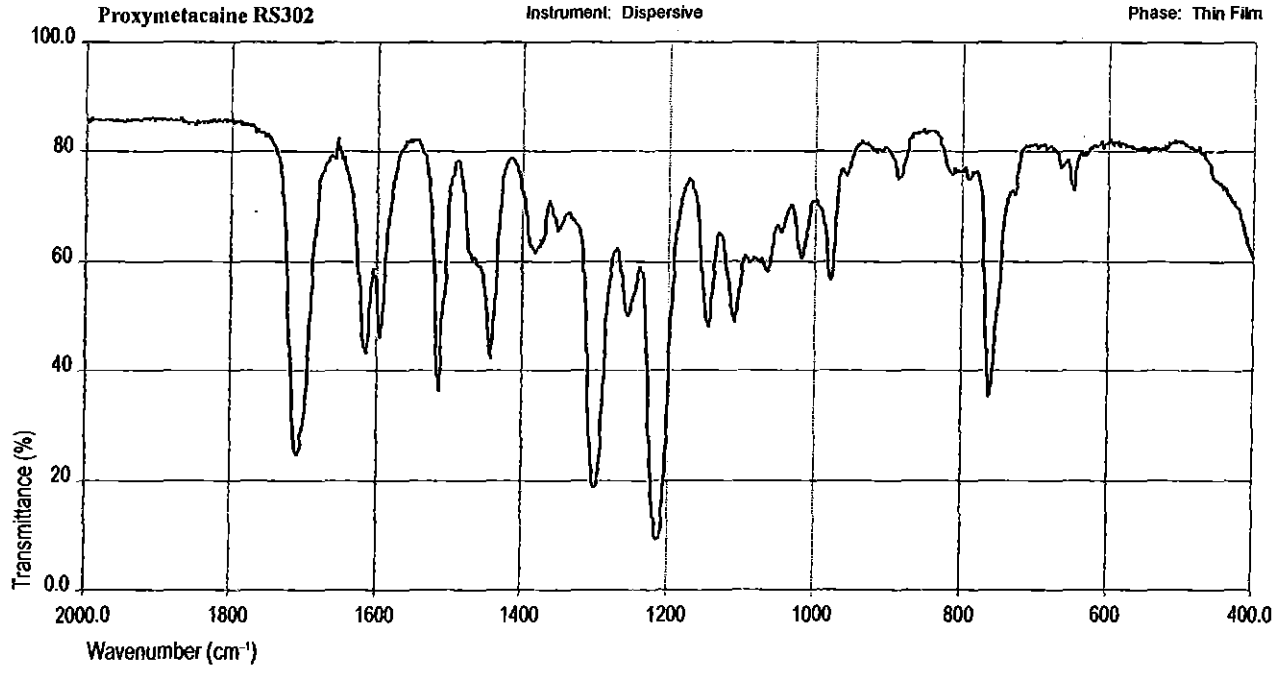


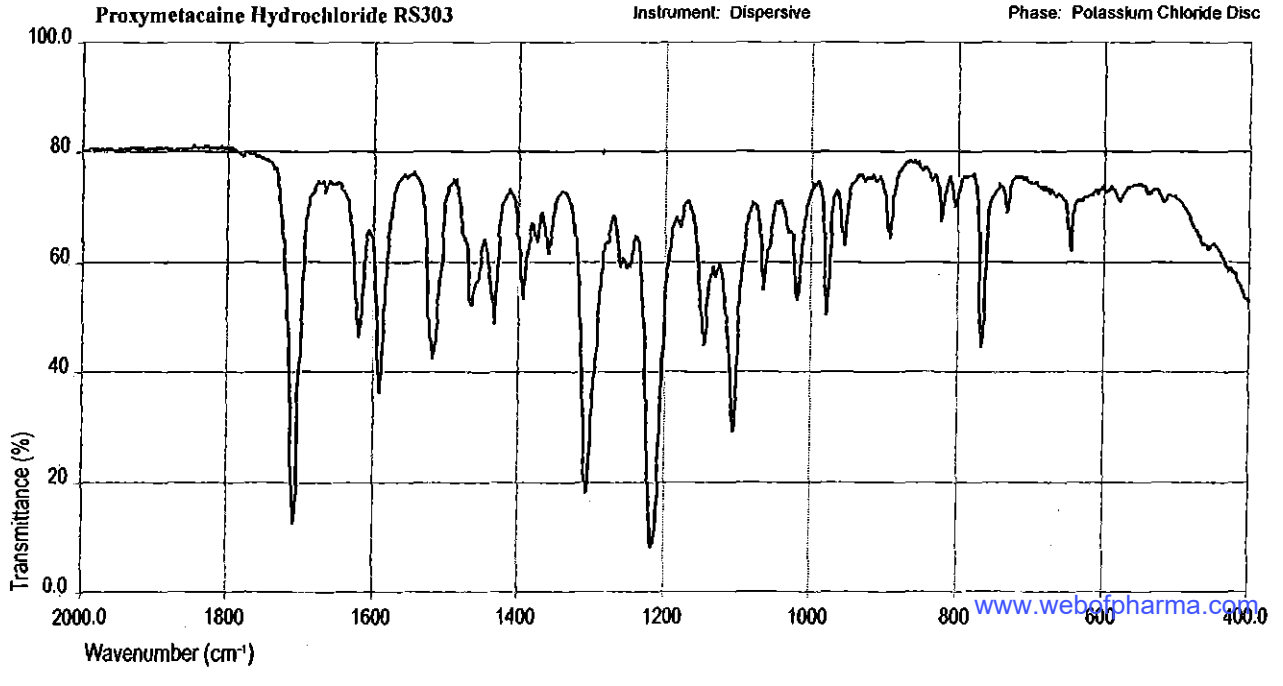


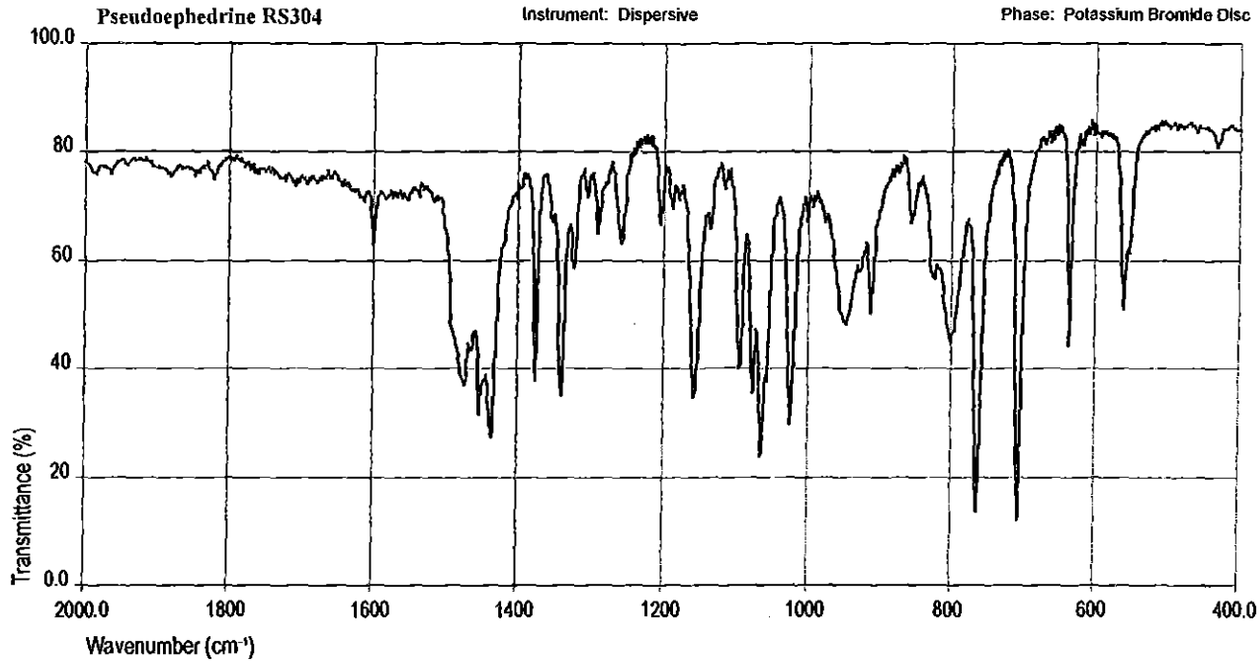


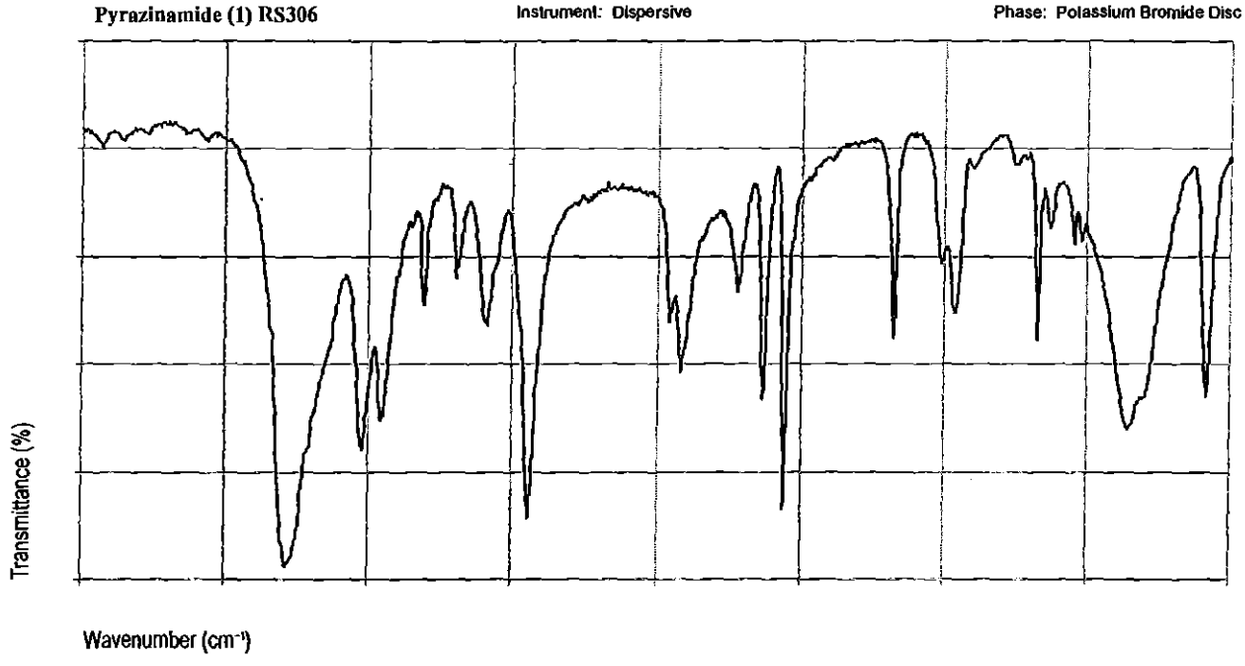


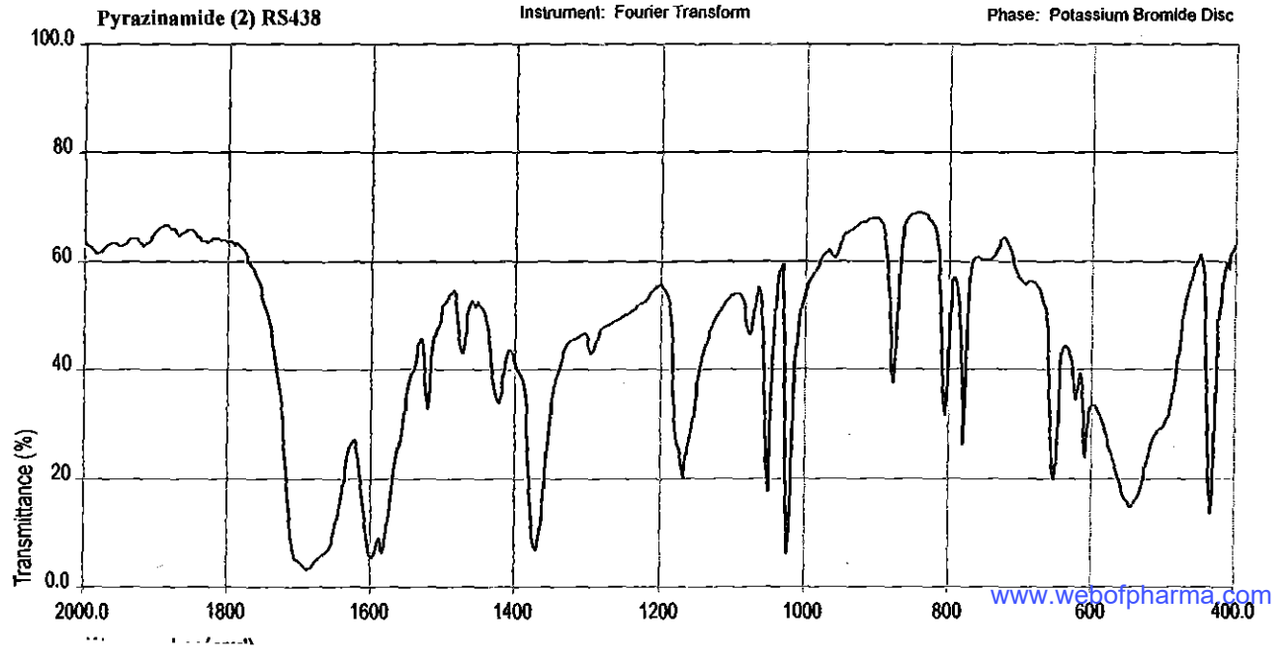


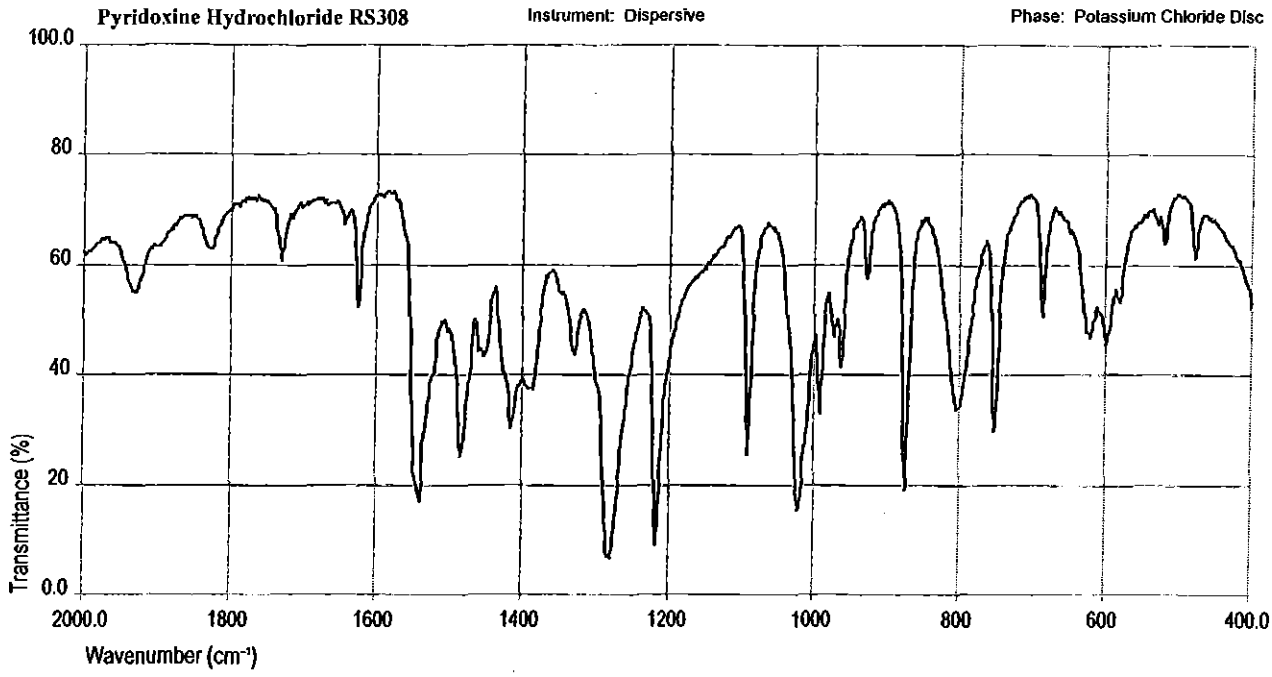


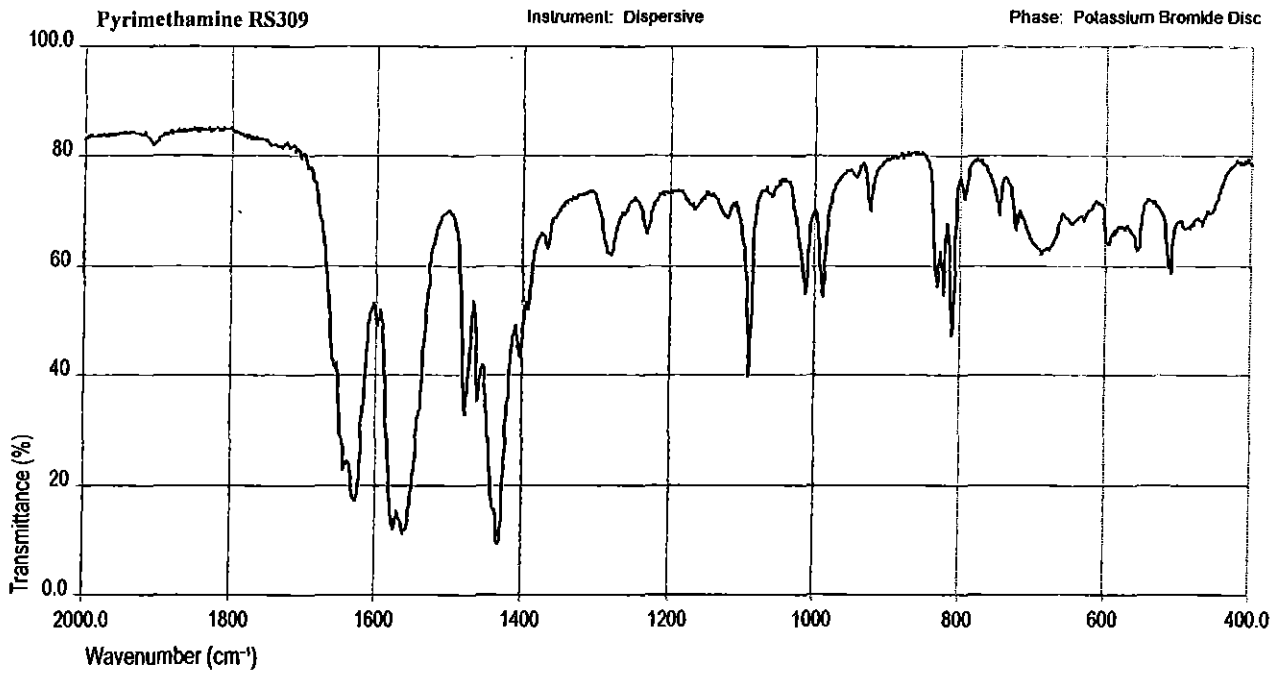


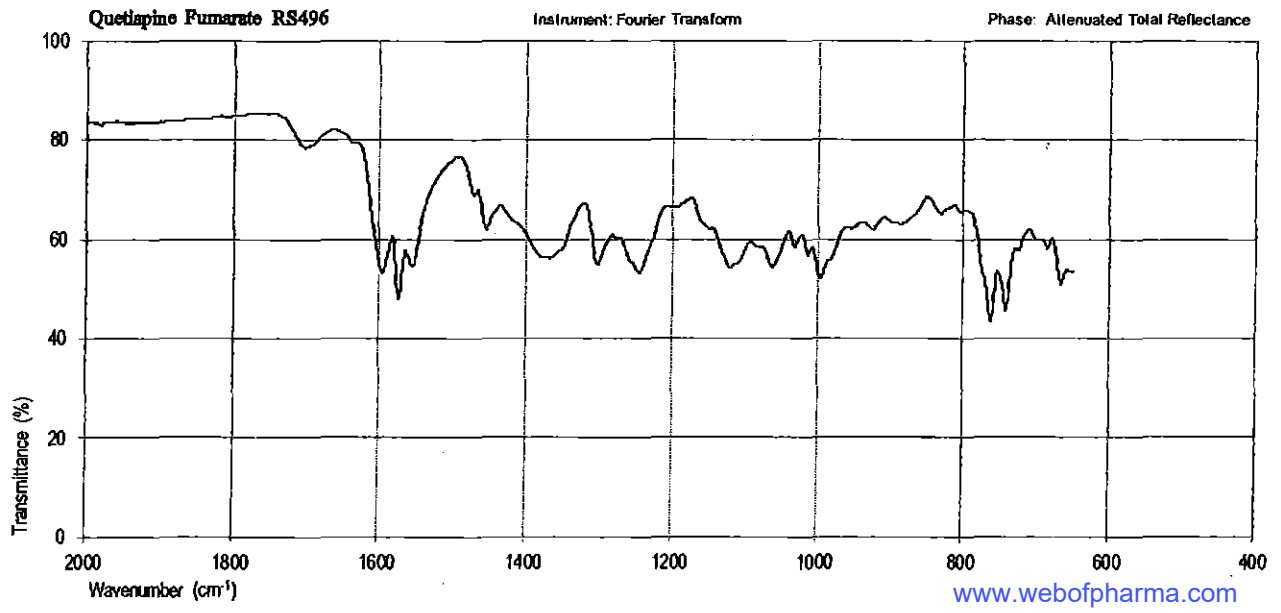


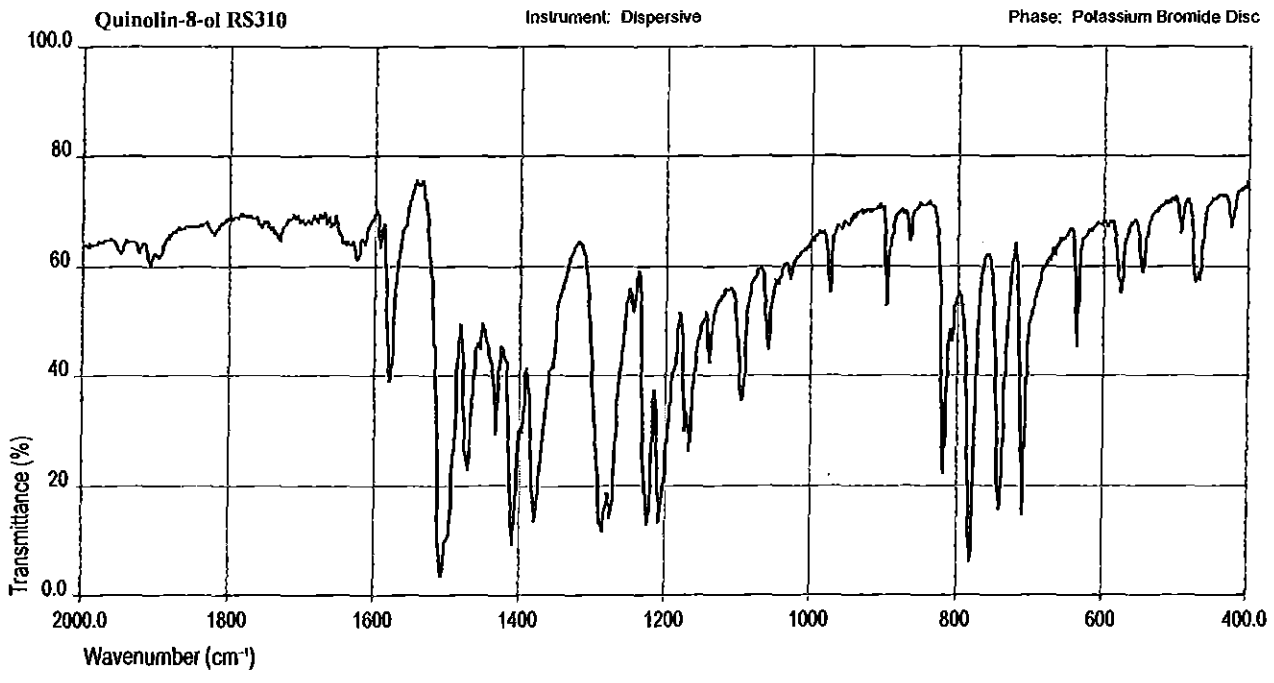


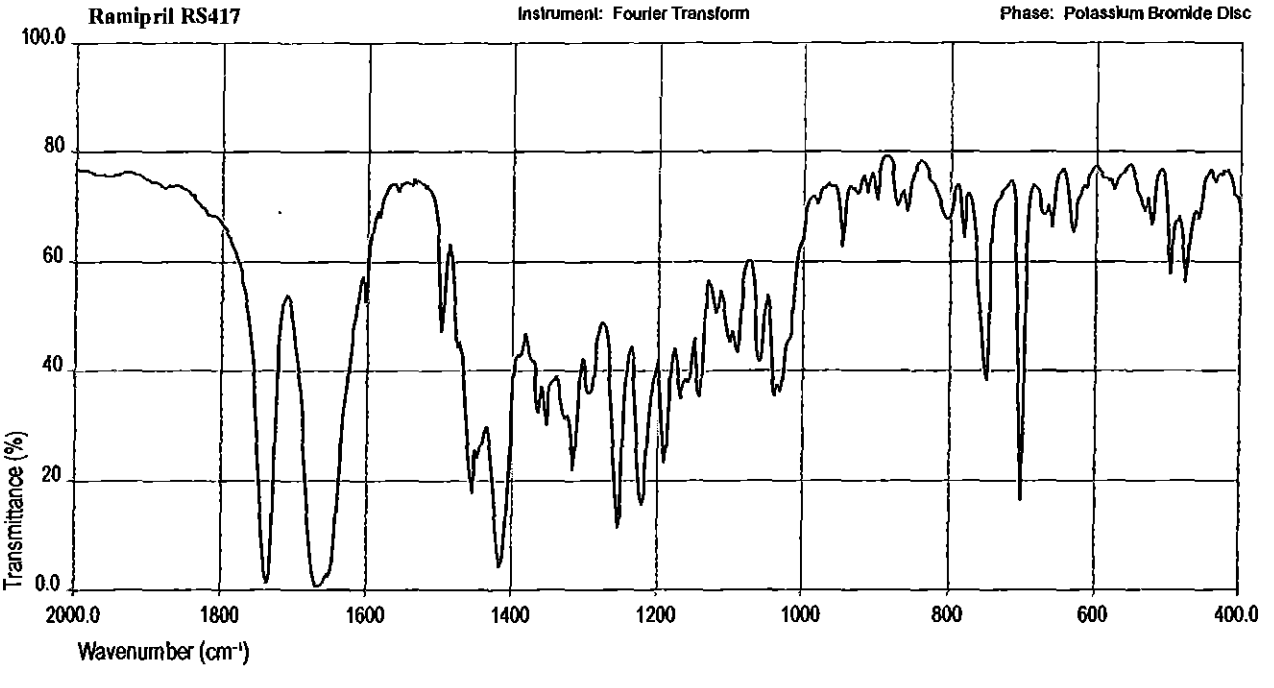


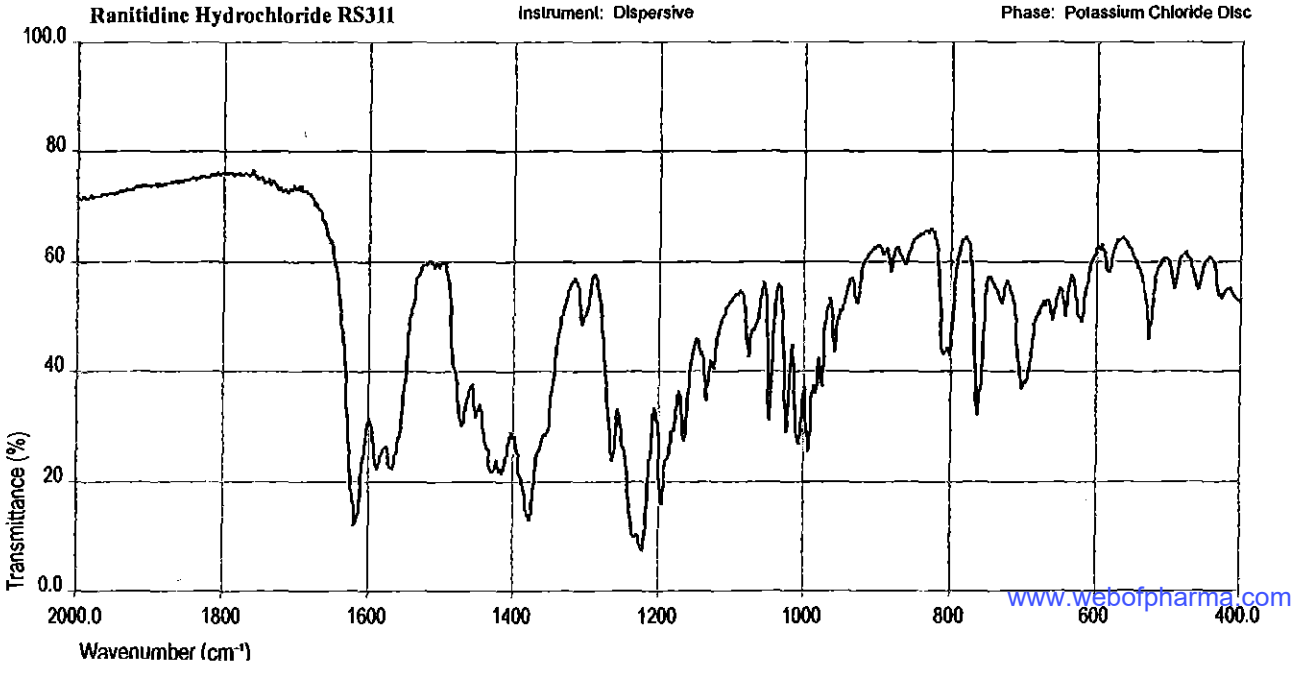








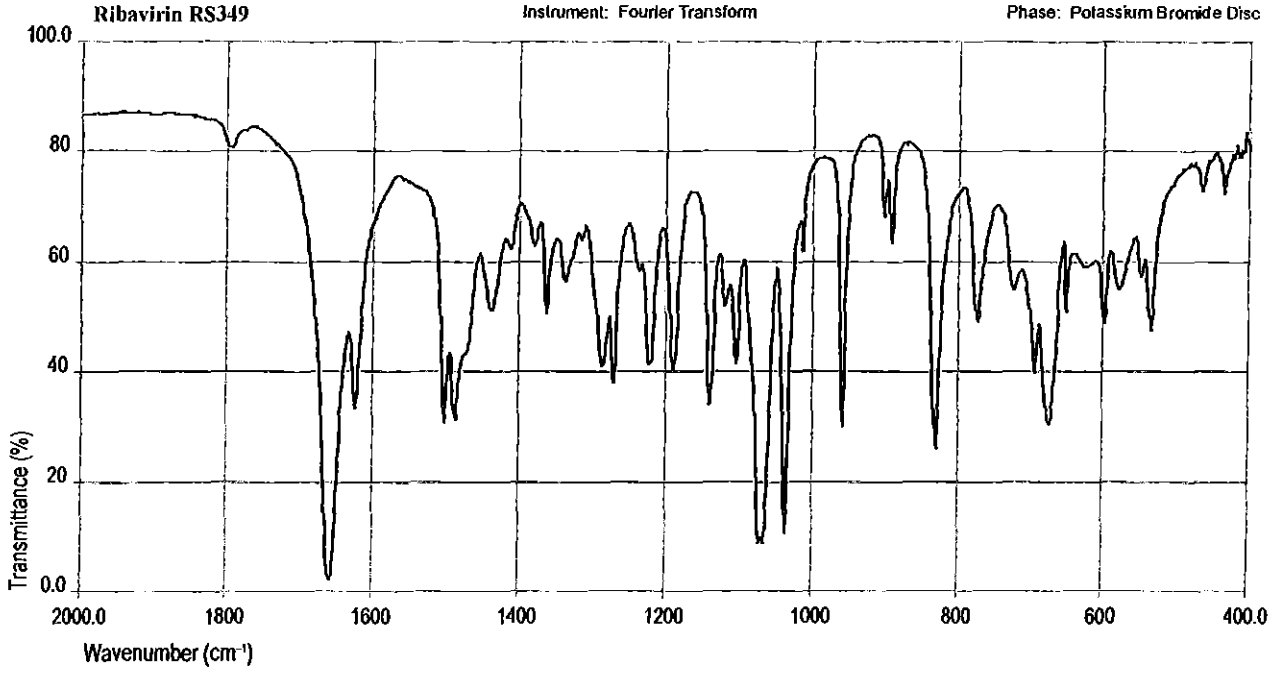




Ribavirin RS349

Instrument: Fourier Transform

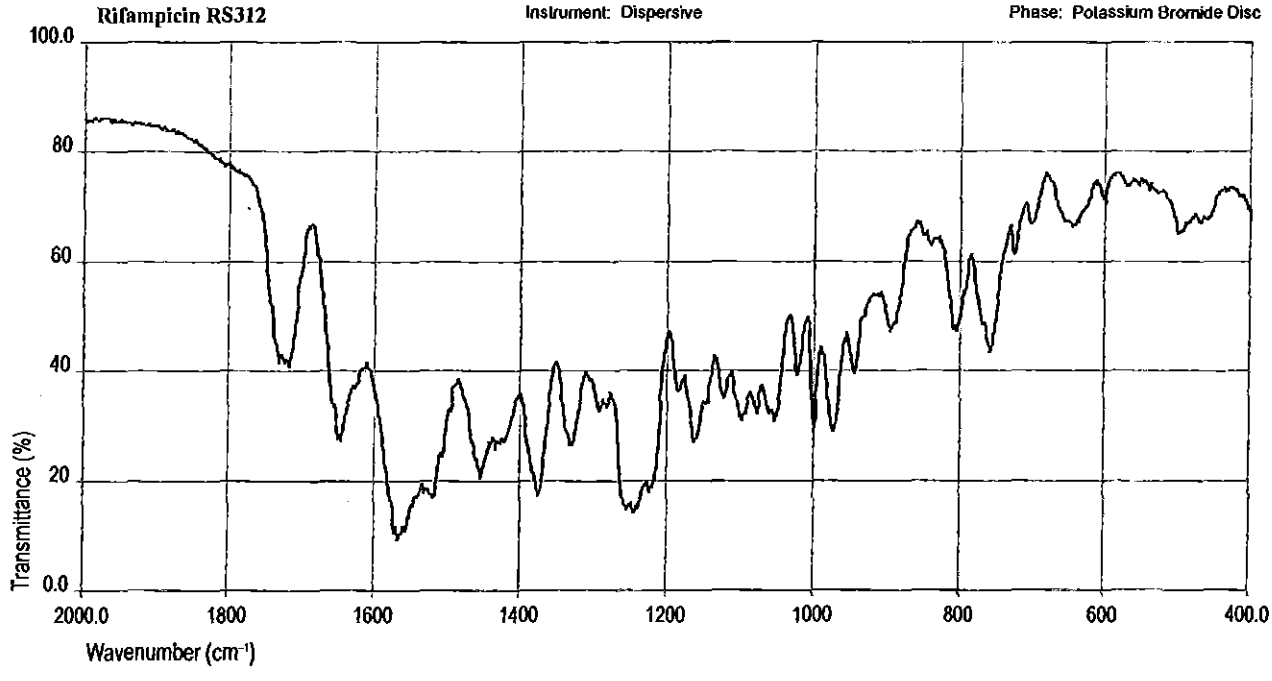
Phase: Potassium Bromide Disc



Rifampicin RS312

Instrument: Dispersive

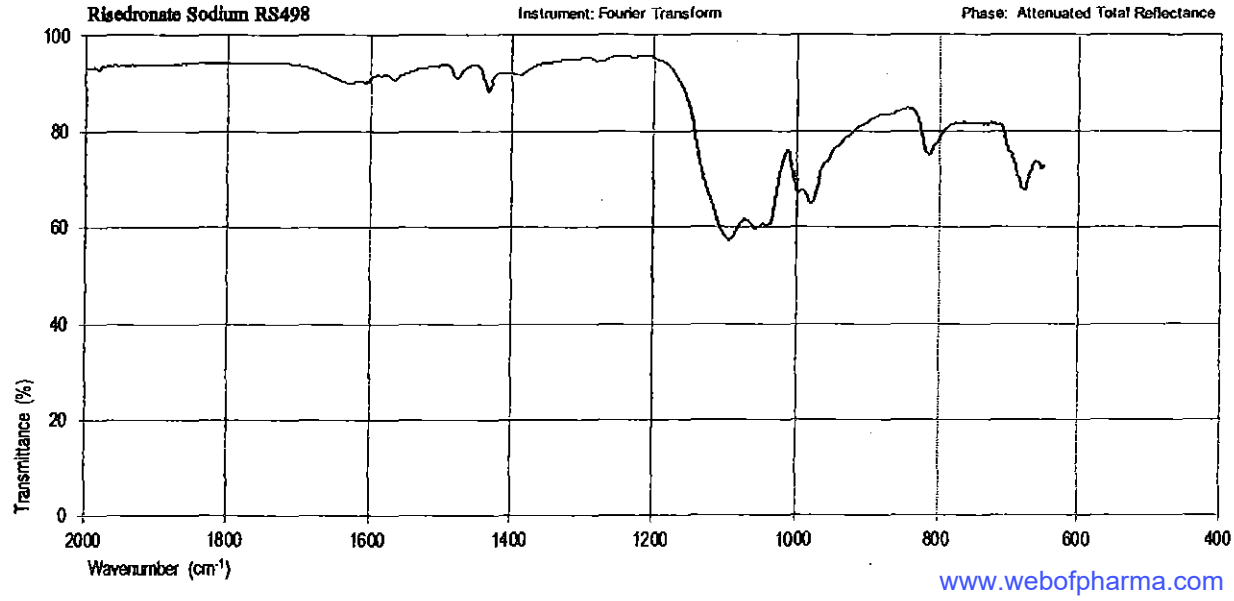
Phase: Potassium Bromide Disc

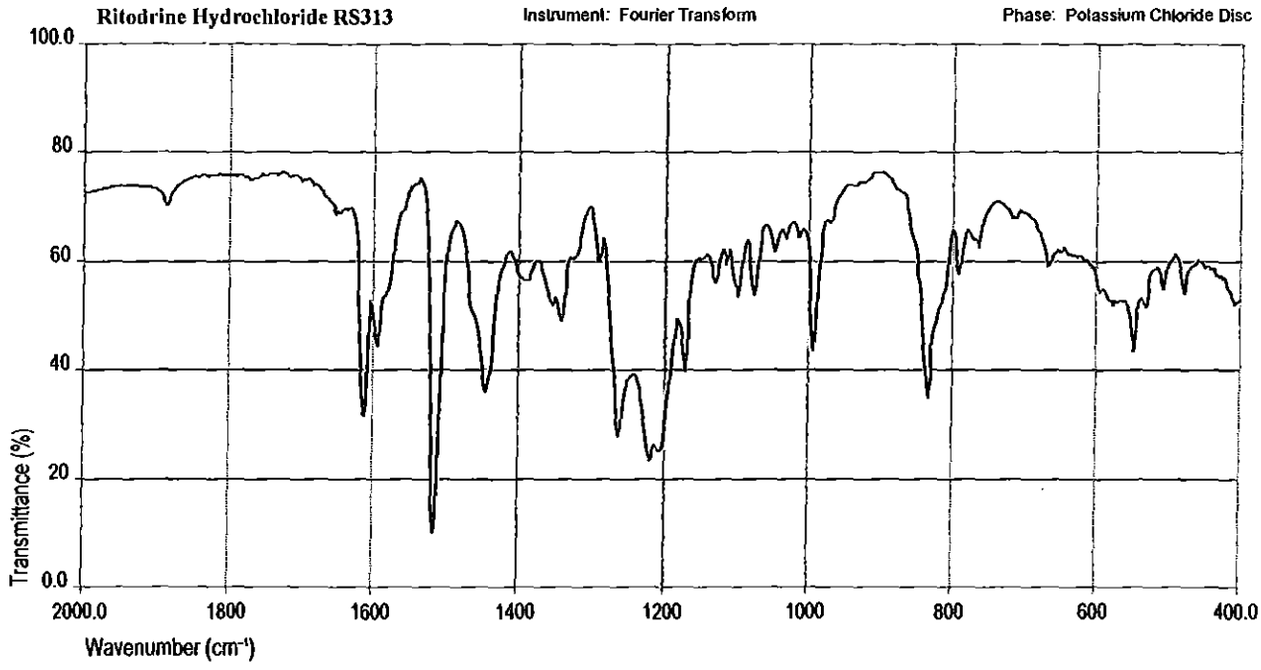


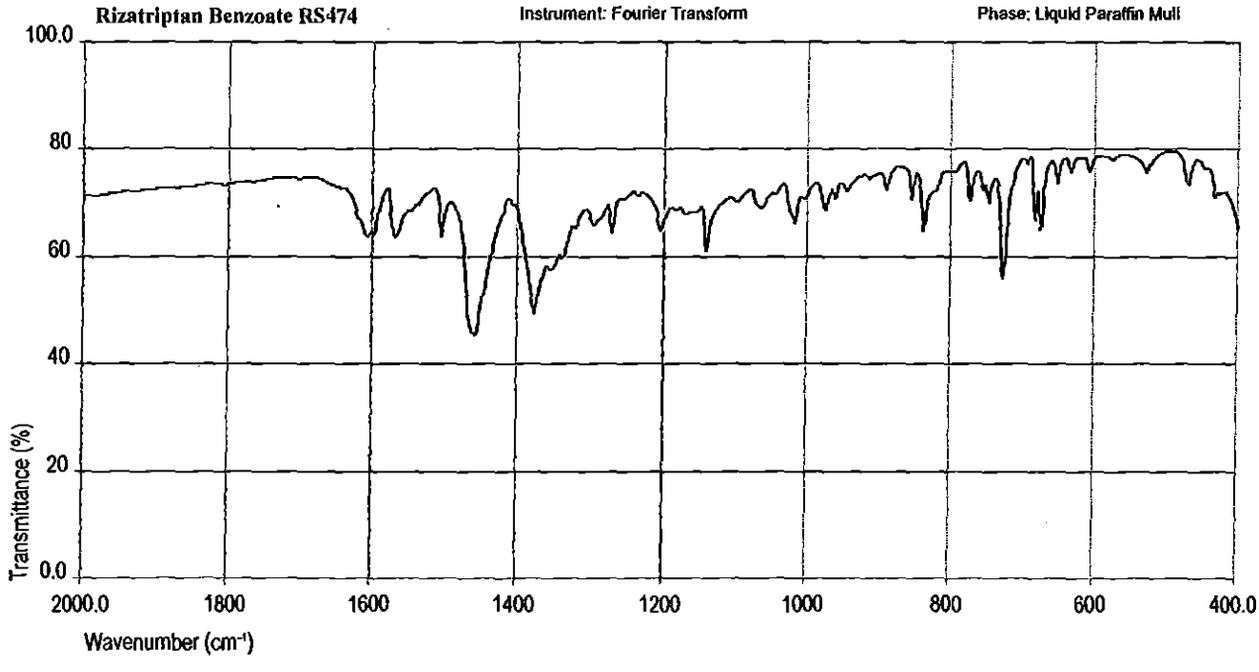
Risedronate Sodium RS498

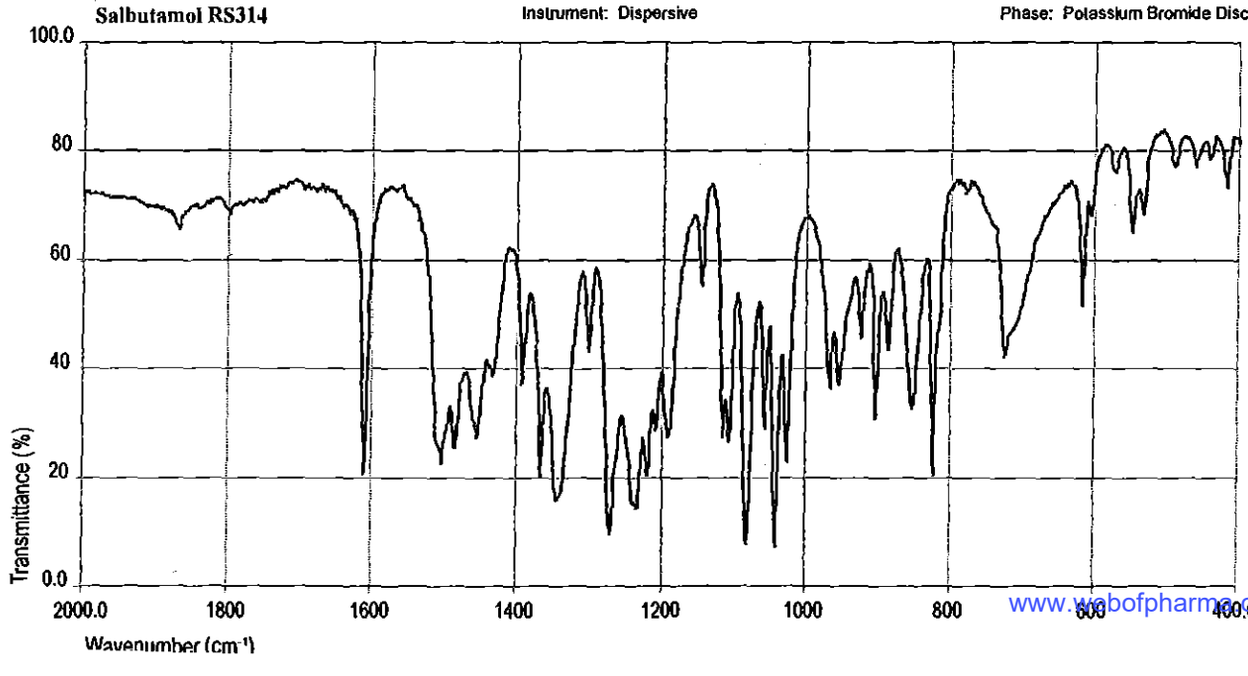
Instrument: Fourier Transform

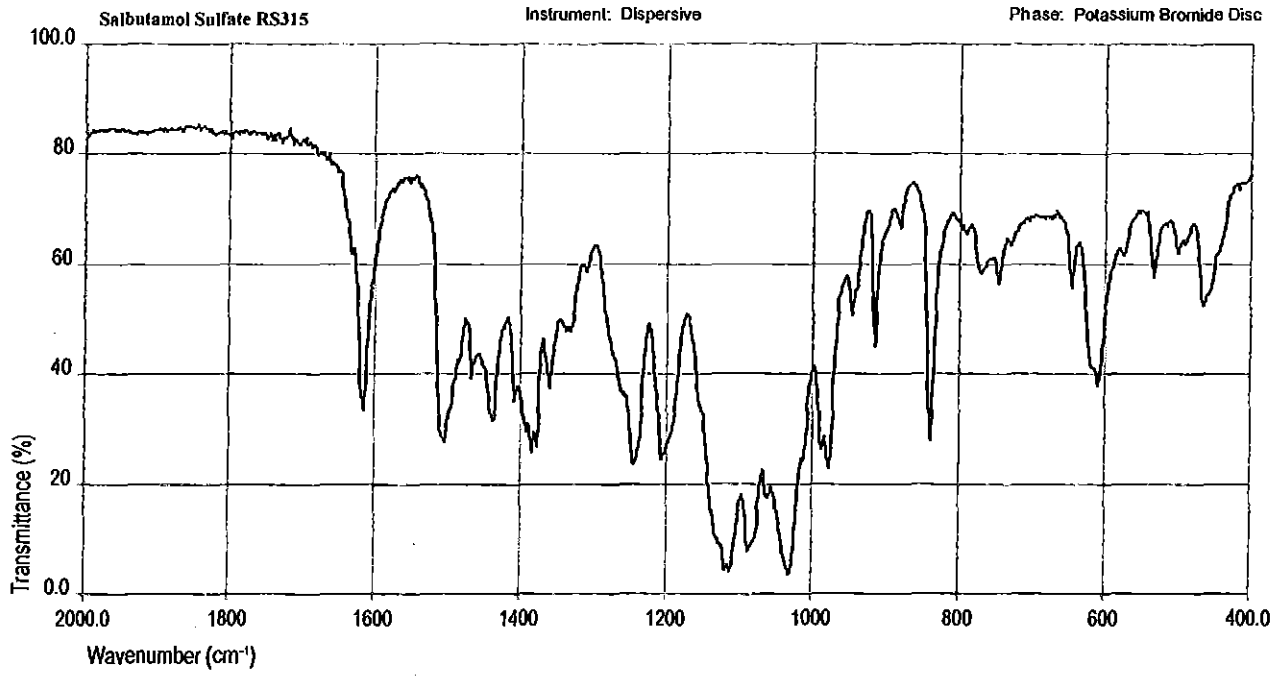
Phase: Attenuated Total Reflectance

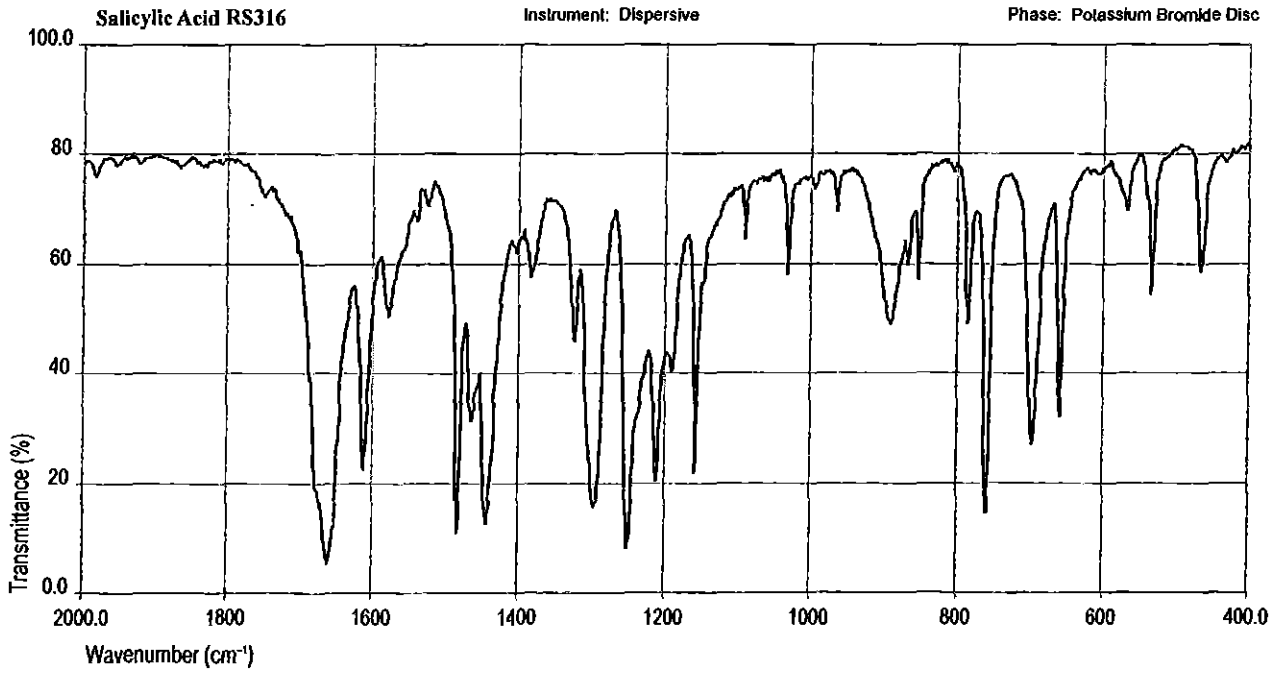


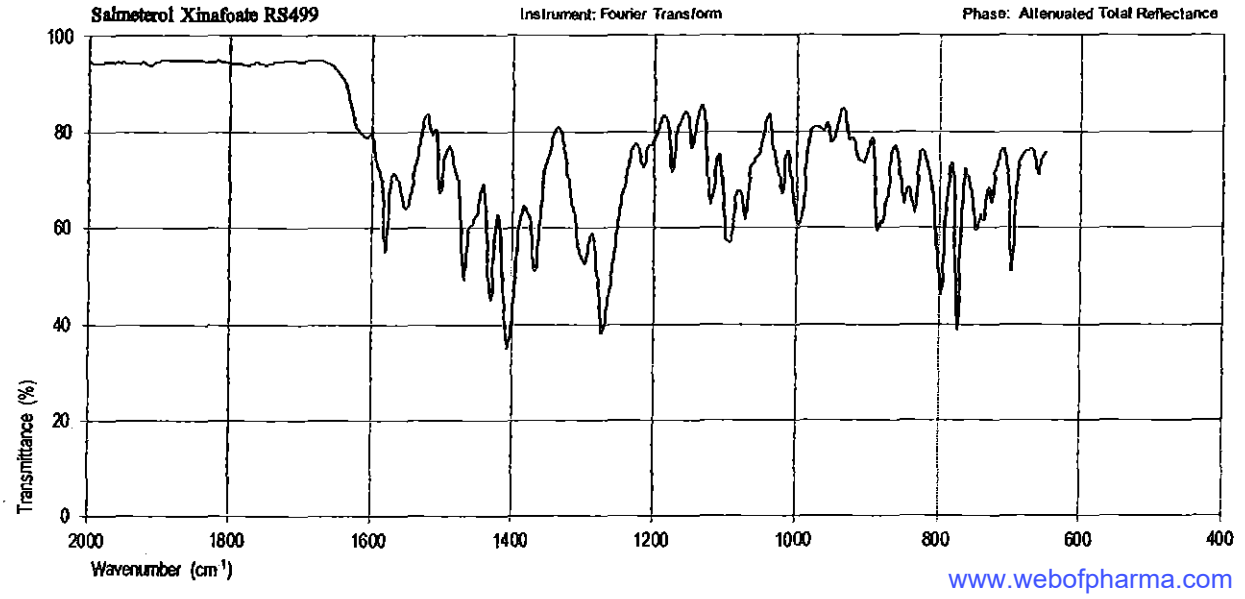


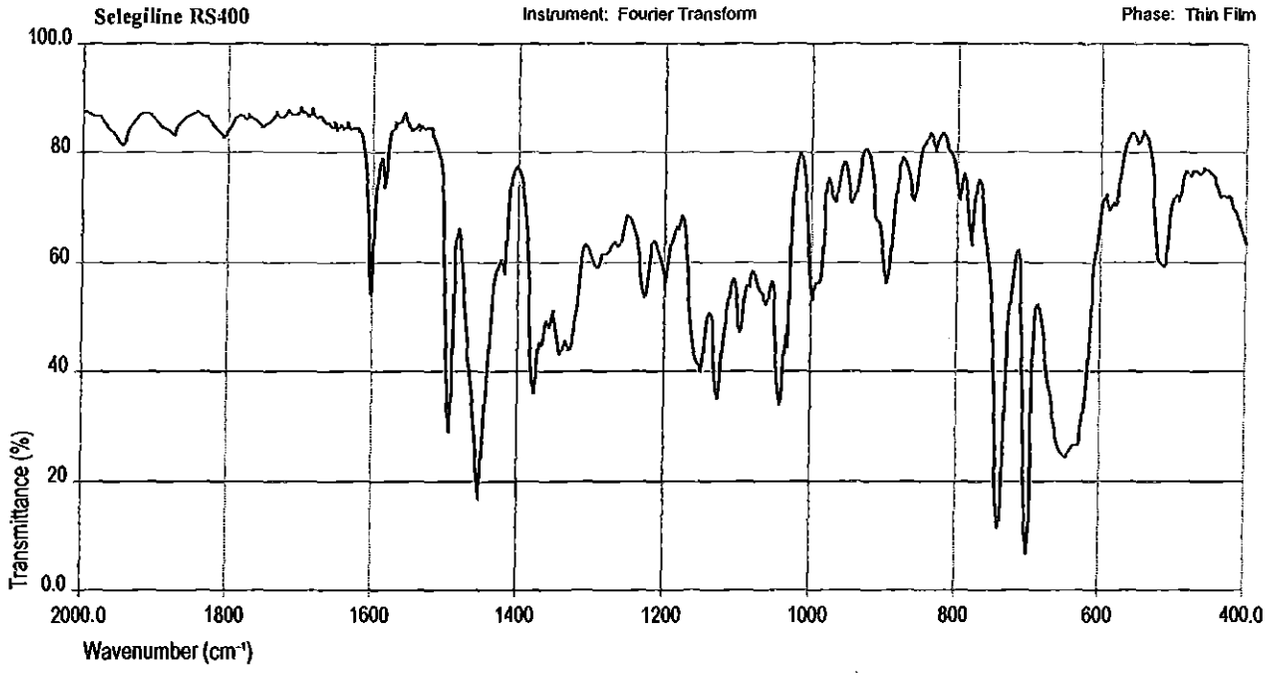


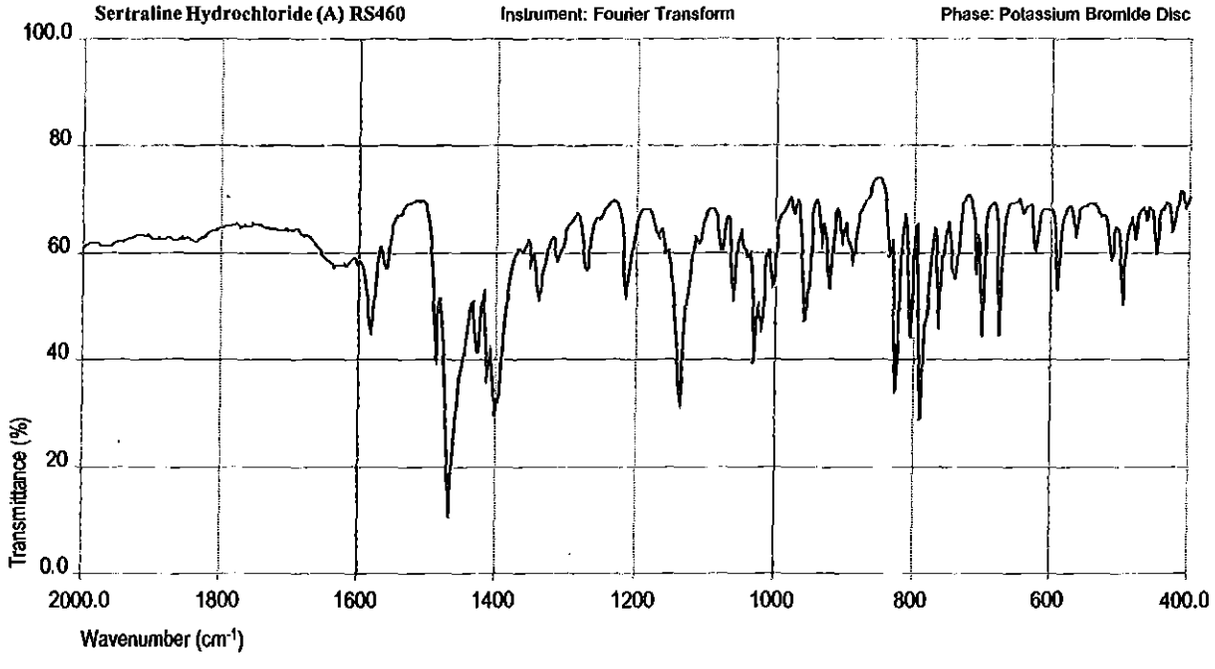


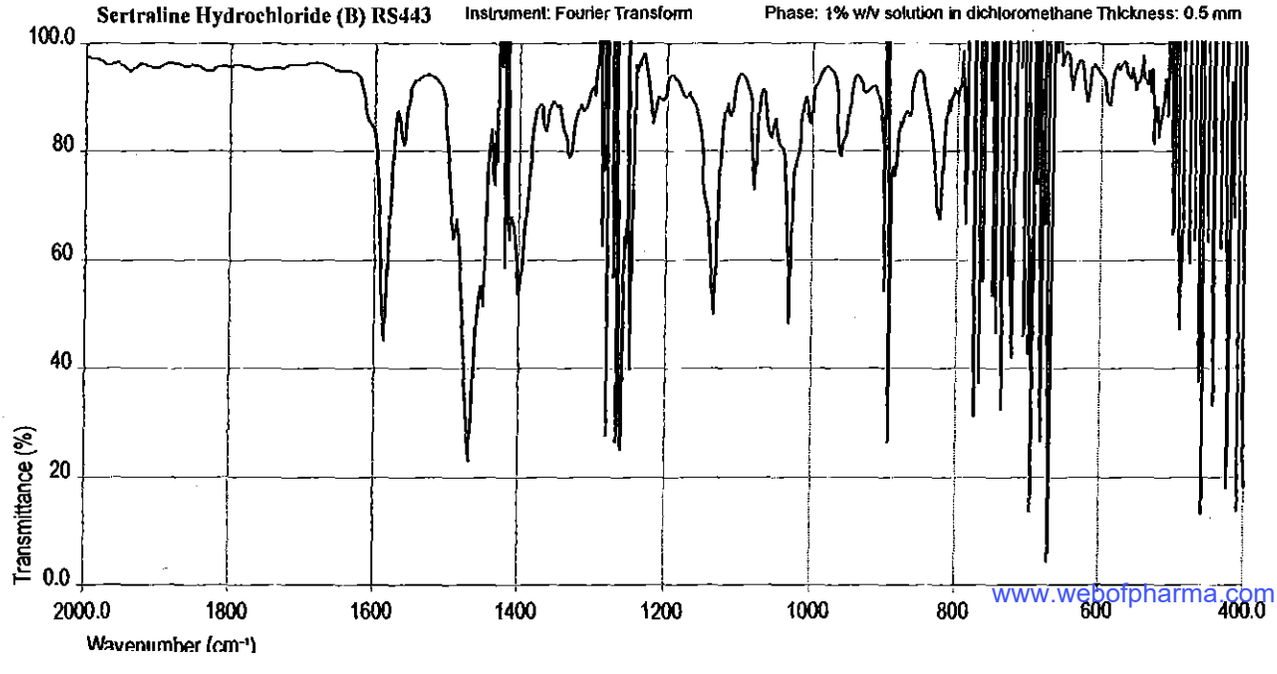


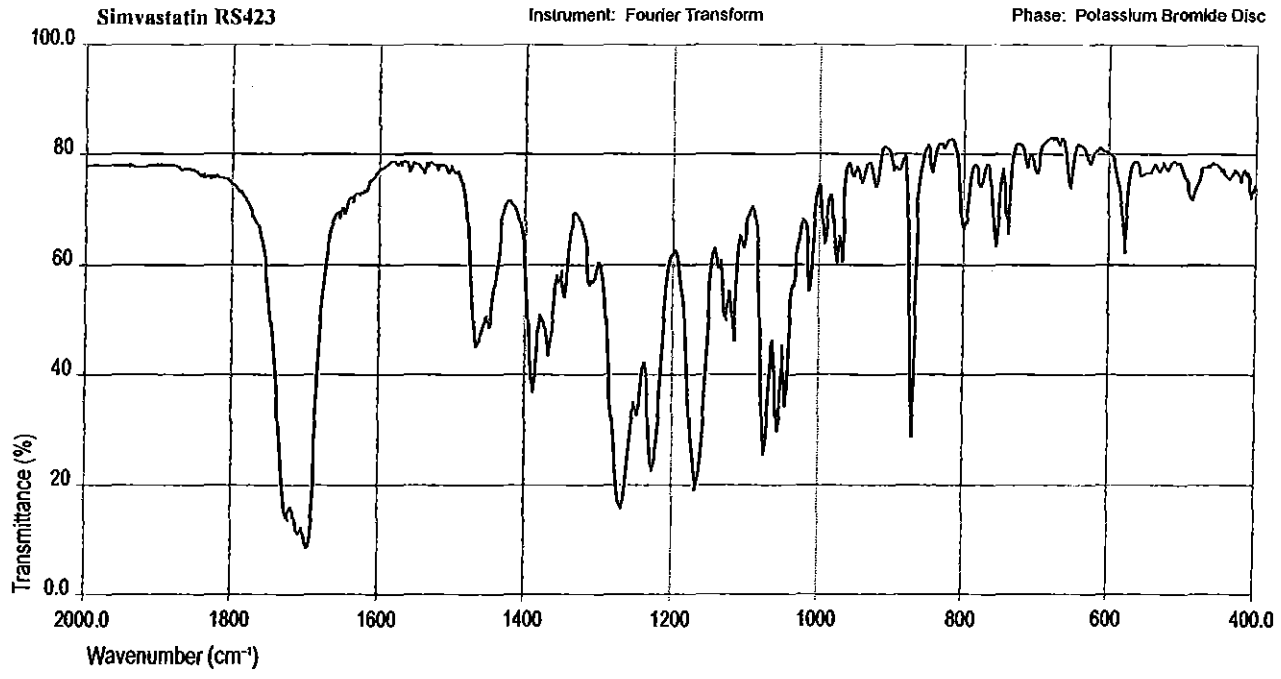


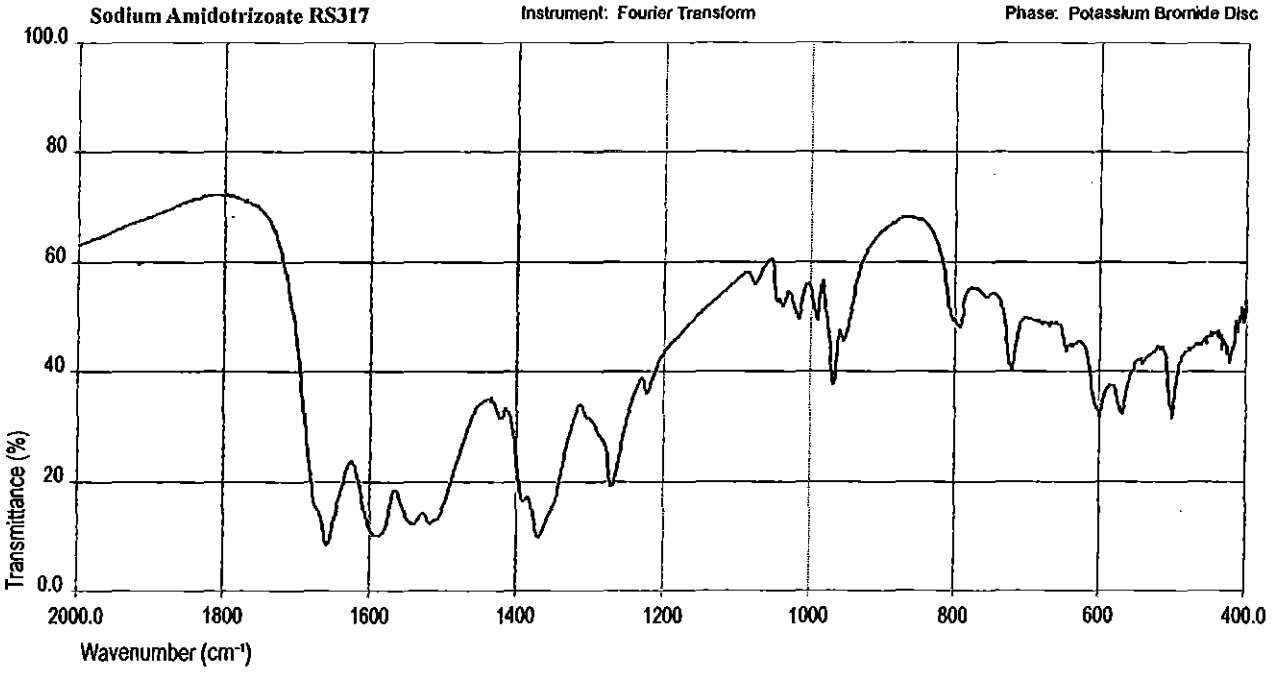


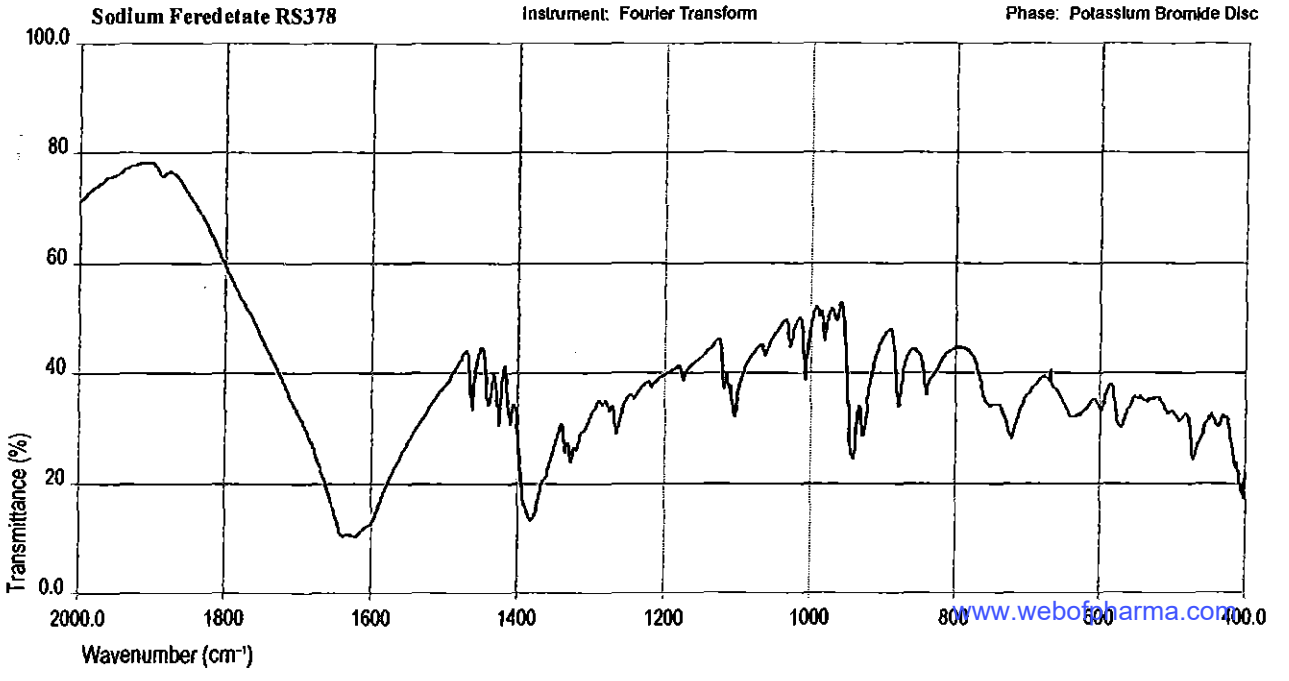




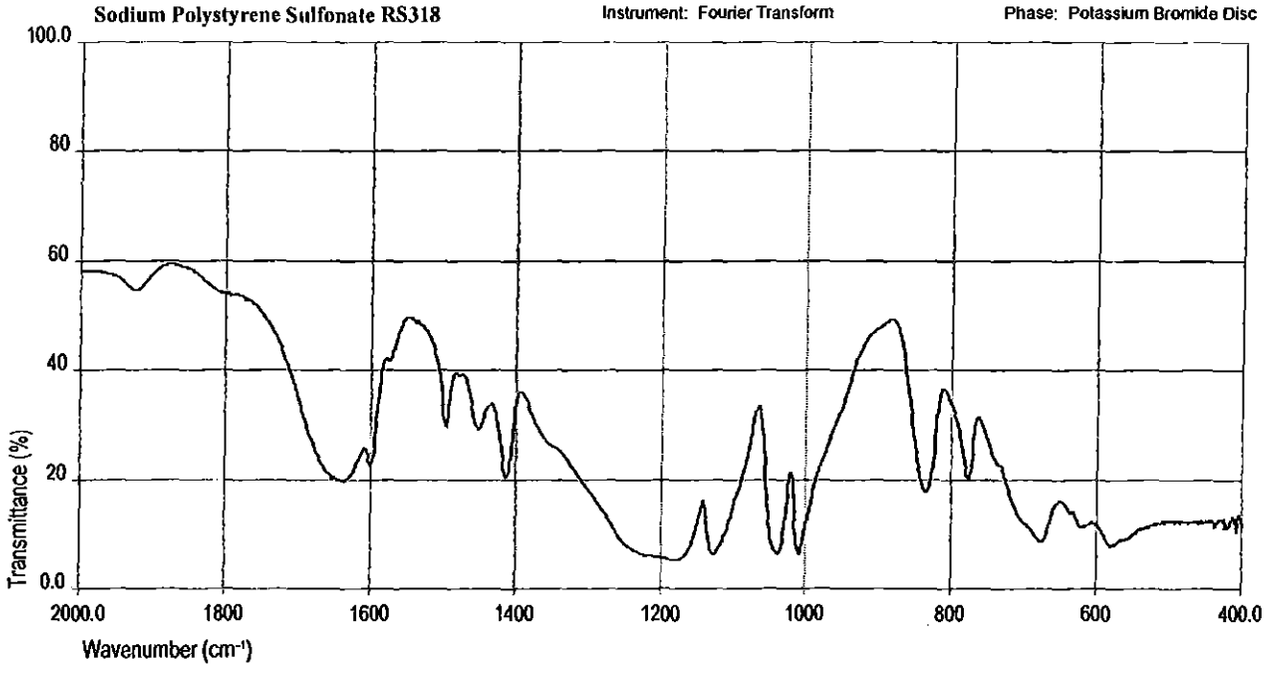




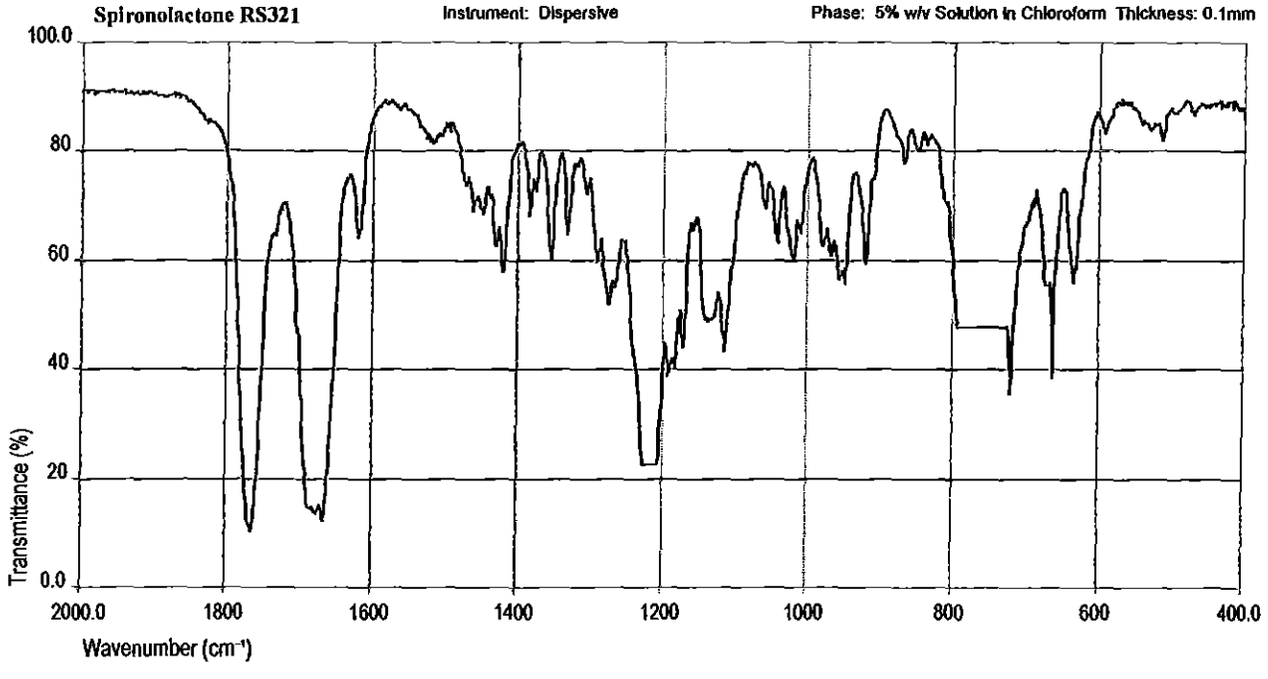




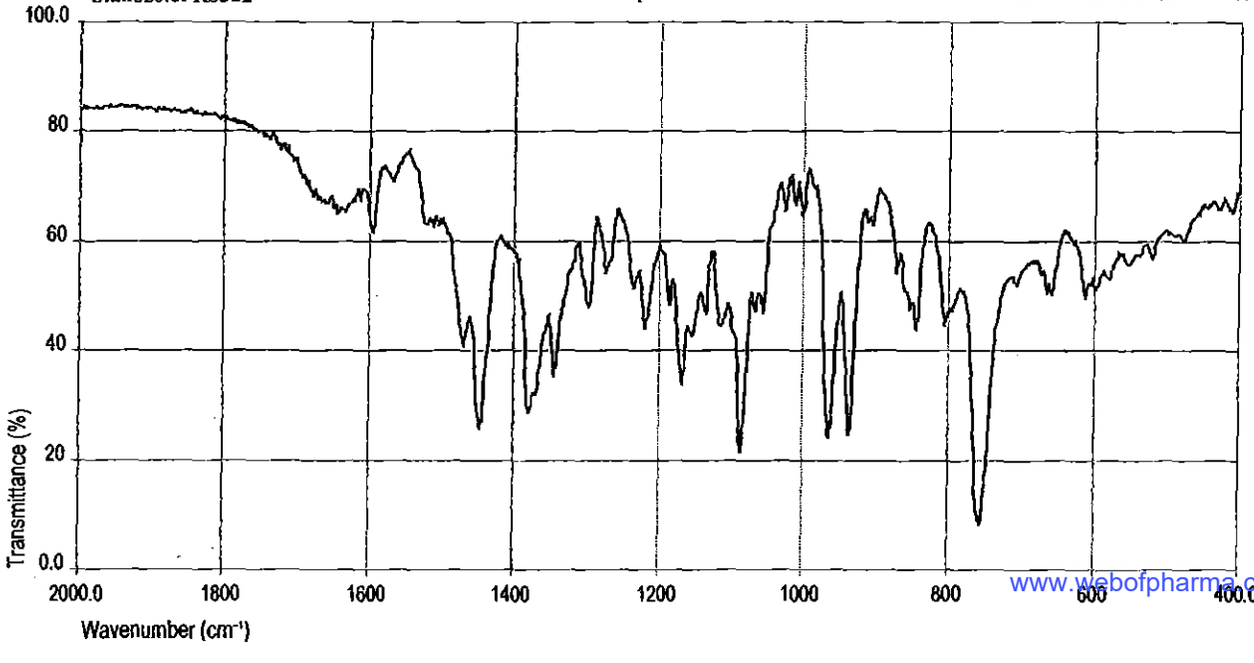
Sodium Polystyrene Sulfonate RS318 Instrument: Fourier Transform Phase: Potassium Bromide Disc

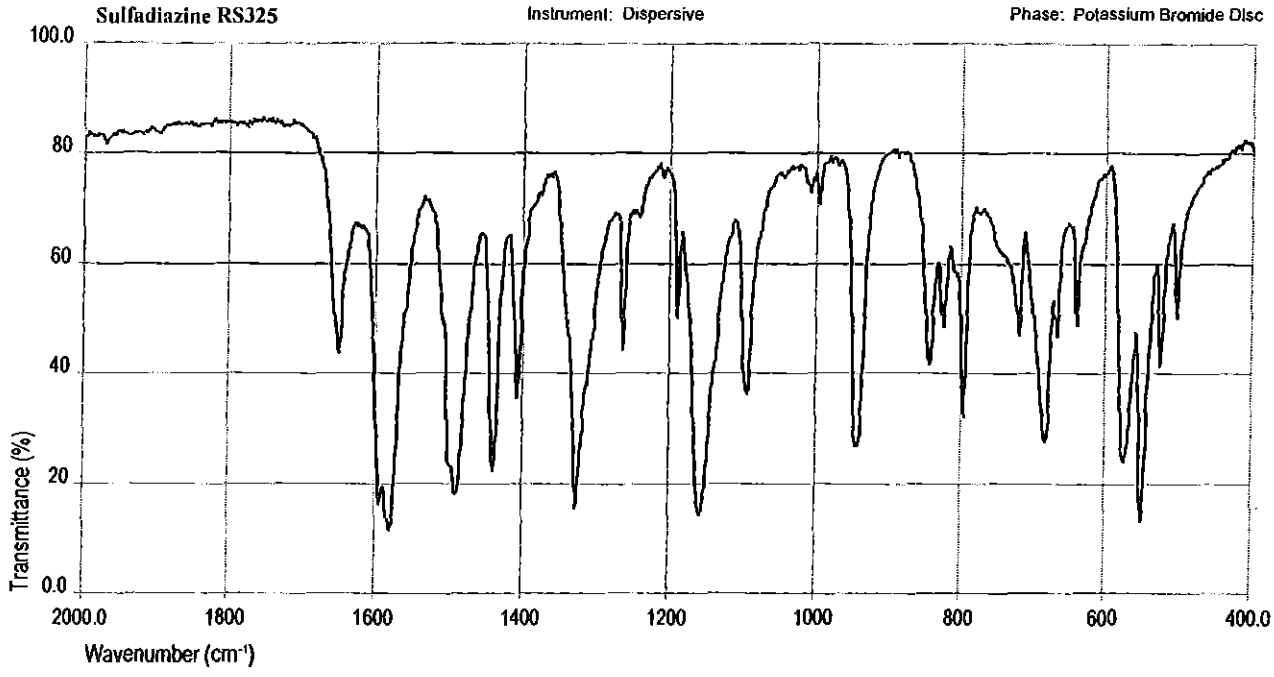


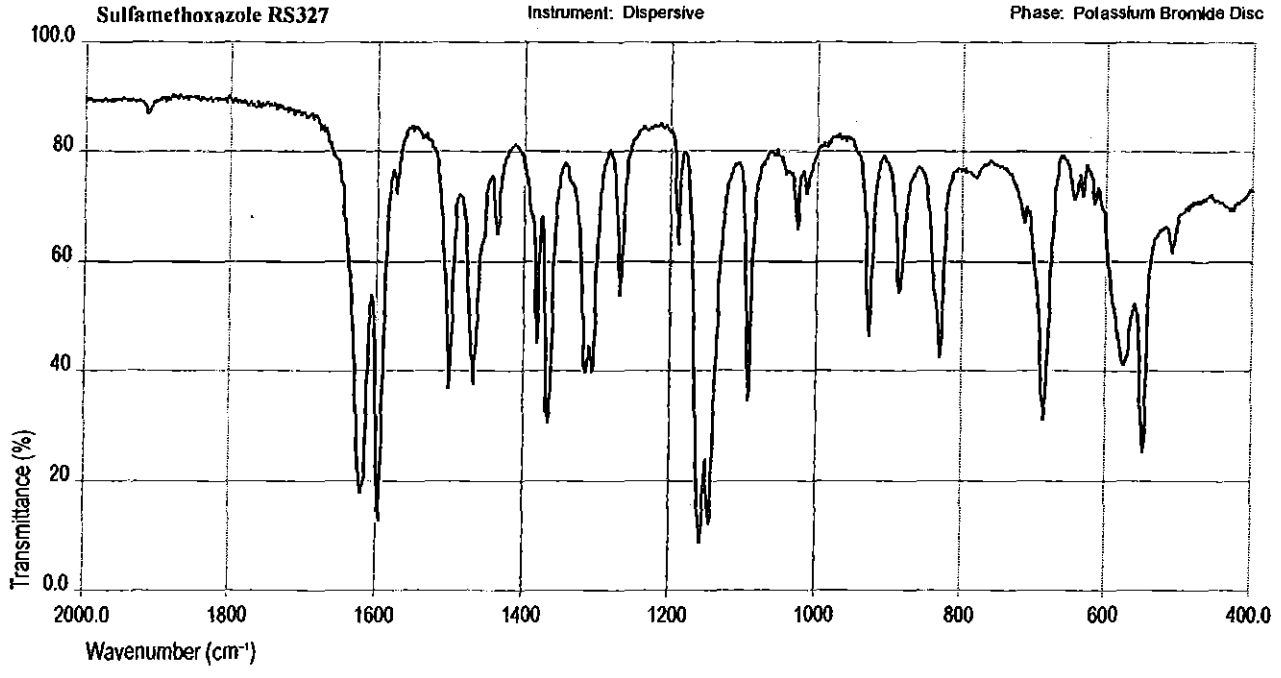
Spironolactone RS321 Instrument: Dispersive Phase: 5% w/v Solution in Chloroform Thickness: 0.1mm

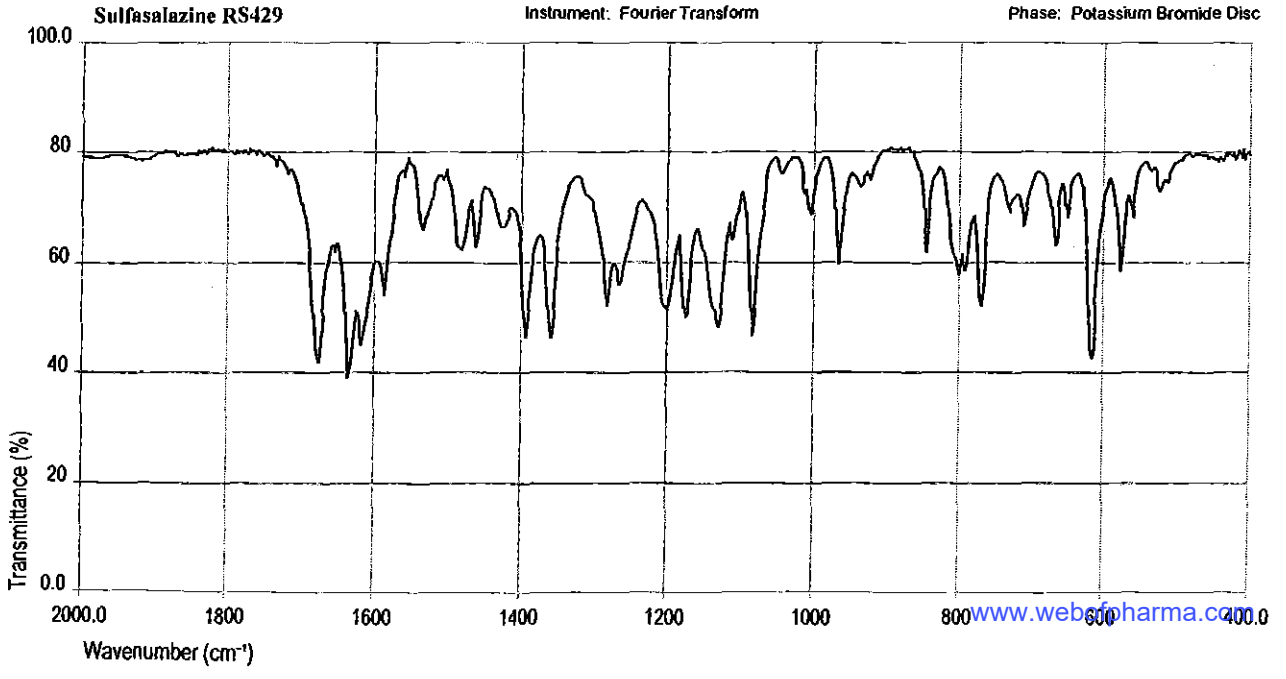


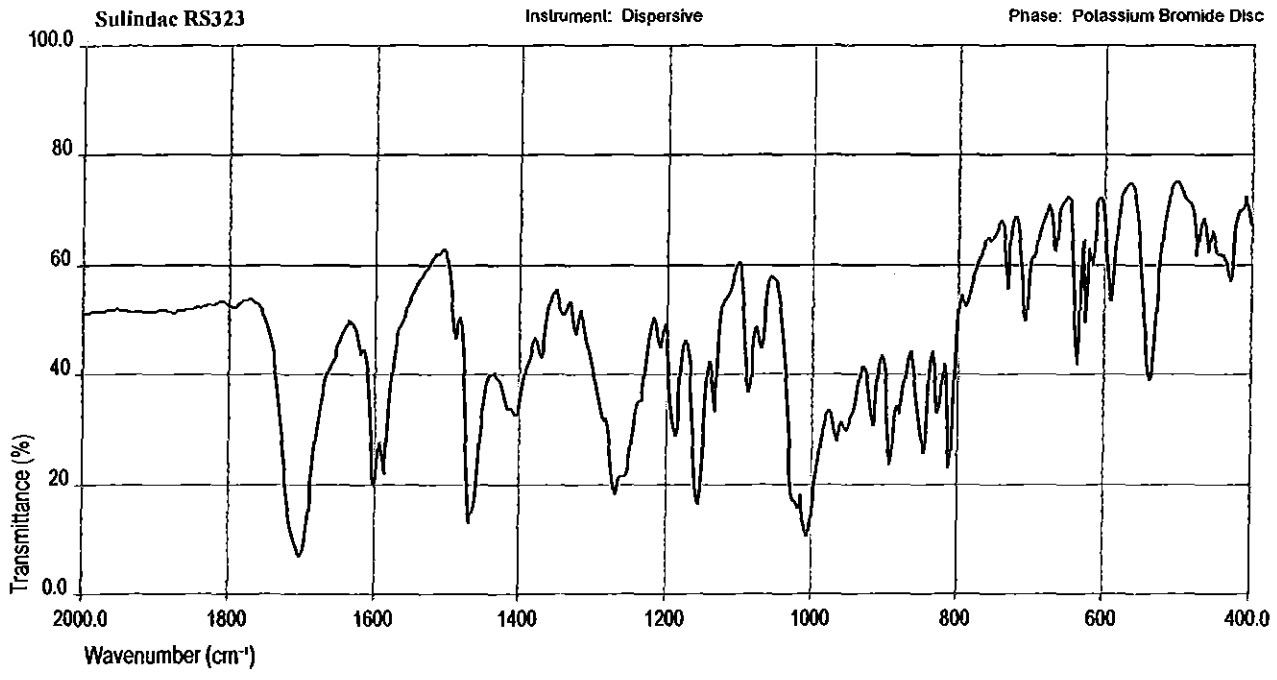
Stanozolol RS322 Instrument: Dispersive Phase: Potassium Bromide Disc

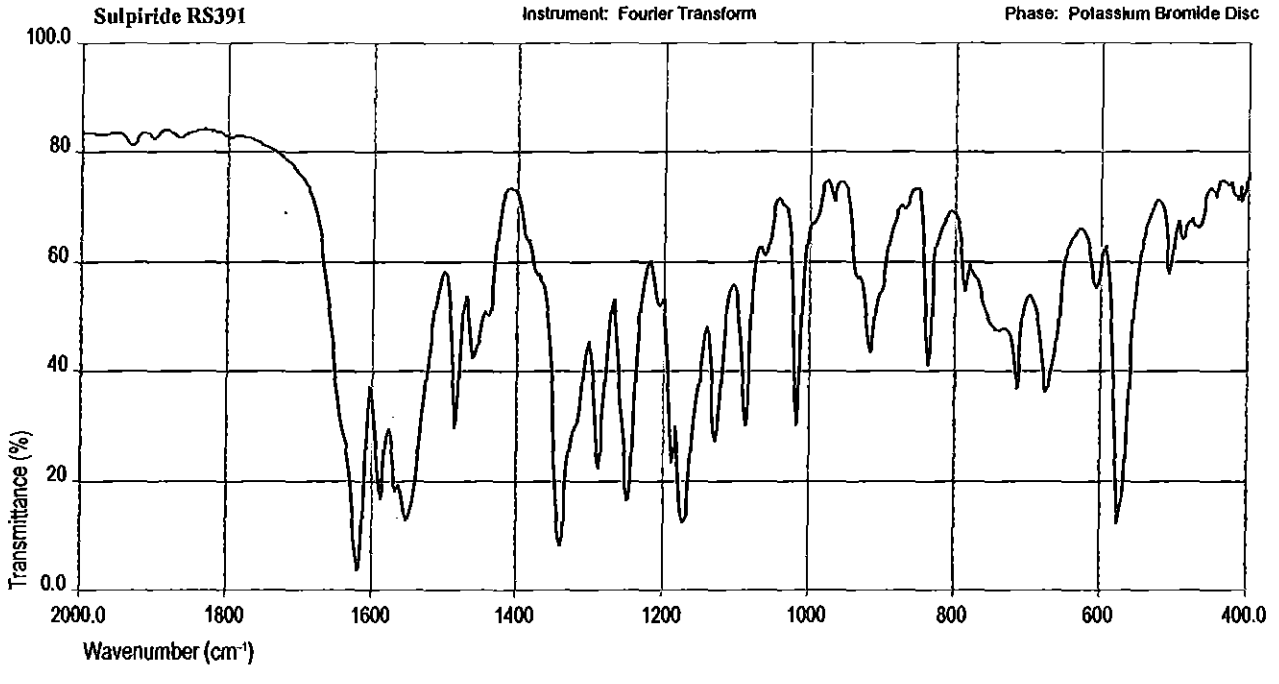


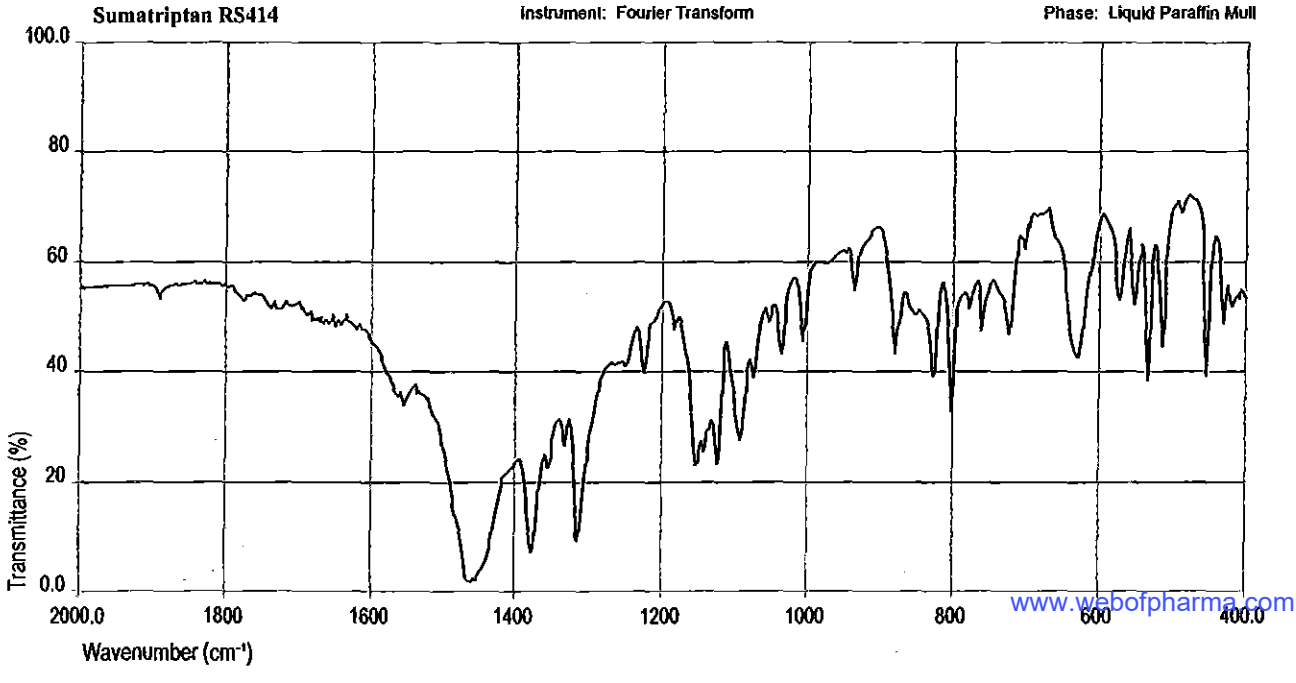


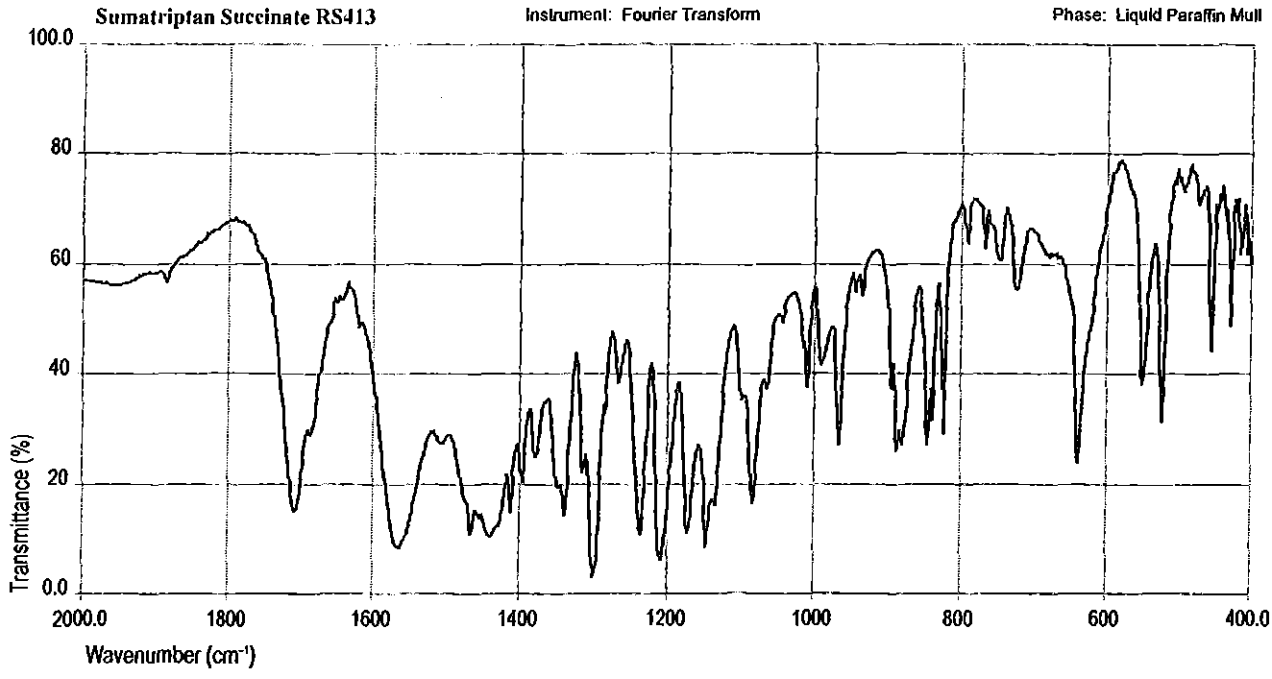


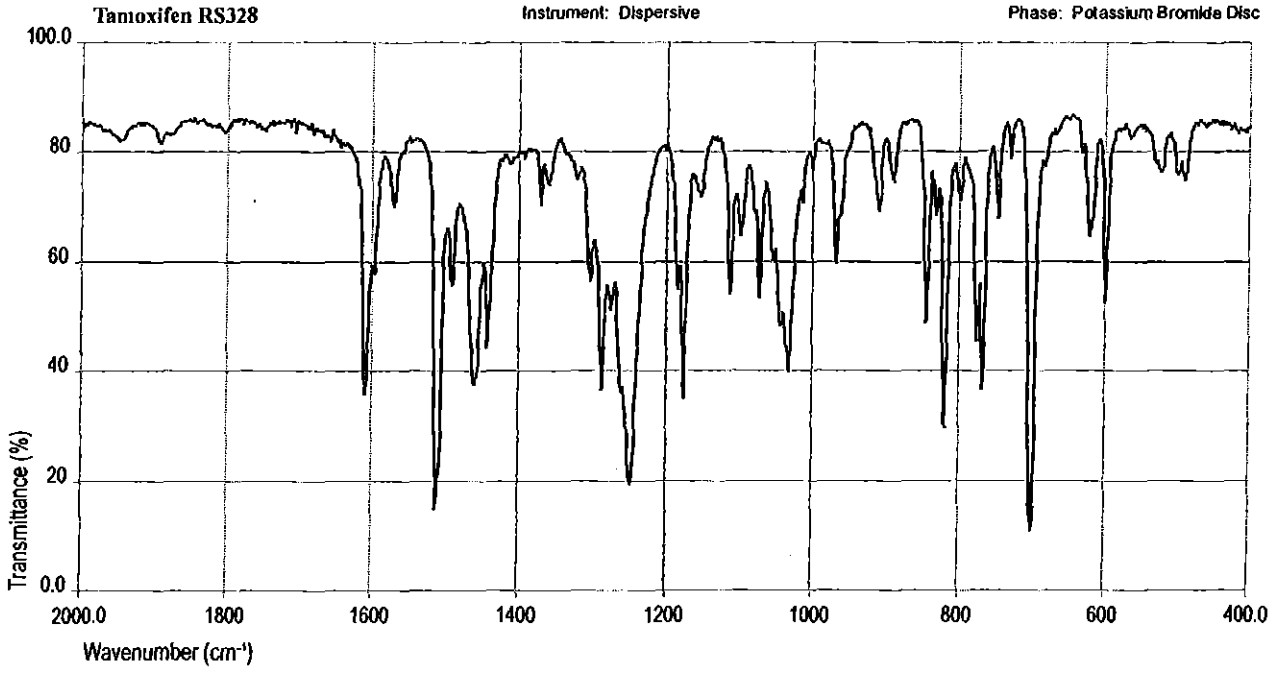


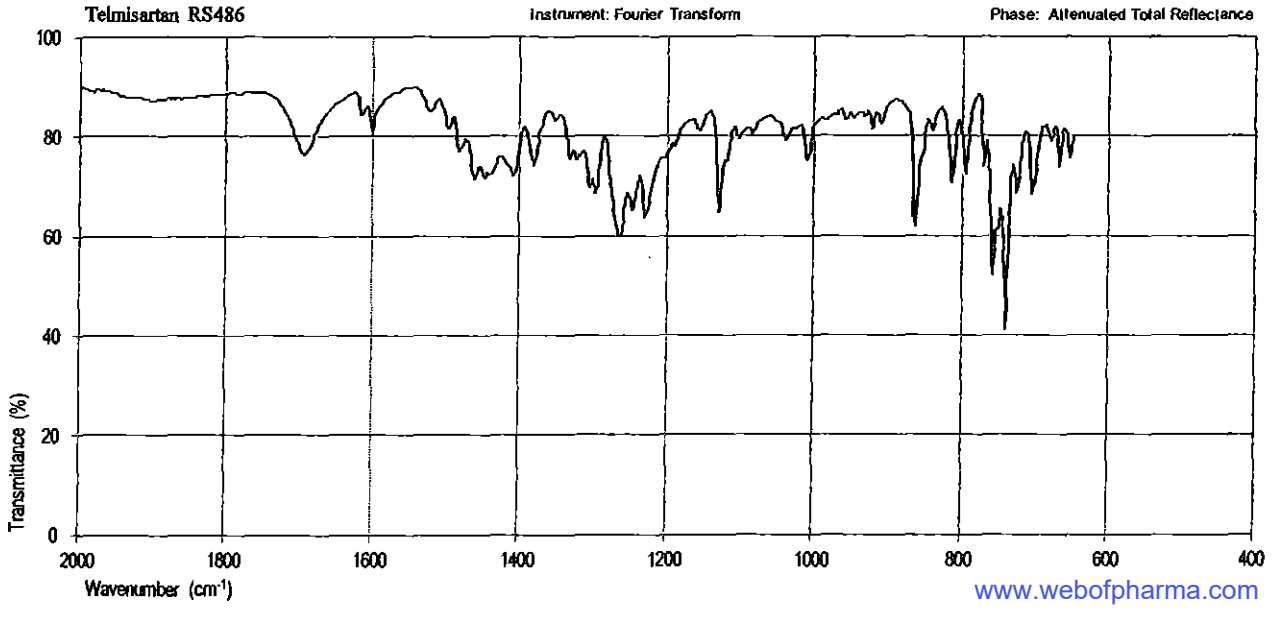


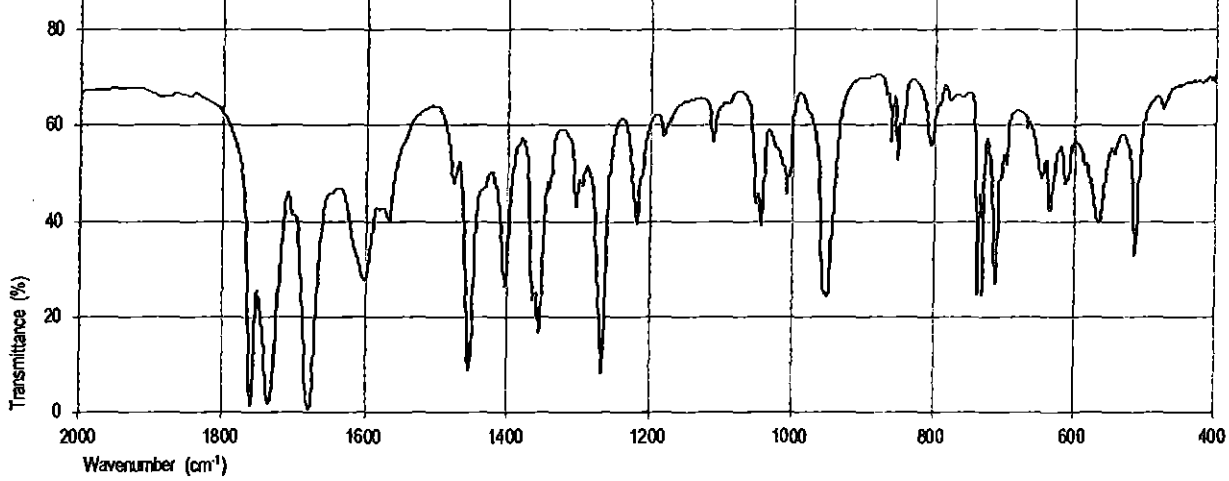


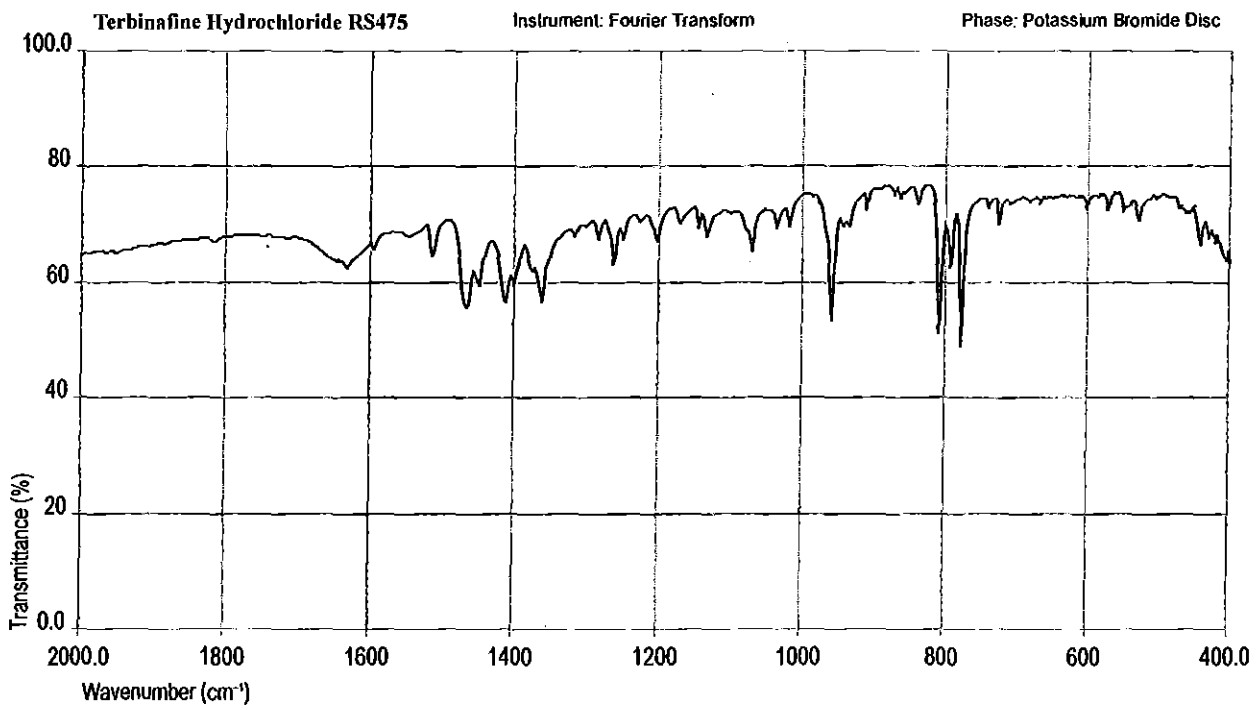


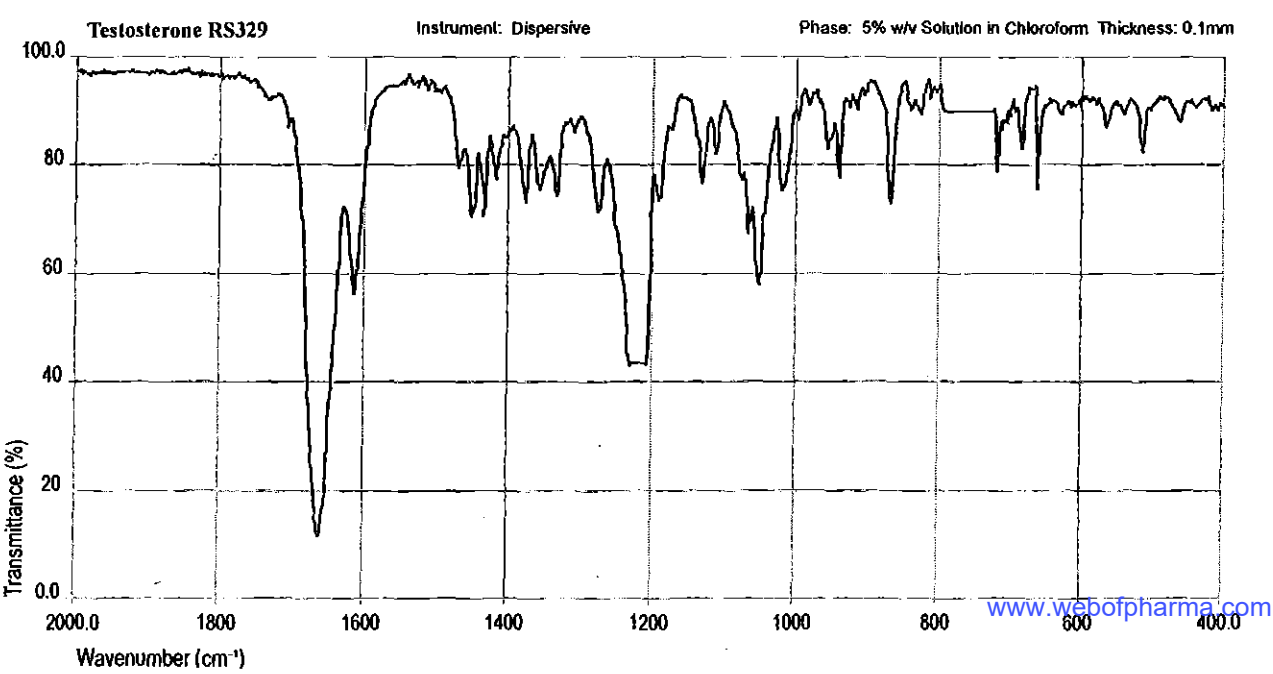


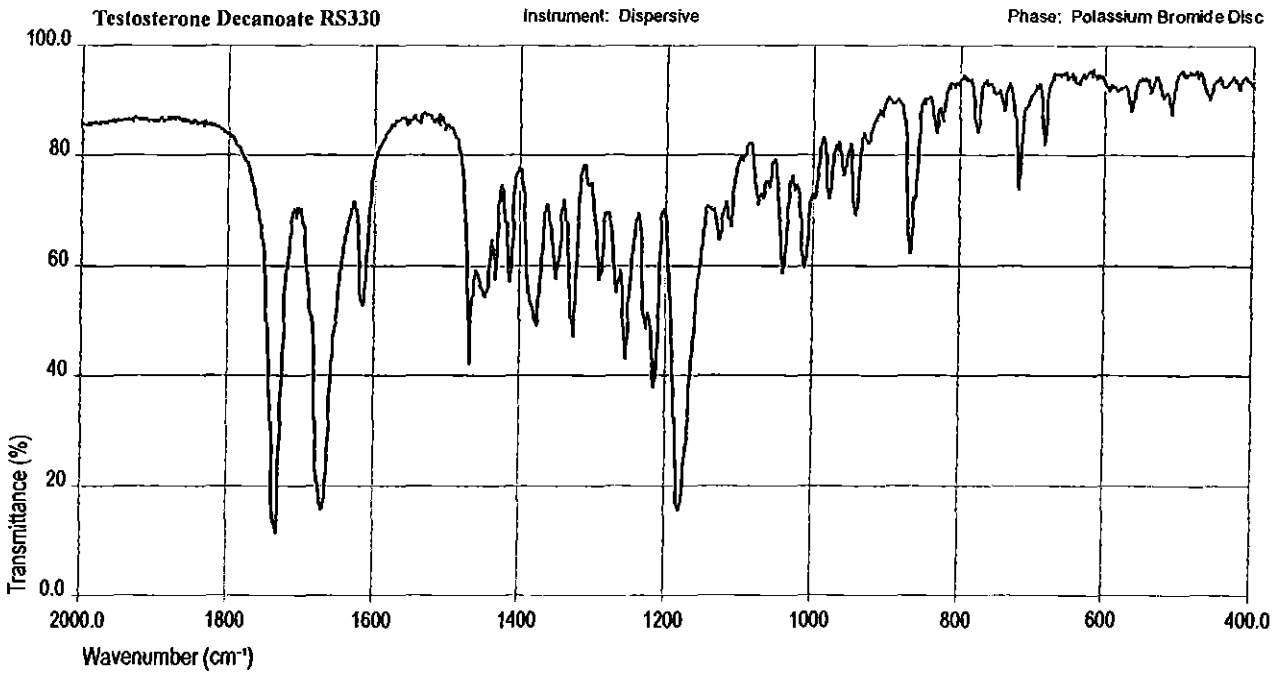


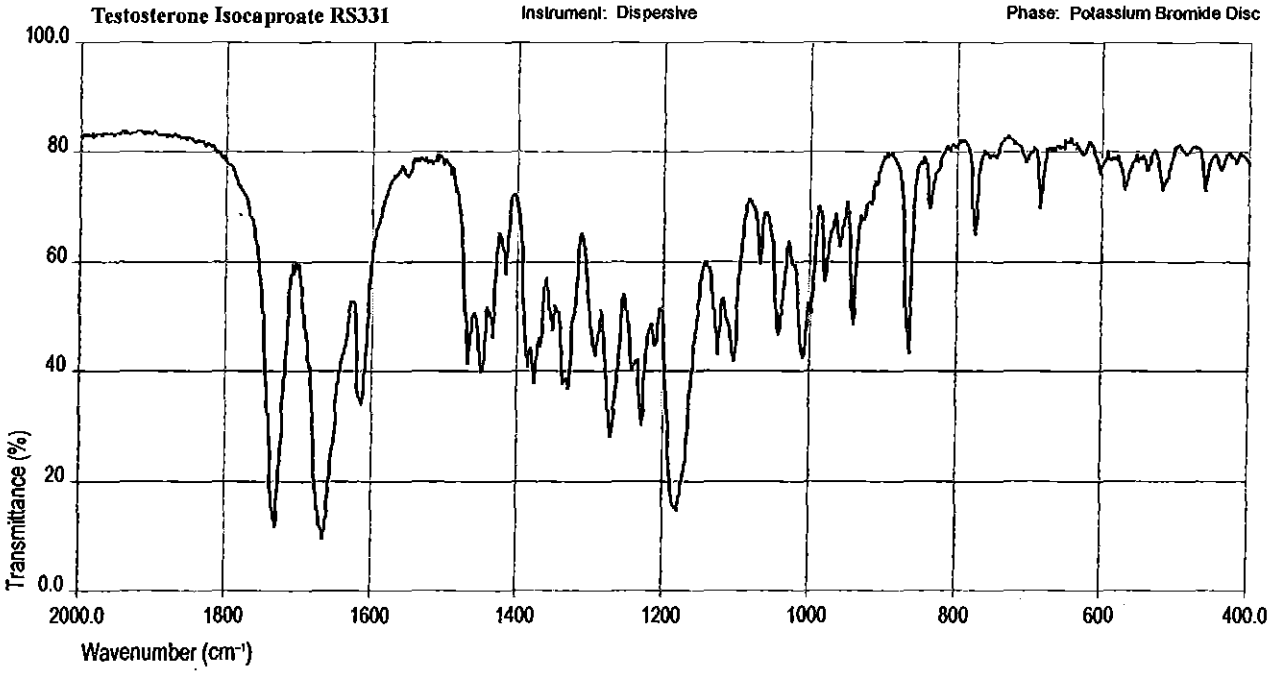


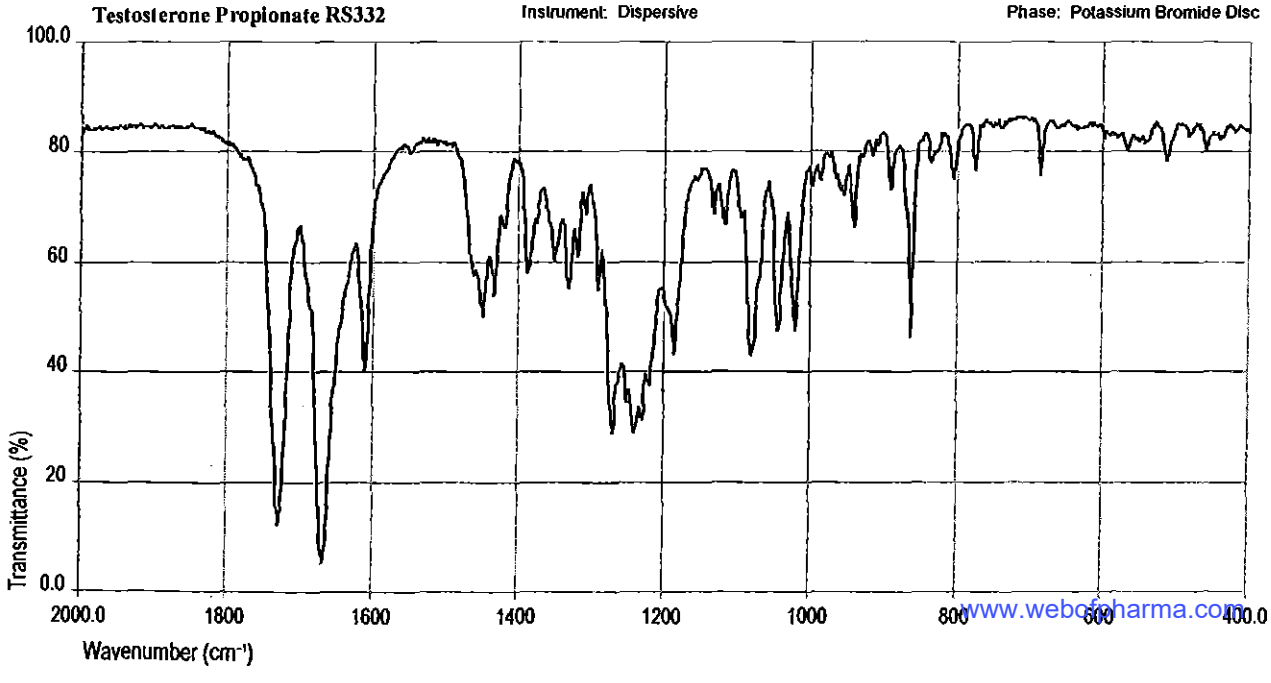


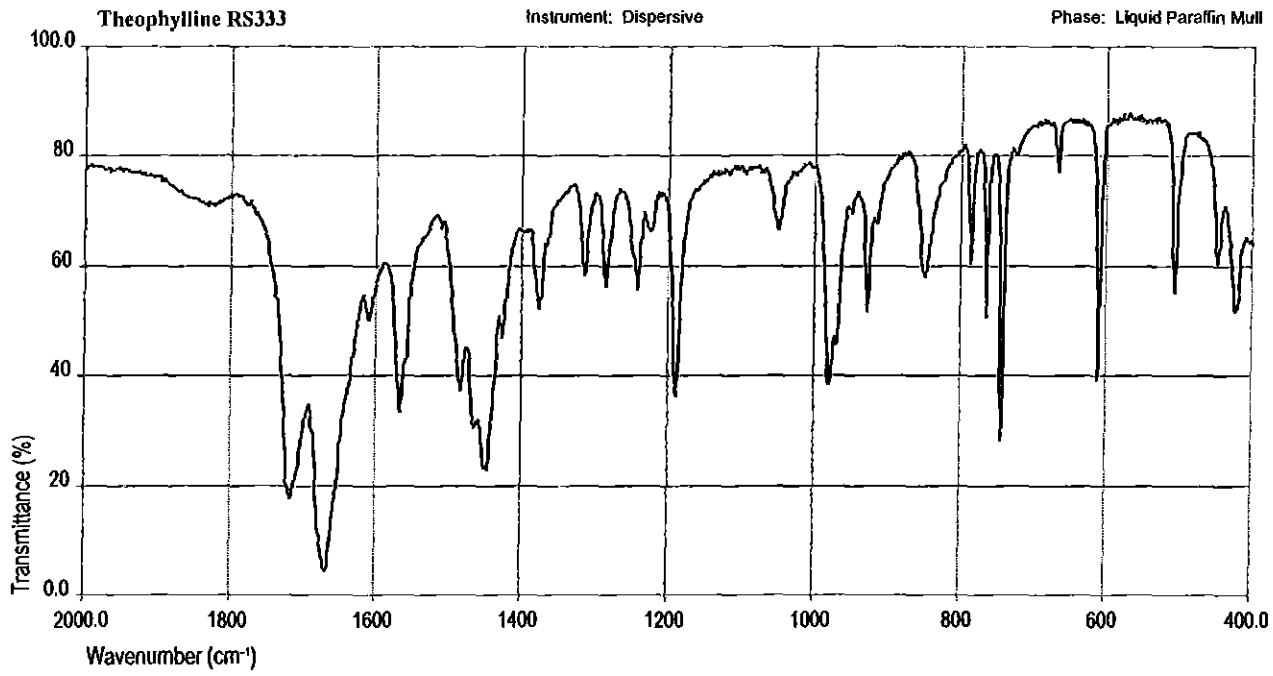


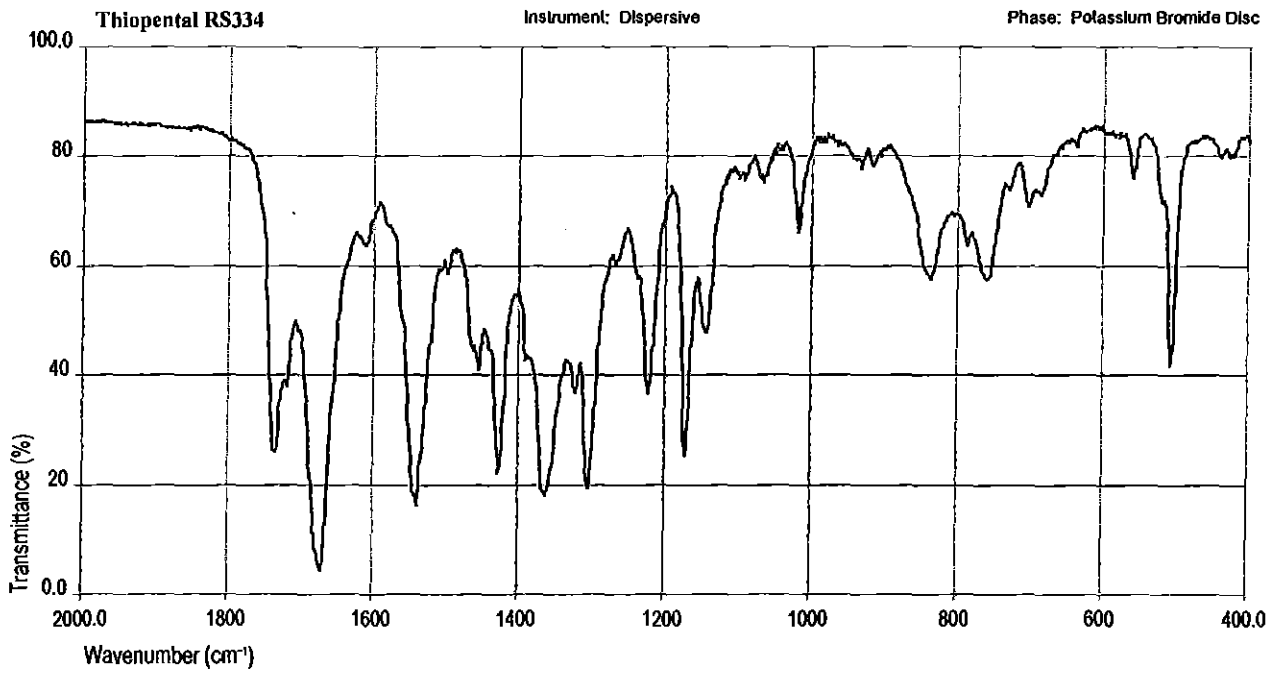


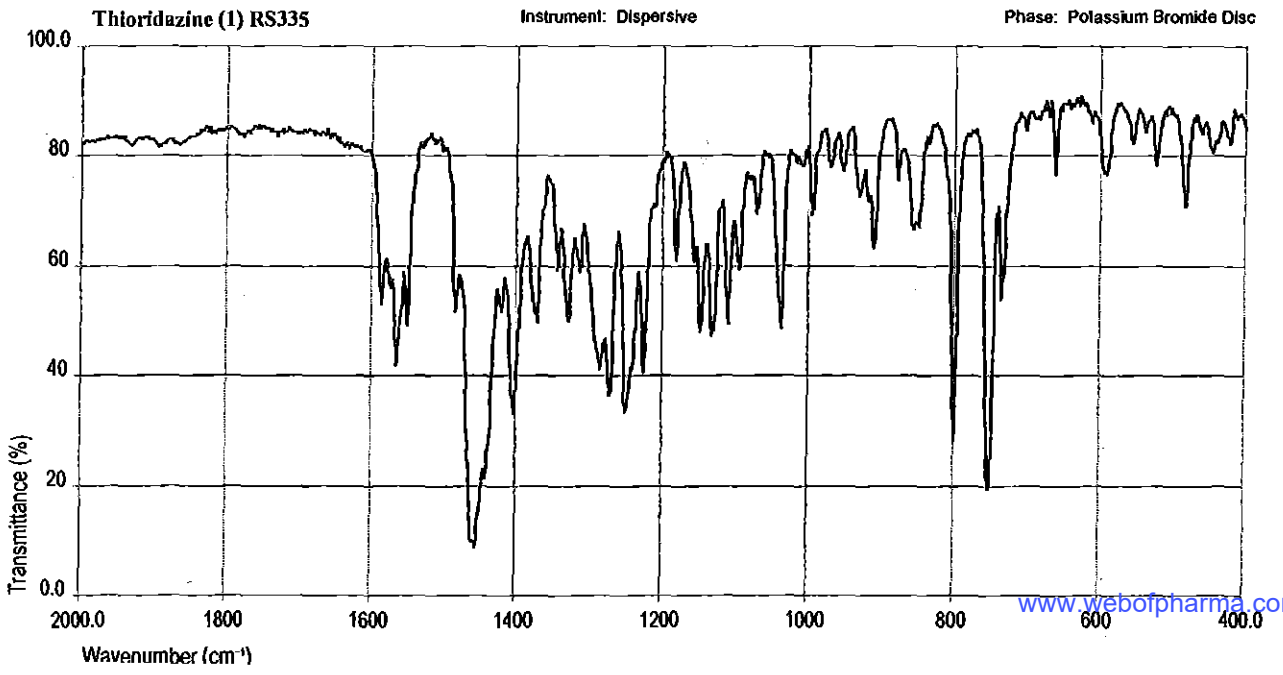


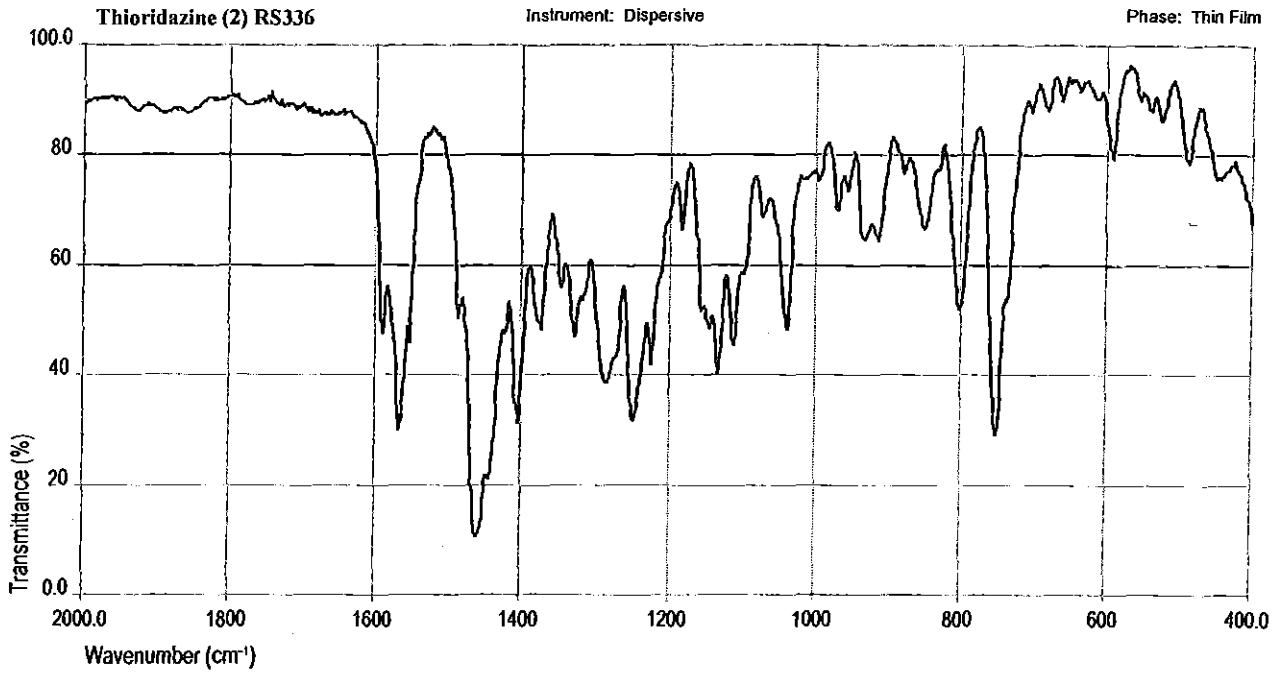


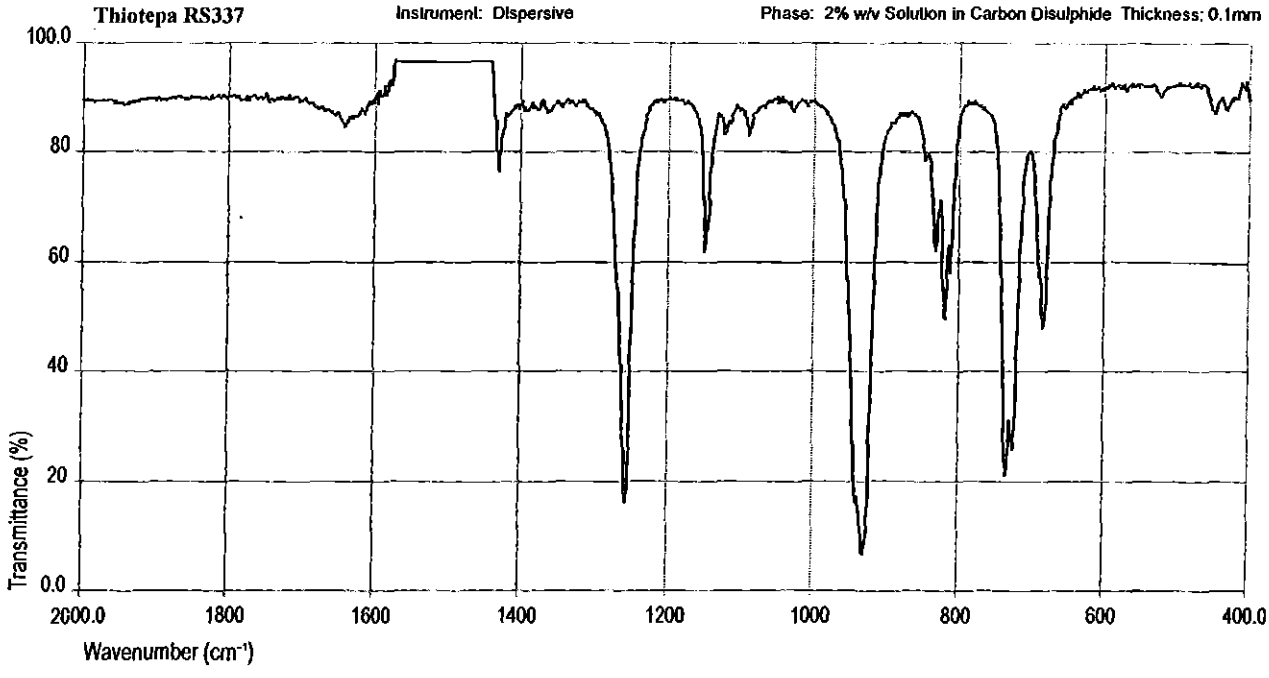


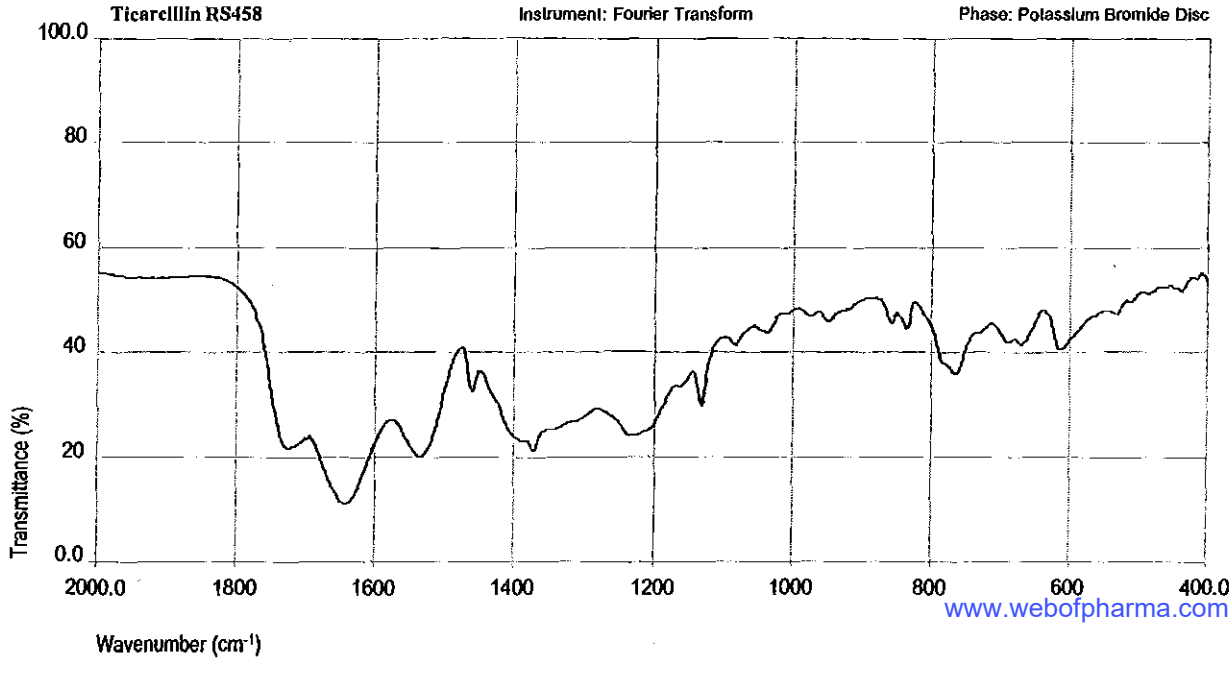


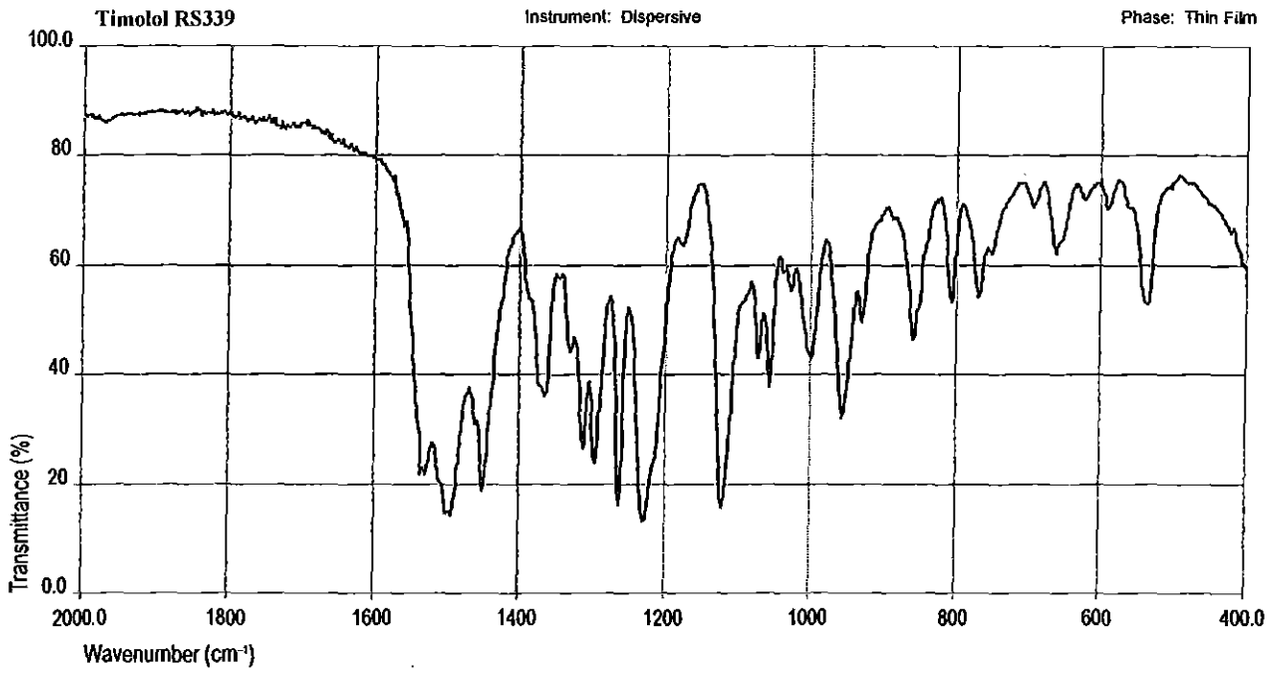


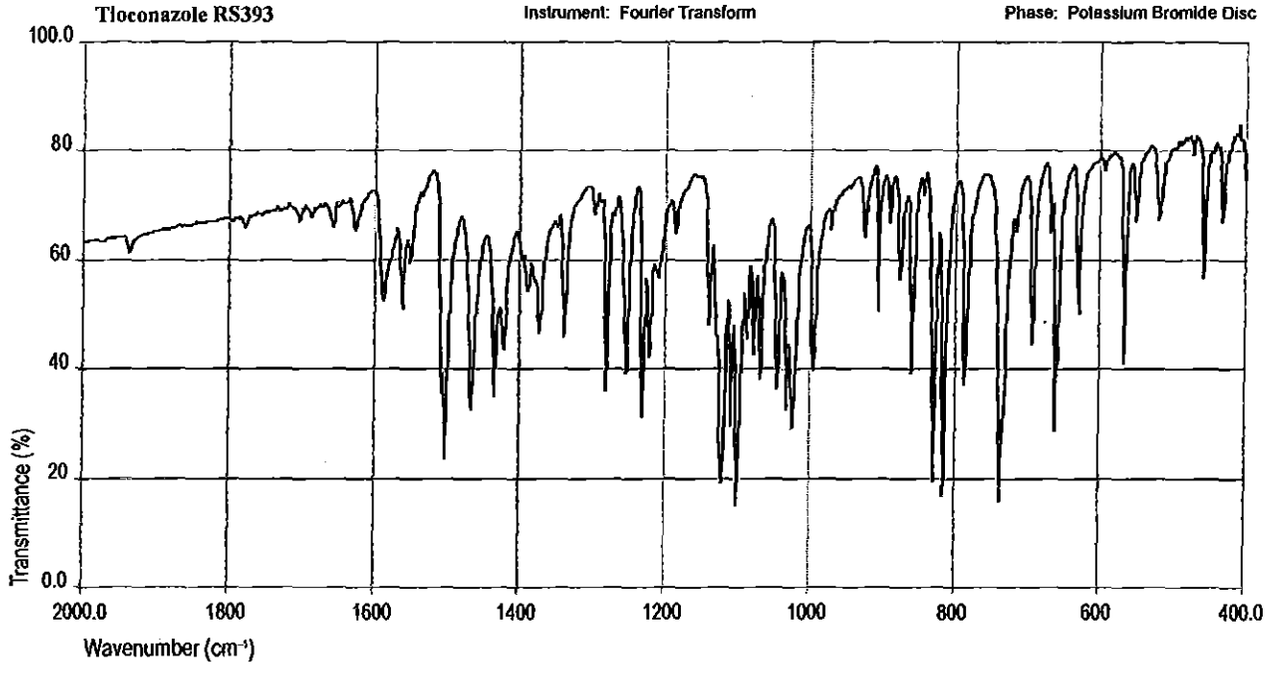


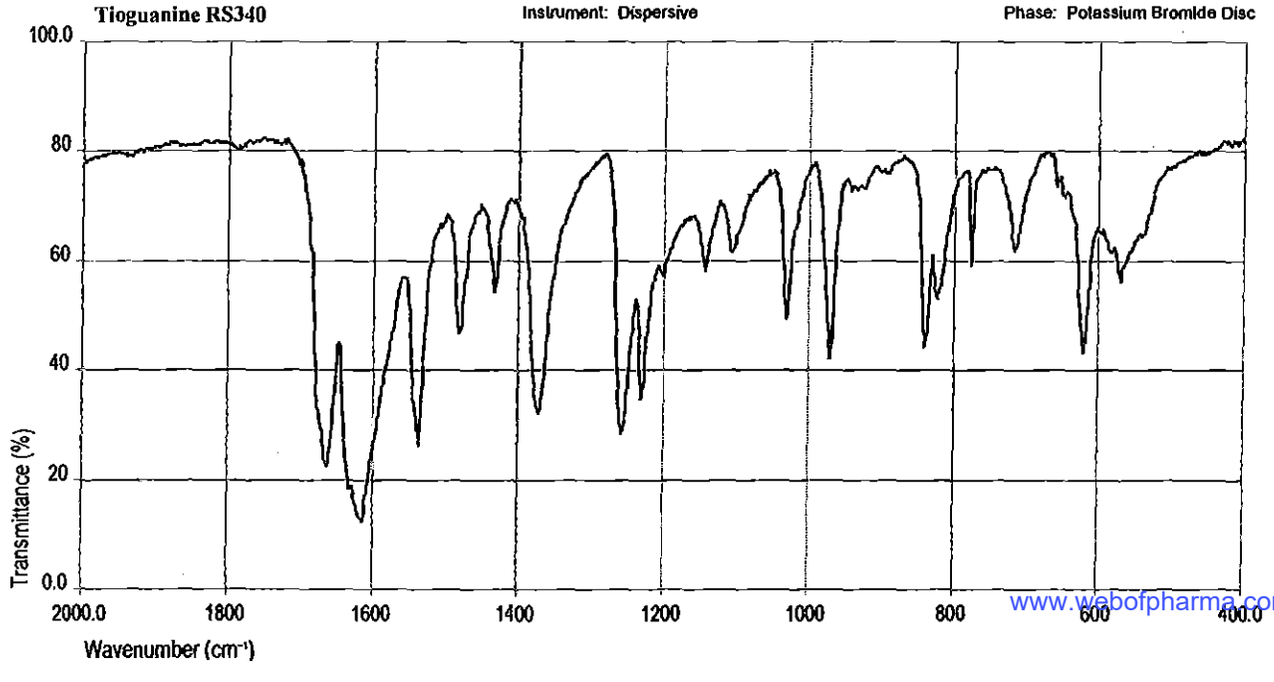


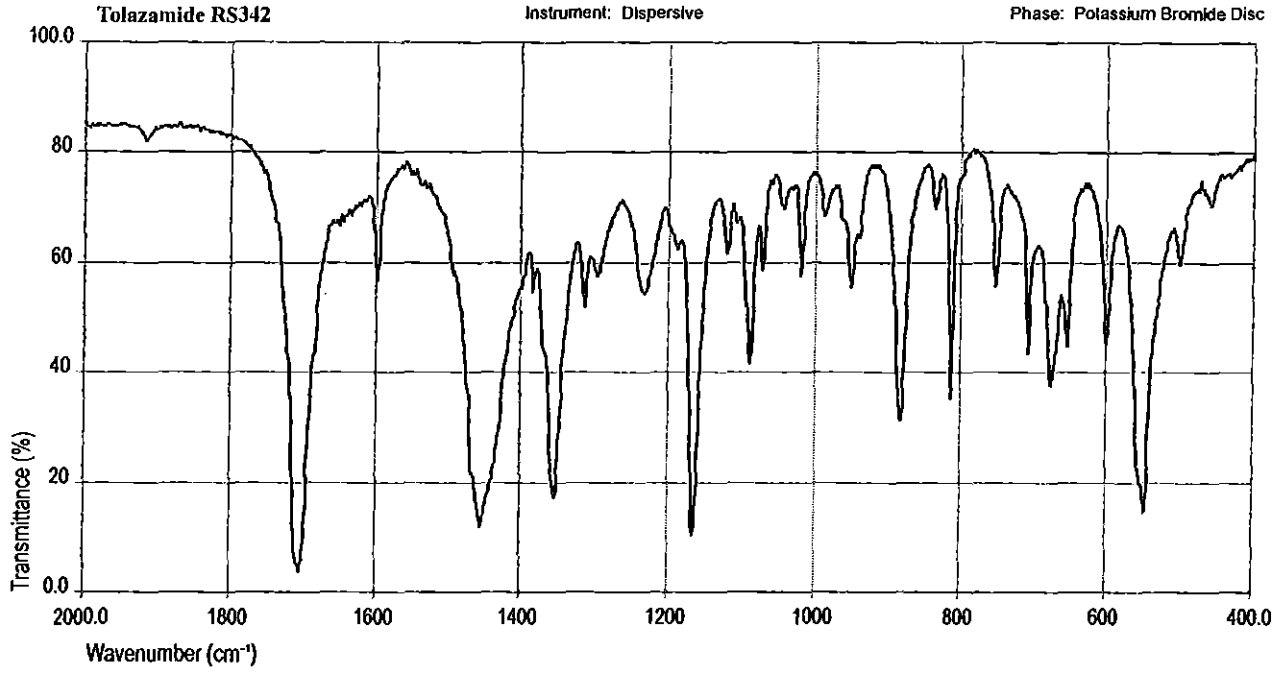


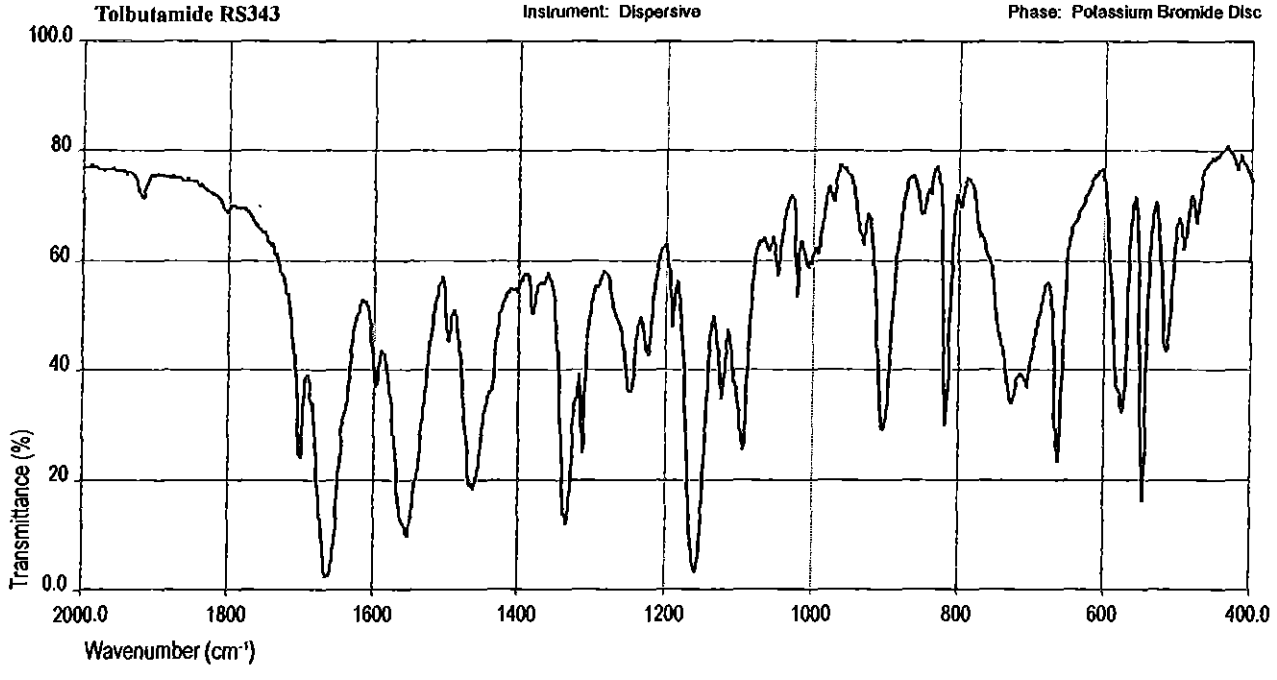


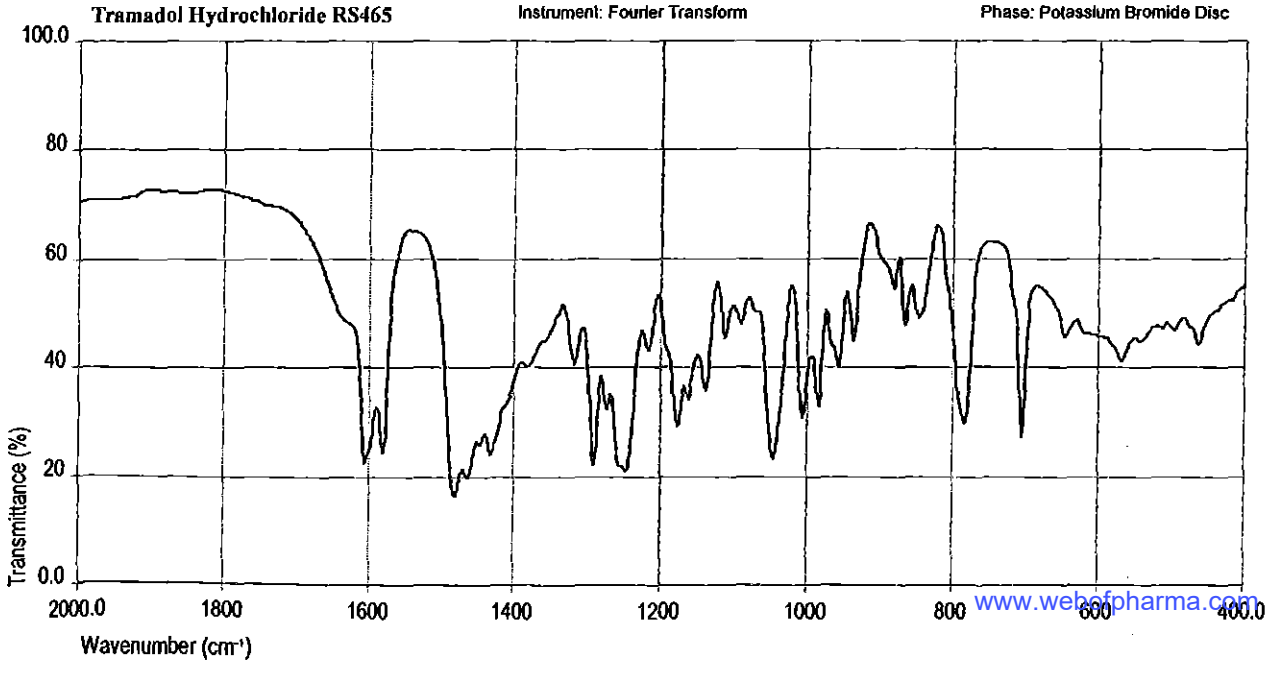


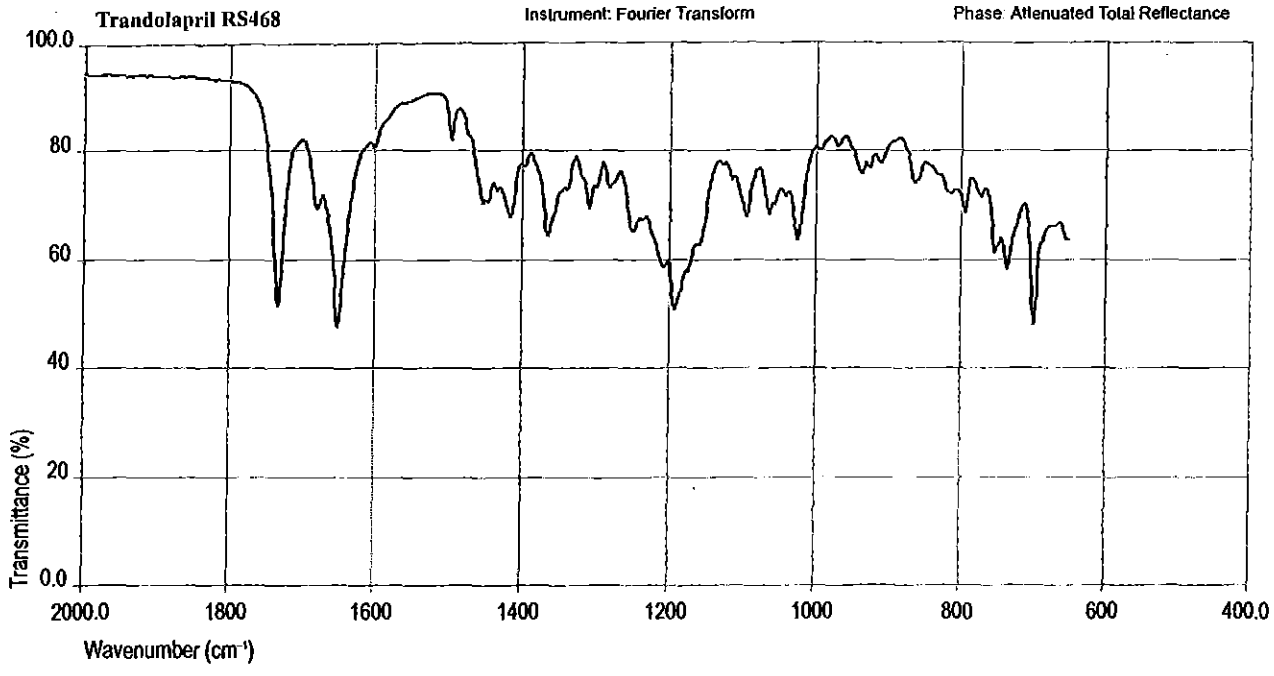


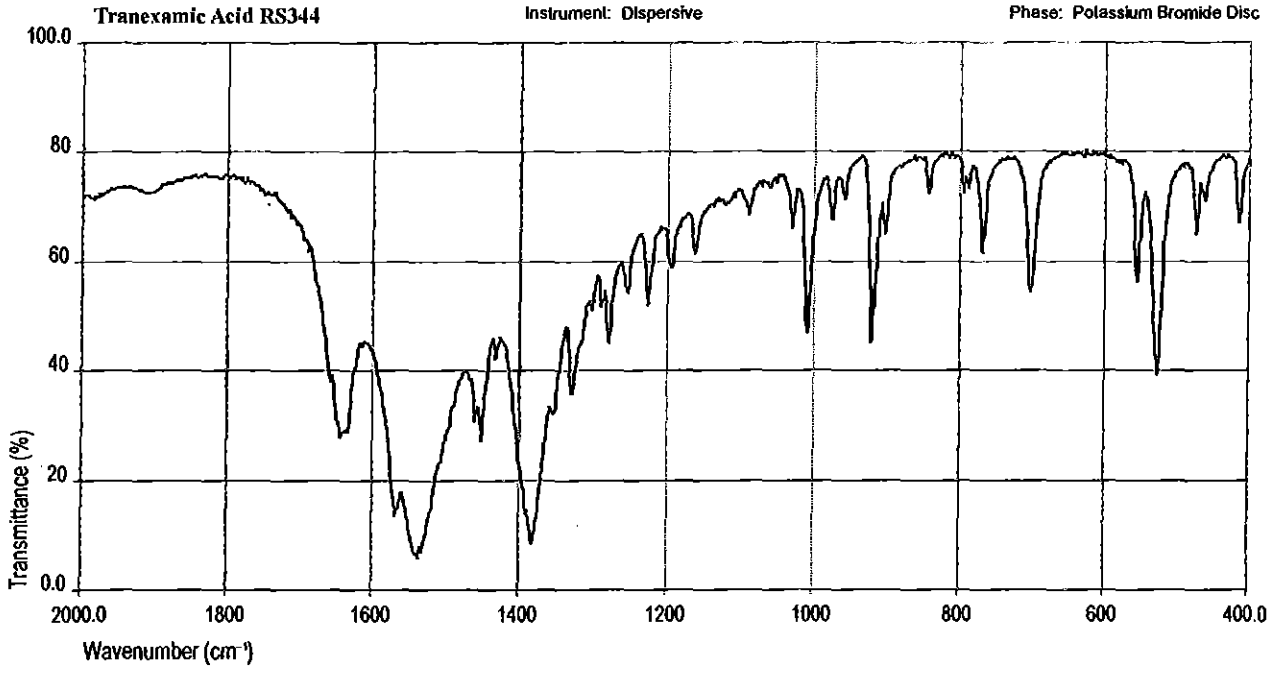


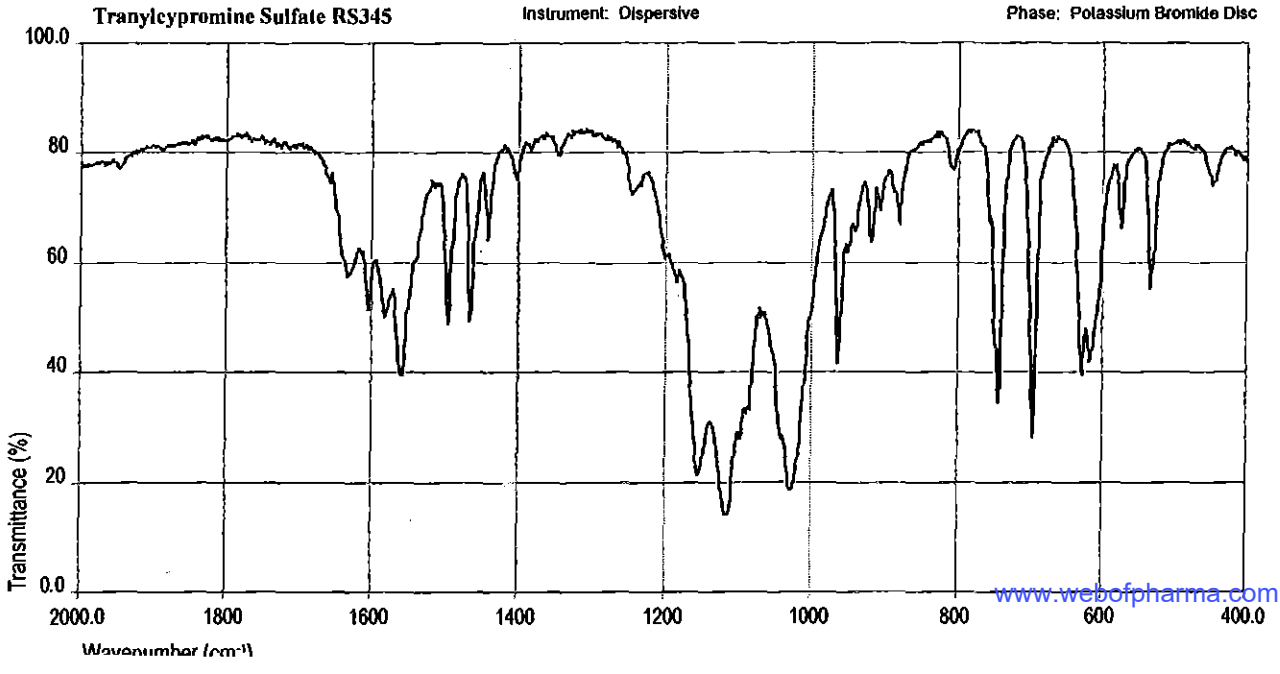


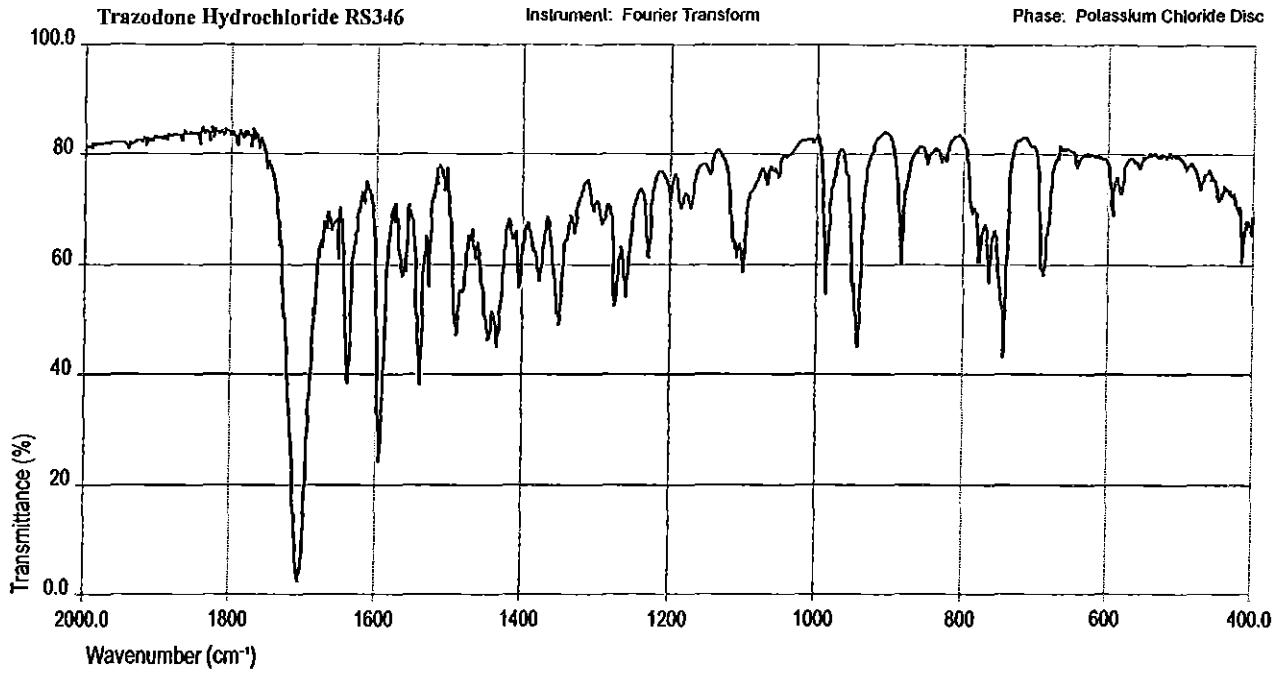


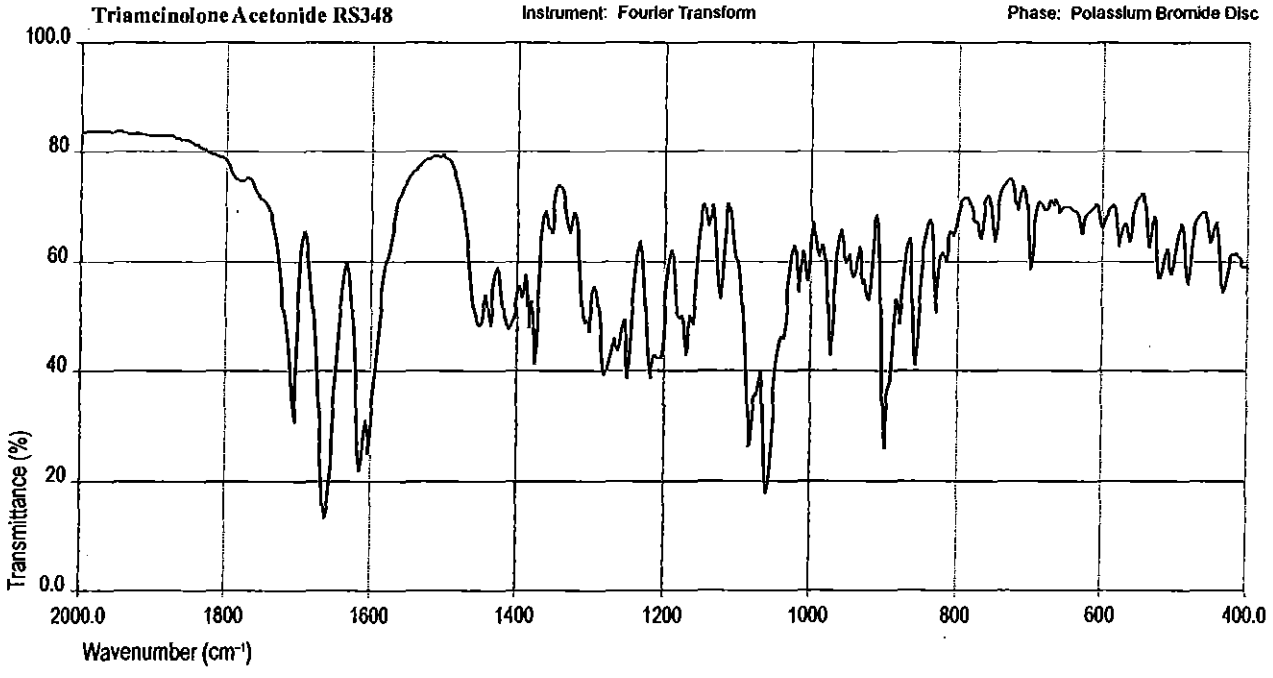


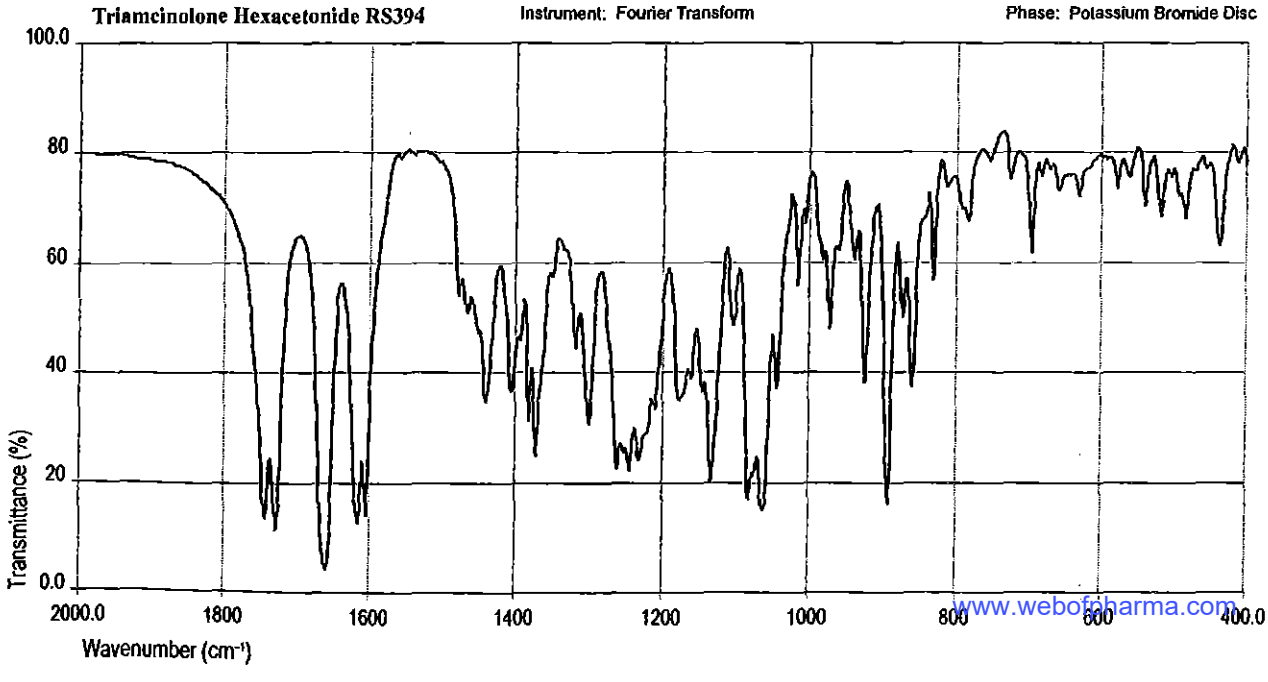


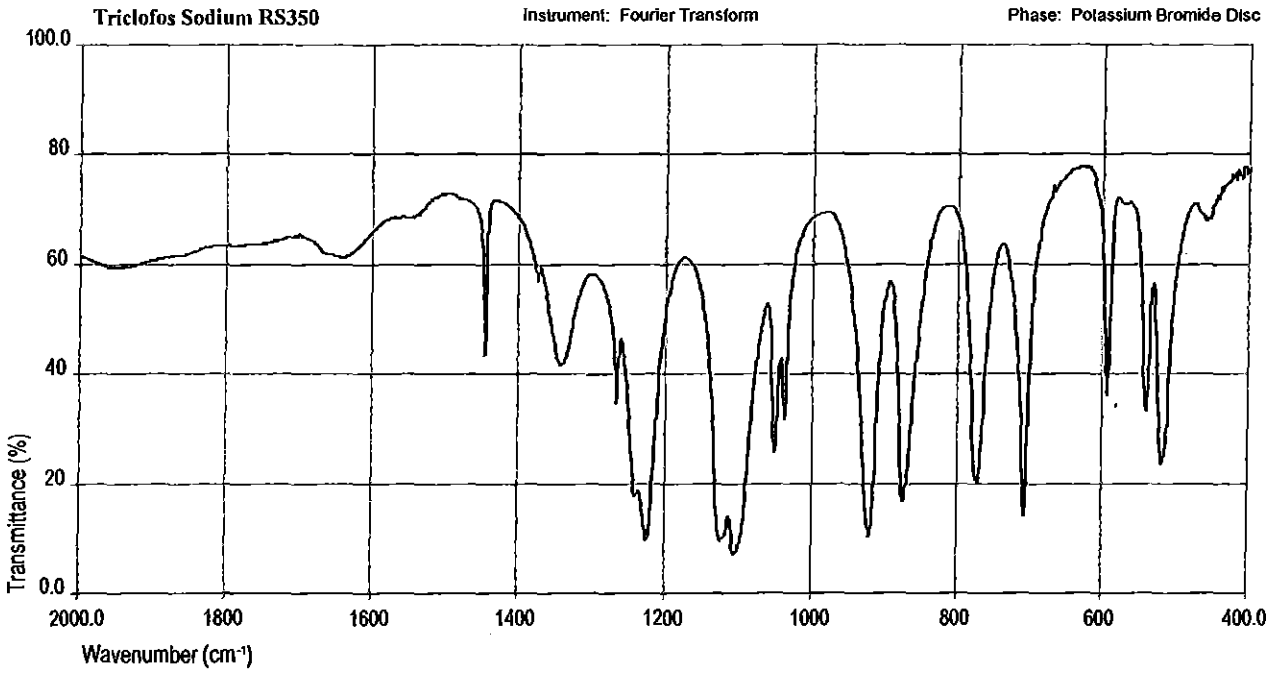


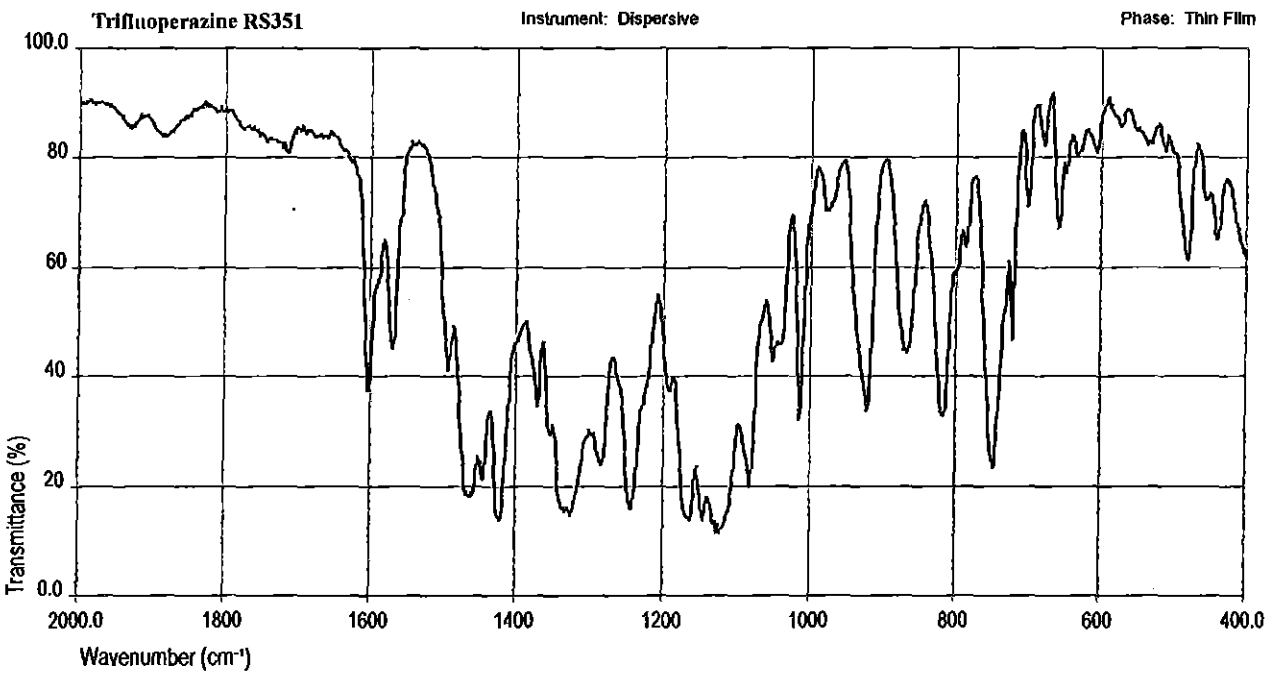


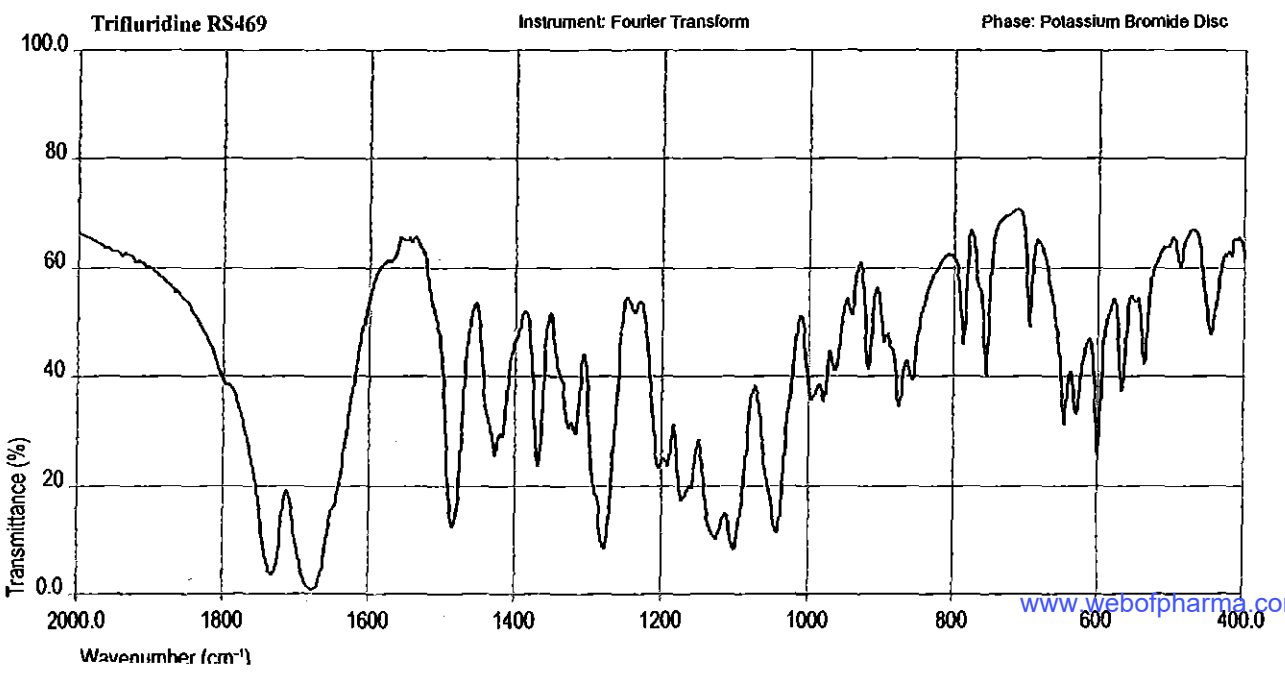


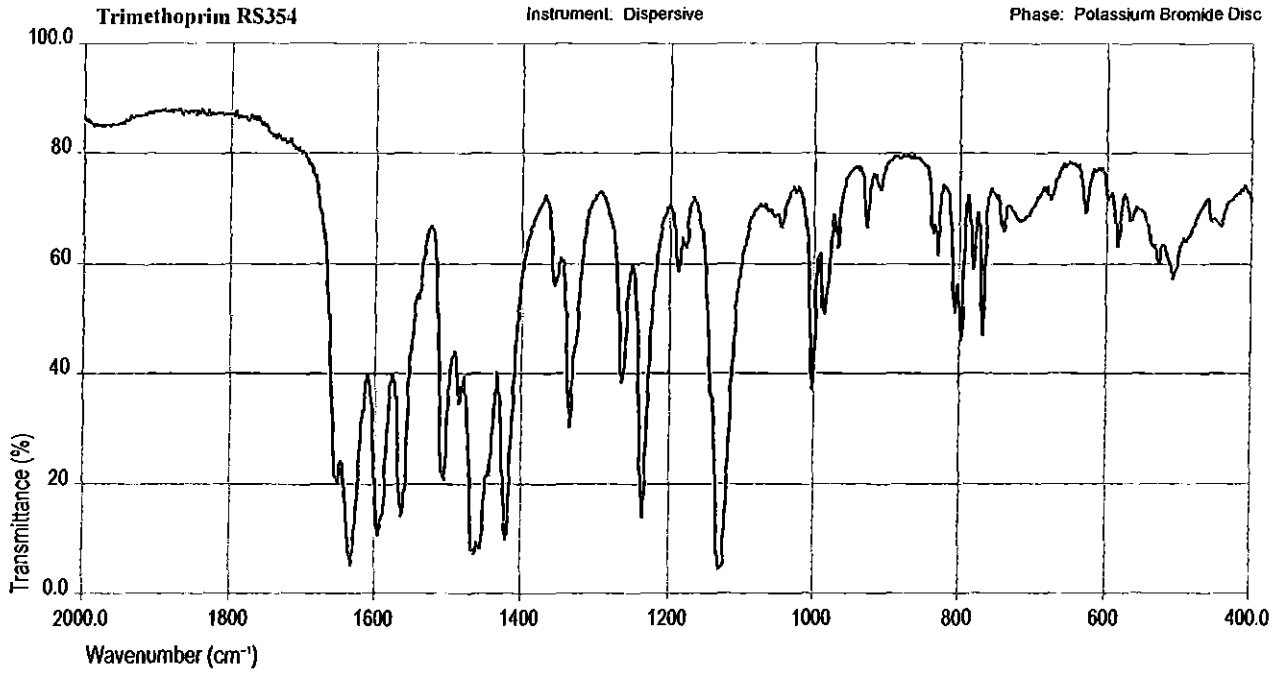


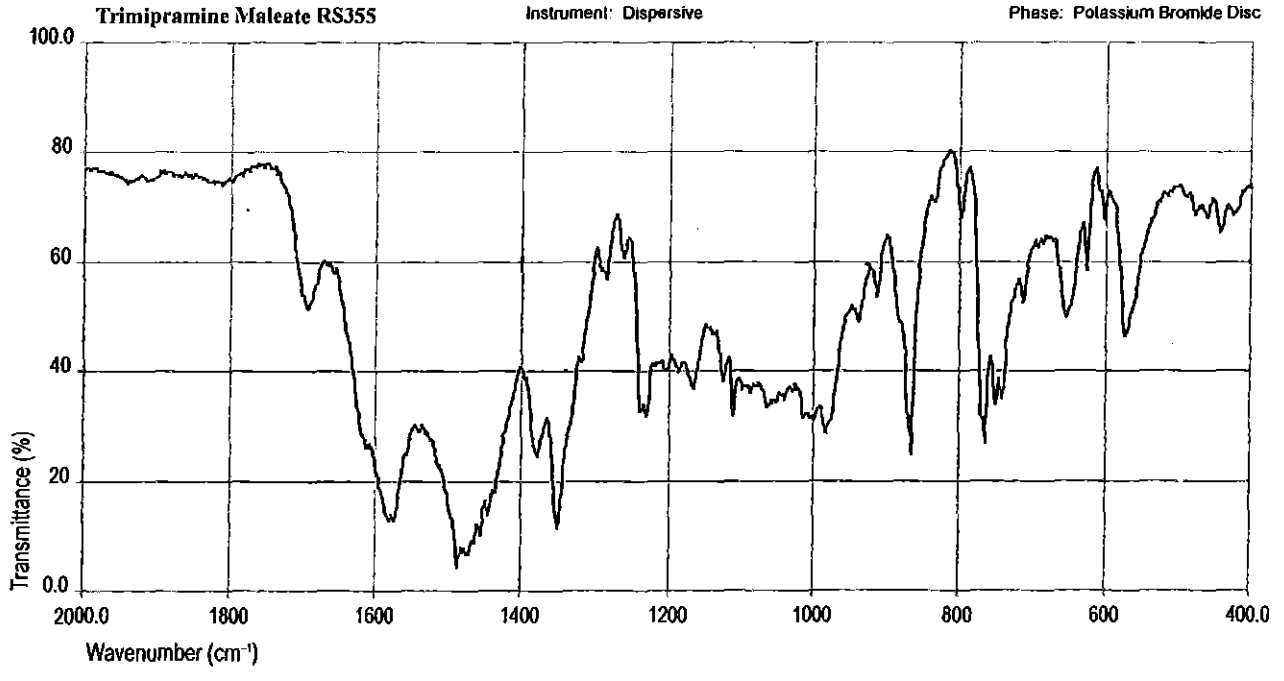


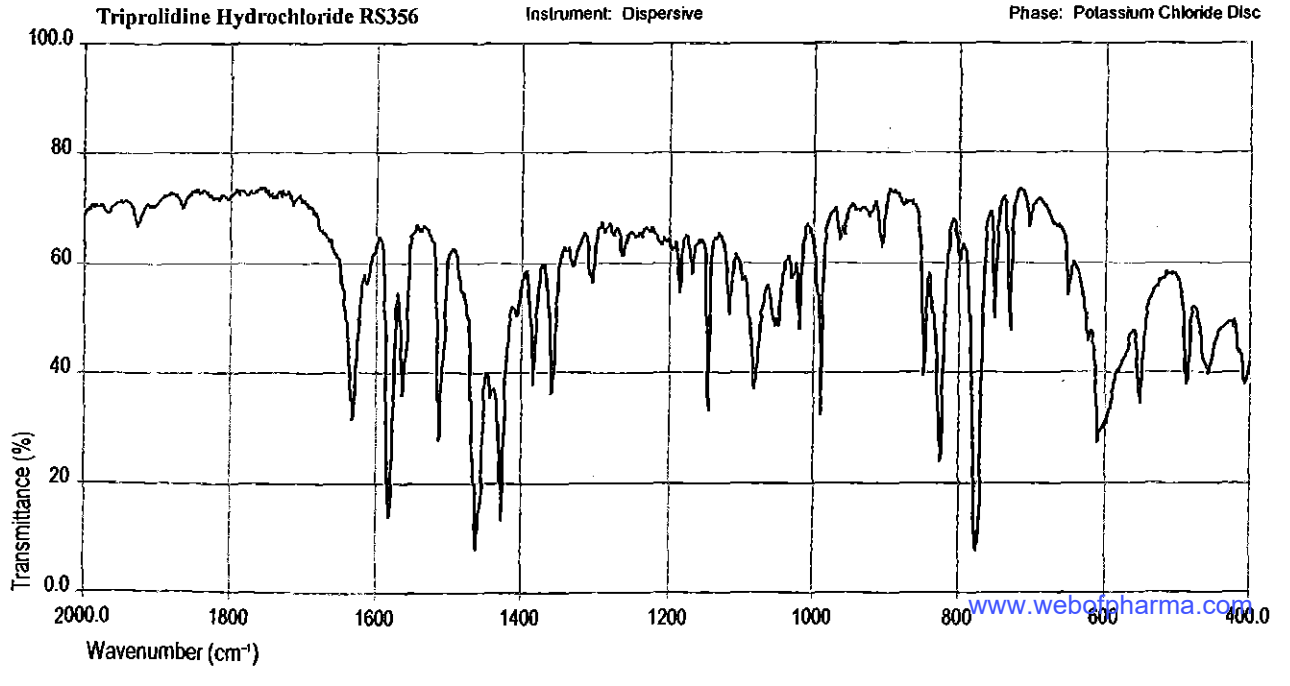


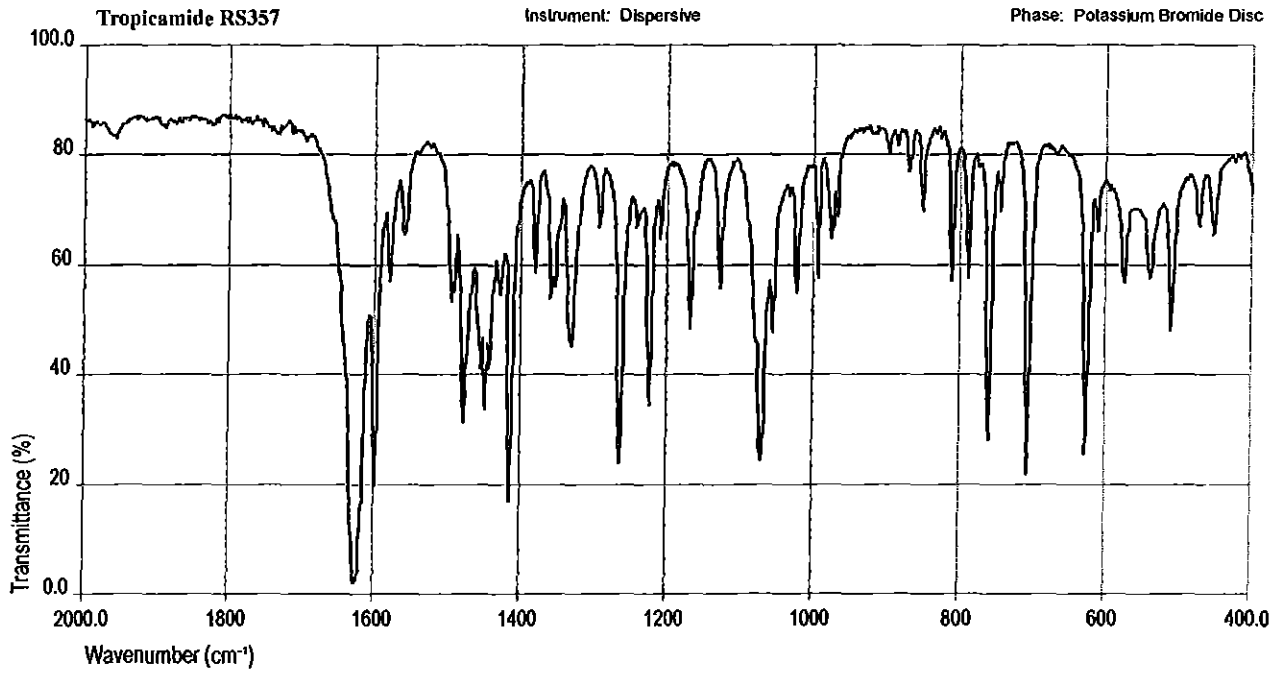


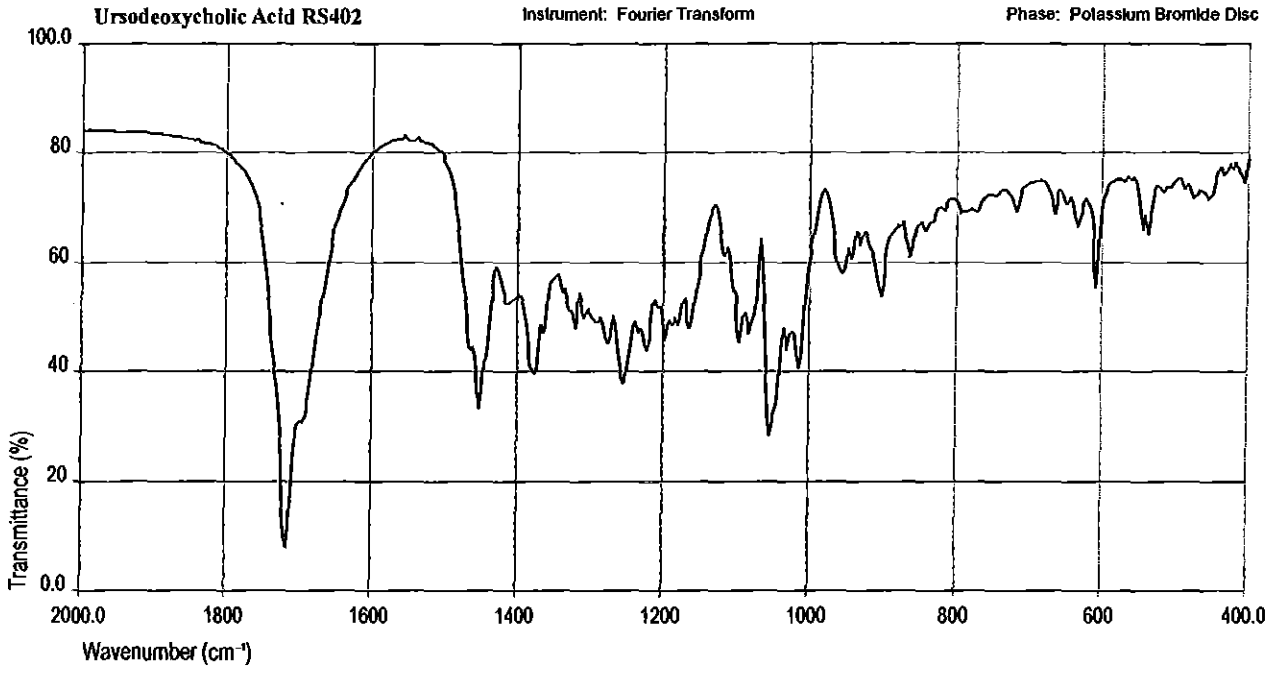


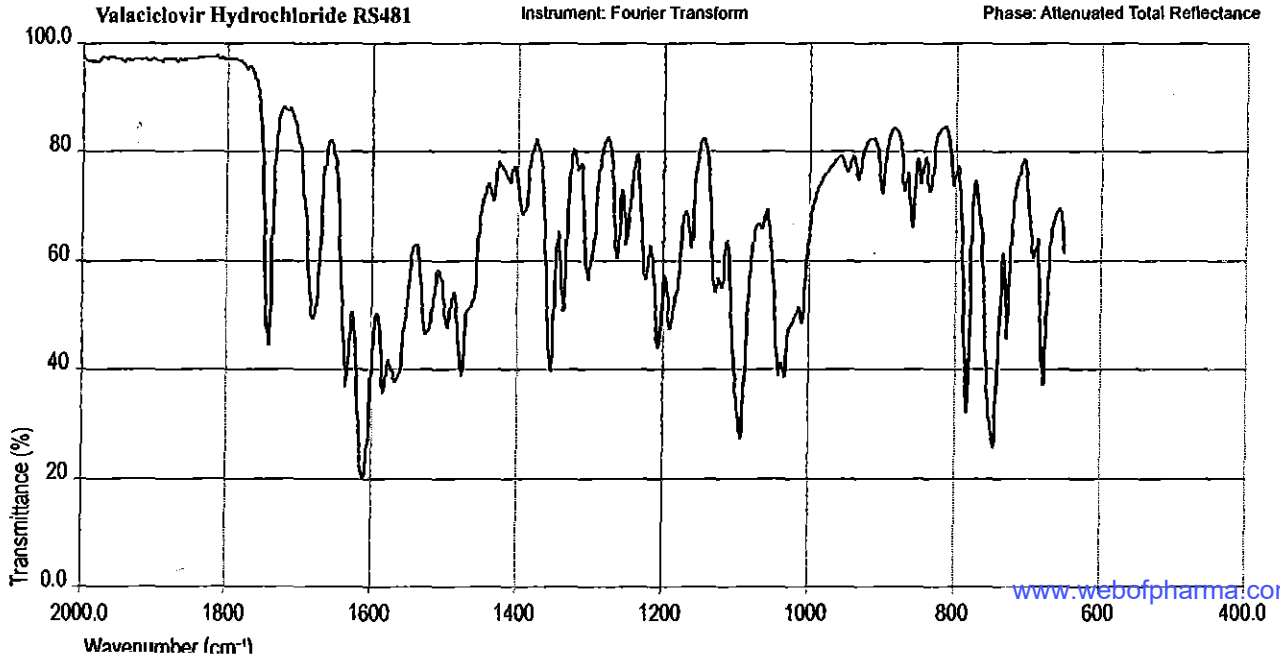


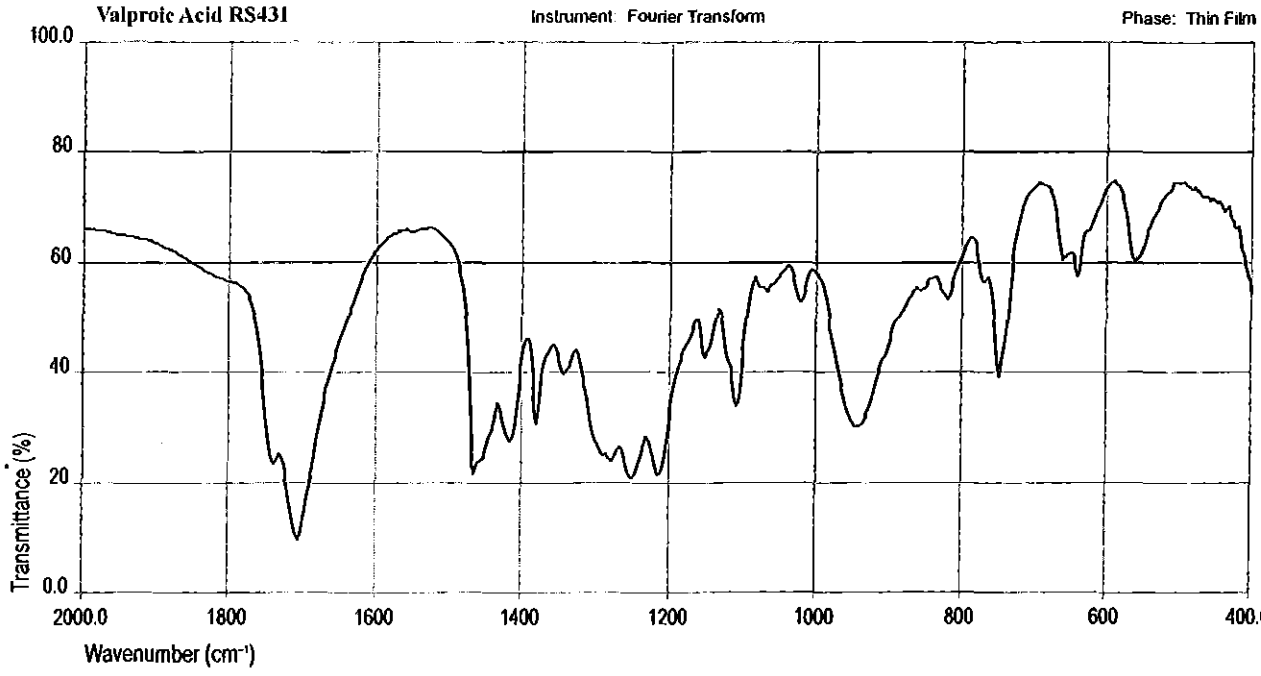


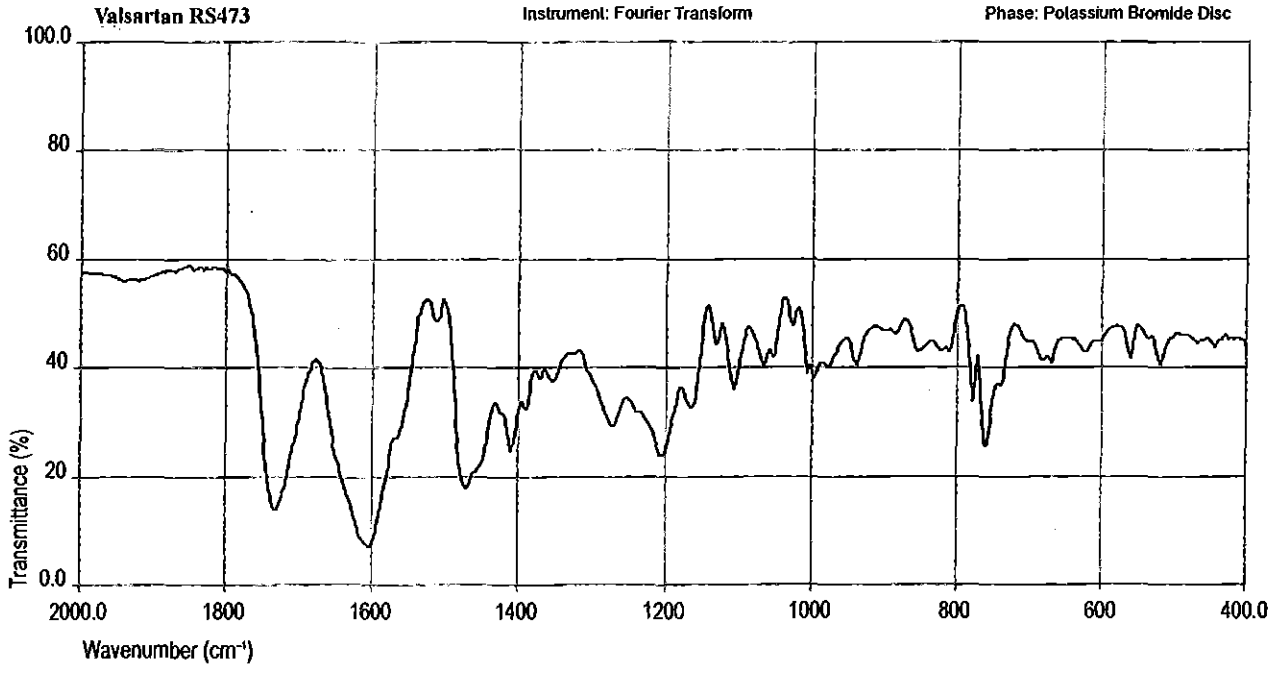


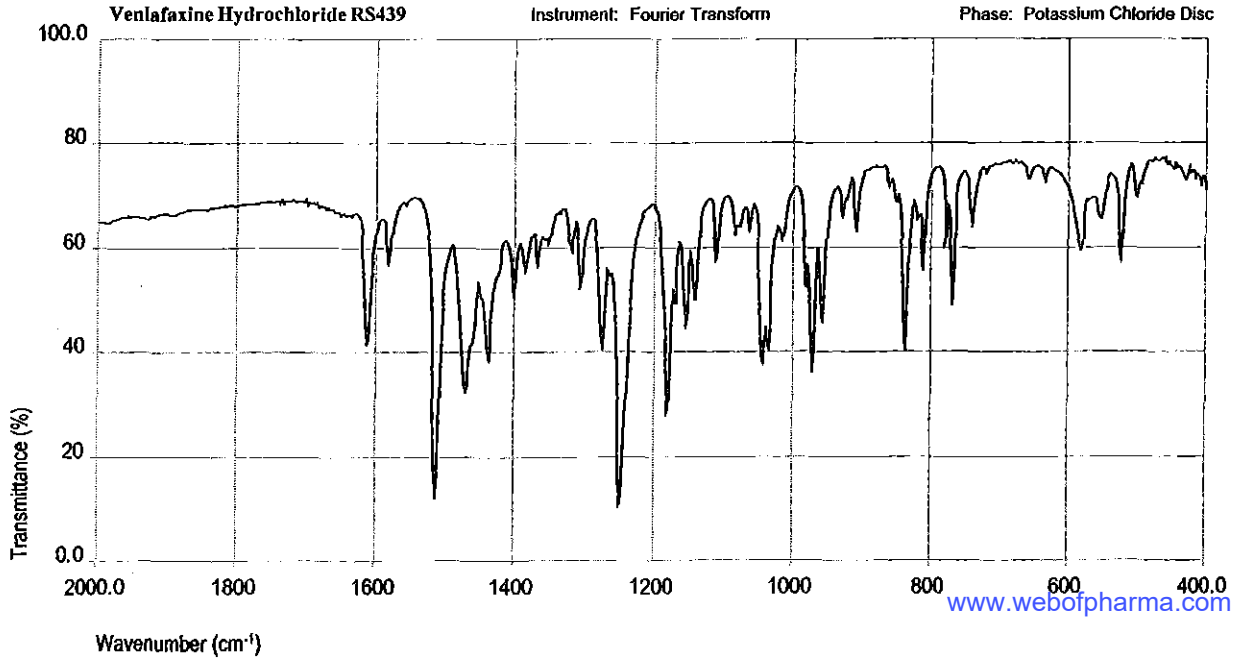


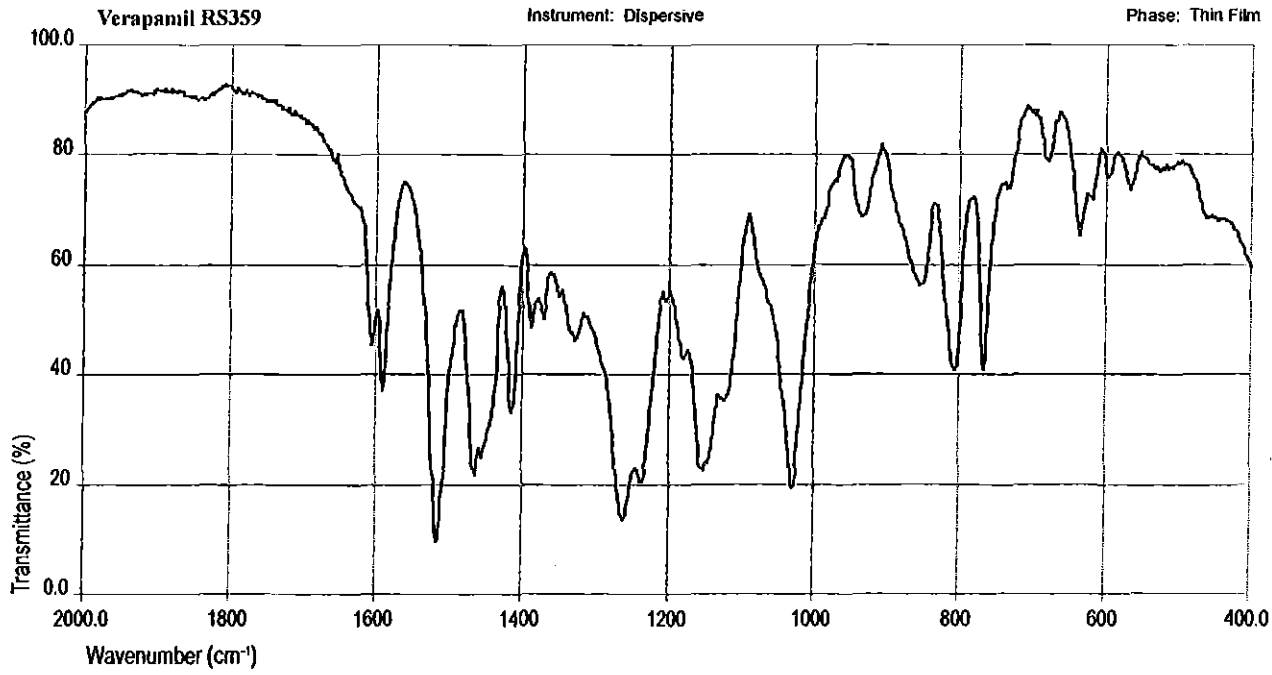


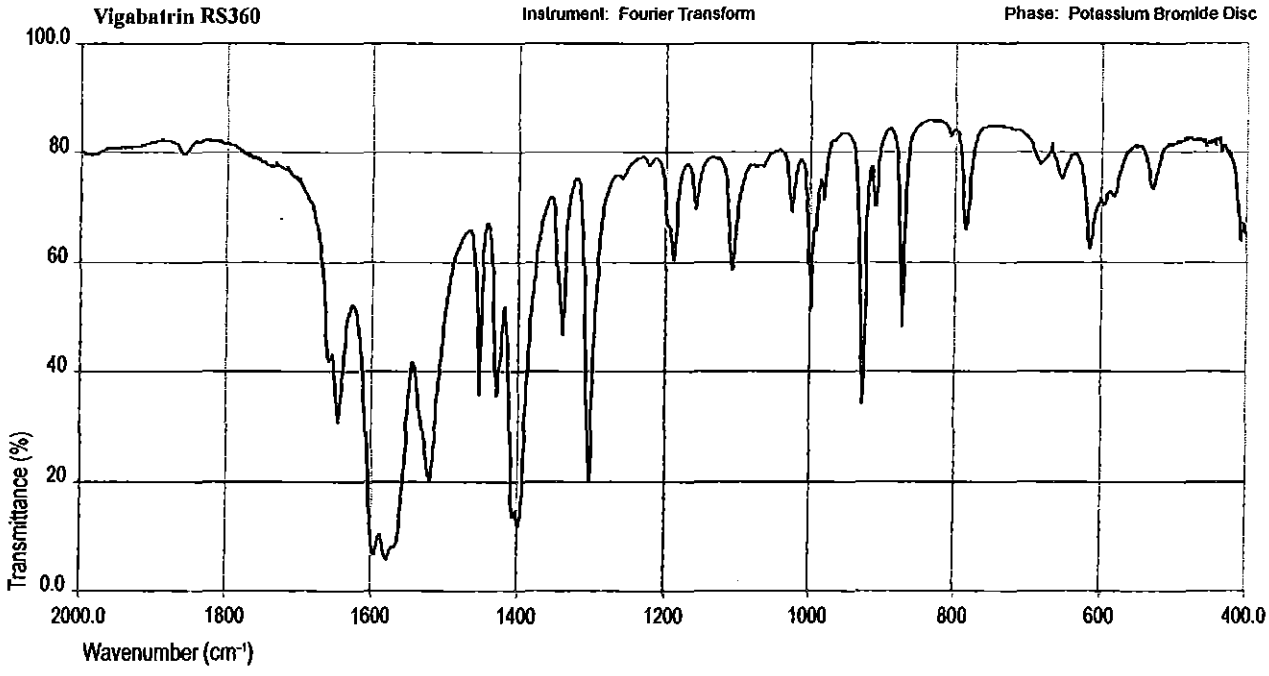


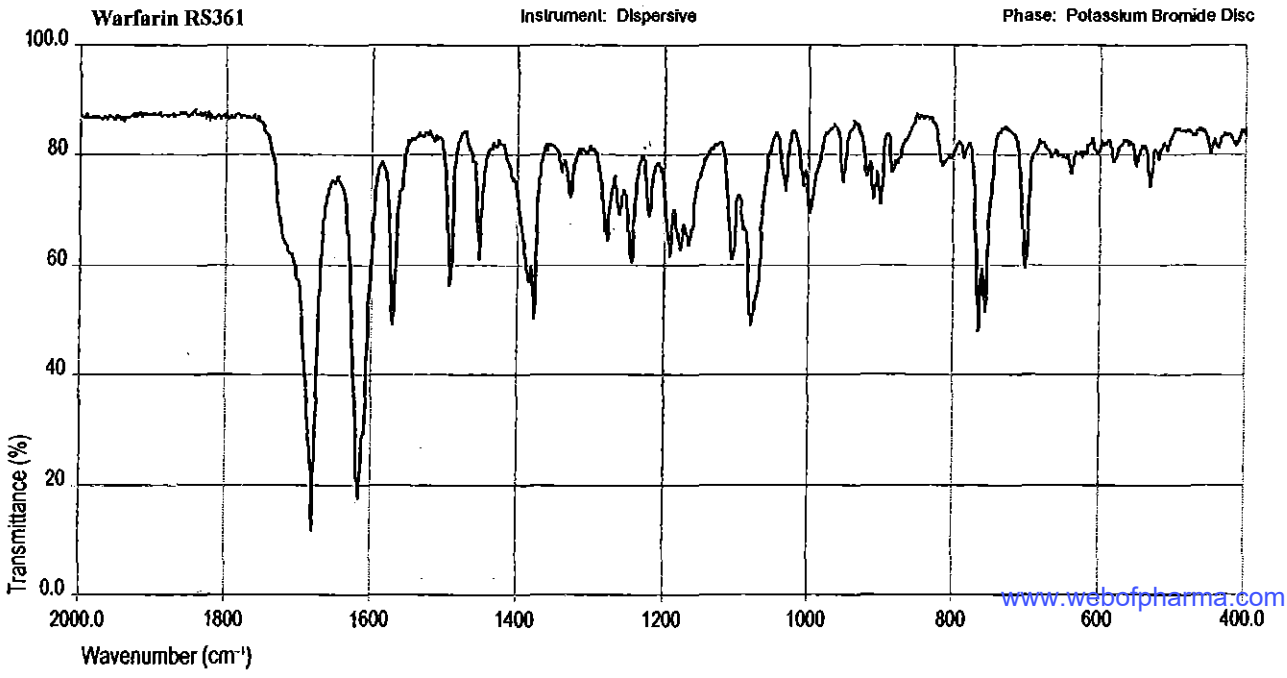


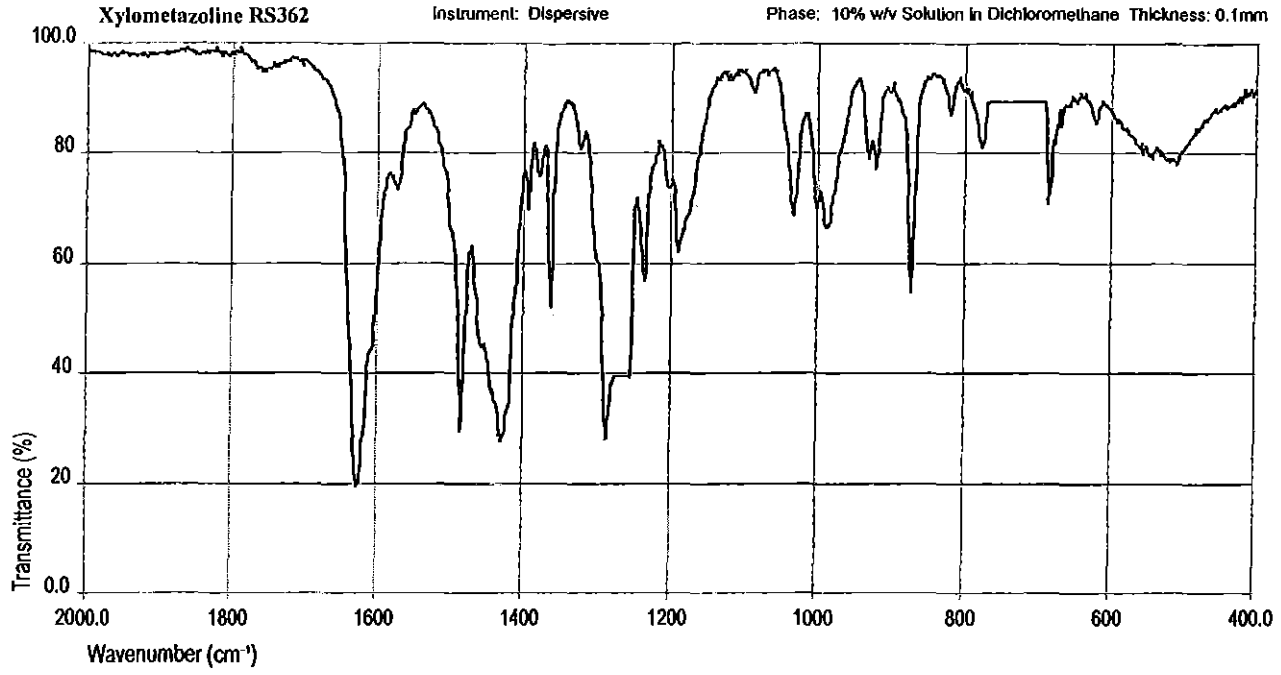


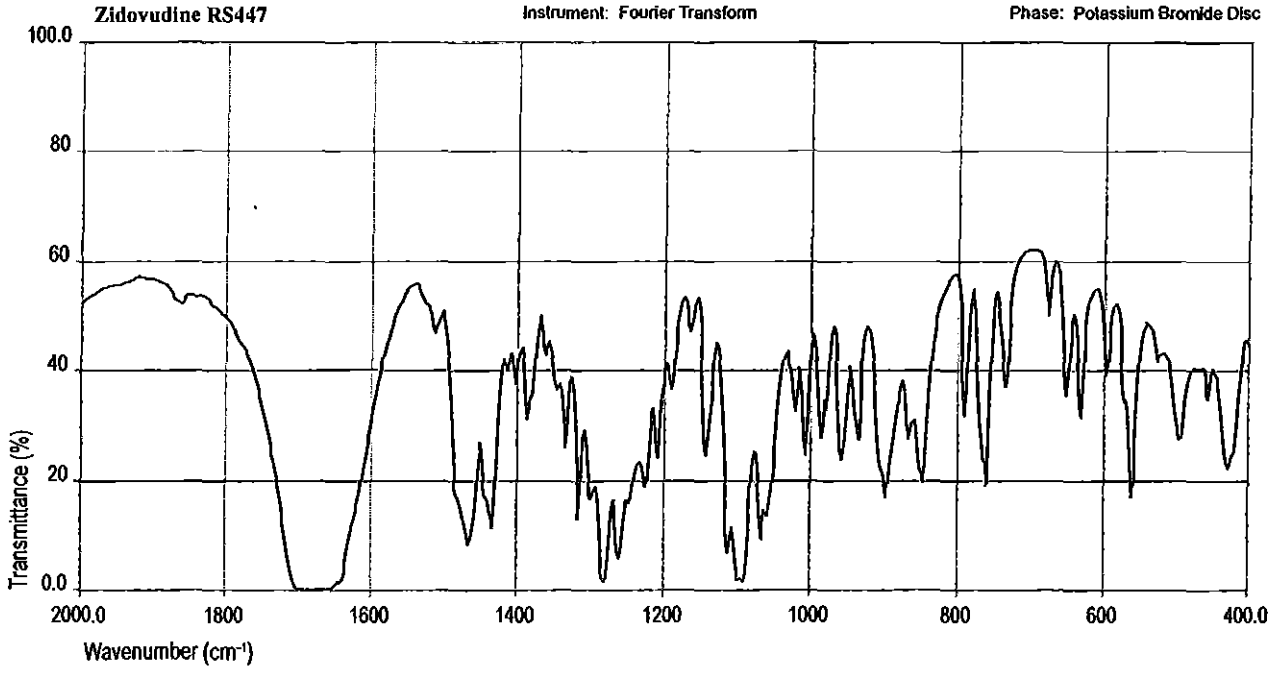


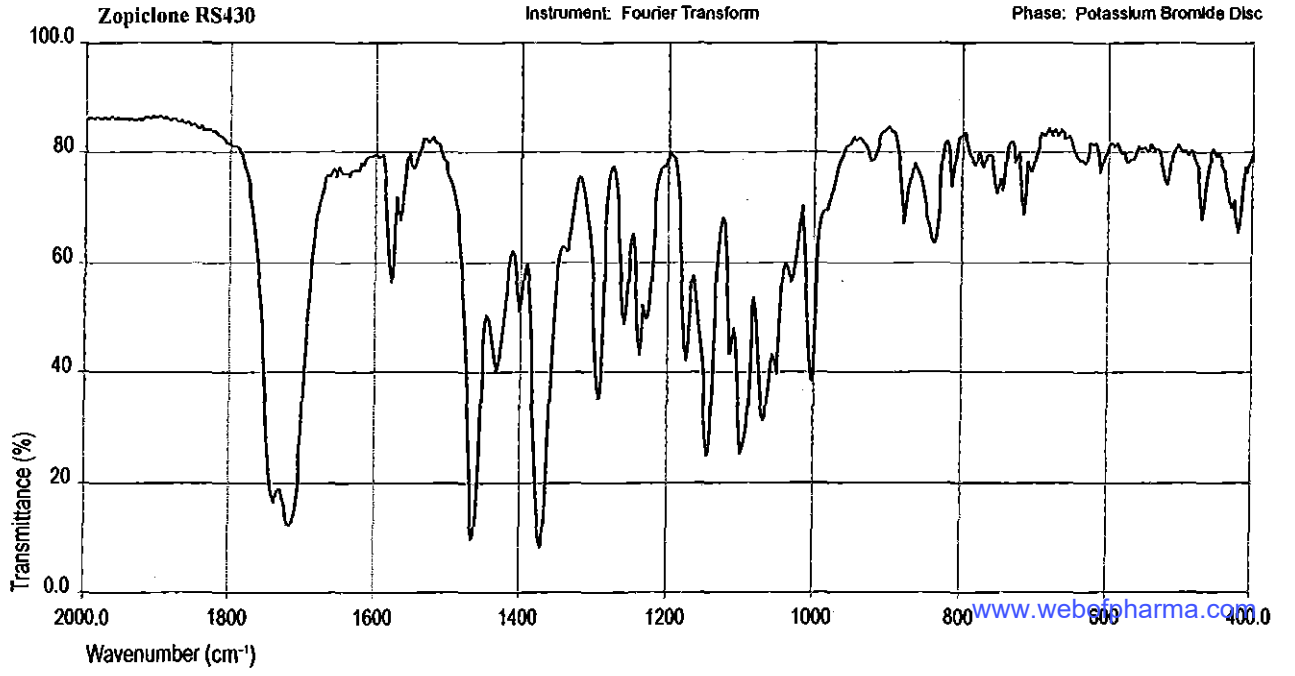


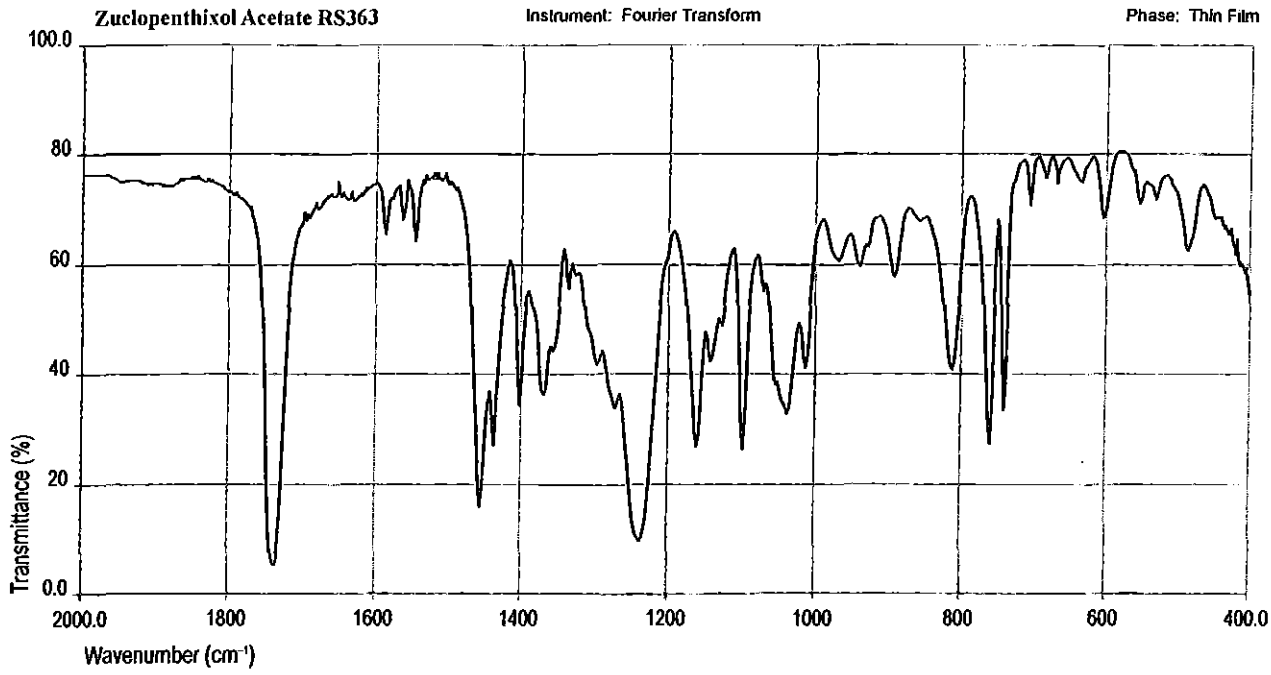


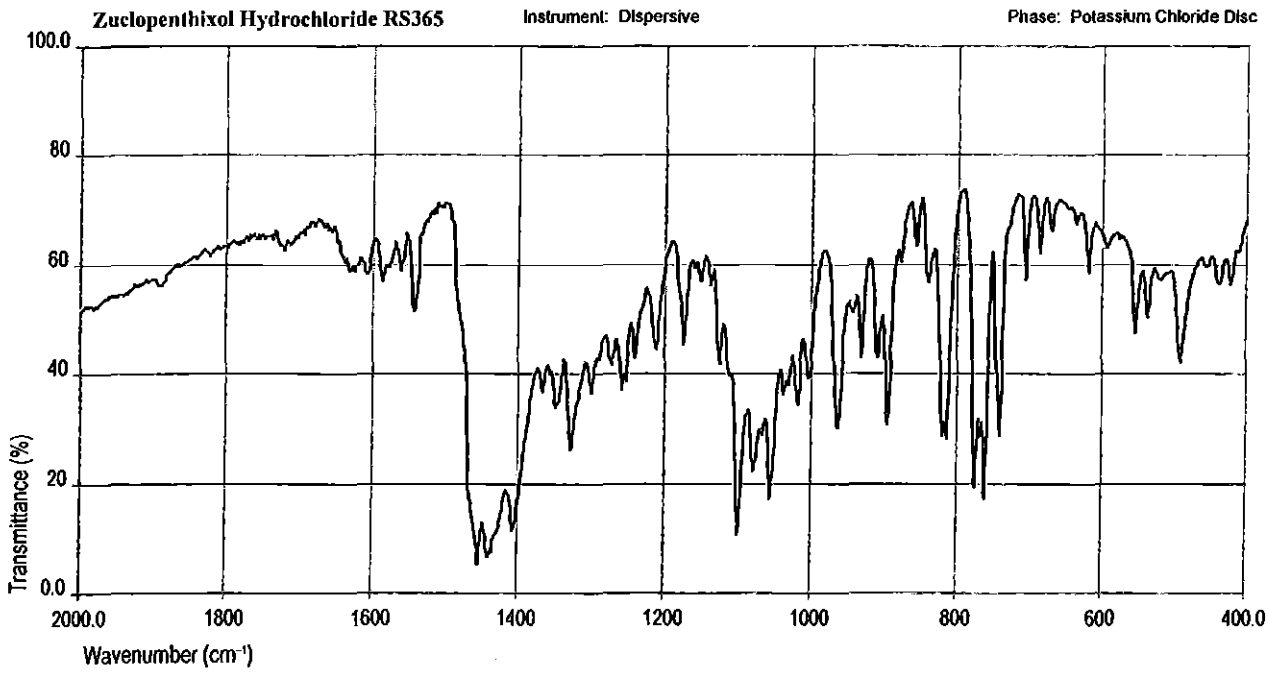












Appendices

Introduction

When a method, test or other matter described in an appendix is invoked in a monograph reproduced from the European Pharmacopoeia, Part III of the General Notices applies. When a method, test or other matter described in an appendix is invoked in any other monograph, Part II of General Notices applies.

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European Pharmacopoeia Equivalent Texts

In monographs reproduced from the European Pharmacopoeia the analytical methods, tests and other supporting texts are invoked by means of the reference number of the text in the General Chapters of the European Pharmacopoeia.

The table below lists the contents of the General Chapters of the European Pharmacopoeia and gives the British Pharmacopoeia or British Pharmacopoeia (Veterinary) equivalents. It is provided for information but it is emphasised that, for texts of the European Pharmacopoeia, in cases of doubt or dispute the text published by the Council of Europe is authoritative. Appendices of the British Pharmacopoeia (Veterinary) are identified by inclusion of '(Vet)' after the Appendix letter, for example, Appendix XV J (Vet)[1].

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[1] *Reproduced in full as Part III of the General Notices of the British Pharmacopoeia and British Pharmacopoeia (Veterinary).*

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Appendix I

Additional Information for Reagents

(Ph. Eur. text 4.0)

Additional information for reagents that can only be fully identified by a trademark or whose availability is limited may be found in the Knowledge database on the EDQM website. This information is given only to make it easier to obtain such reagents and this does not suggest in any way that the mentioned suppliers are especially recommended or certified by the European Pharmacopoeia Commission or the Council of Europe. It is therefore acceptable to use reagents from another source provided that they comply with the standards of the Pharmacopoeia.

A. General Reagents

(Ph. Eur. text 4.1)

Where the name of a substance or a solution is followed by the letter R (the whole in italics), this indicates a reagent included in the following list. The specifications given for reagents do not necessarily guarantee their quality for use in medicines.

Within the description of each reagent there is a 7-digit reference code in italics (for example, *1002501*). This number, which will remain unchanged for a given reagent during subsequent revisions of the list, is used for identification purposes by the Secretariat, and users of the Pharmacopoeia may also find it useful, for example in the management of reagent stocks. The description may also include a CAS number (Chemical Abstract Service Registry Number) recognisable by its typical format, for example 9002-93-1.

Some of the reagents included in the list are toxic and are to be handled in conformity with good quality control laboratory practice.

Reagents in aqueous solution are prepared using *water R*. For liquid chromatography, *water for chromatography R* is used for the preparation of mobile phases when water, or an aqueous solution, is one of the components. Where a reagent solution is described using an expression such as 'hydrochloric acid (10 g/L HCl)', the solution is prepared by an appropriate dilution with *water R* of a more concentrated reagent solution specified in this chapter. Reagent solutions used in the limit tests for barium, calcium and sulfates are prepared using *distilled water R*. Where the name of the solvent is not stated, an aqueous solution is intended.

The reagents and reagent solutions are to be stored in well-closed containers. The labelling should comply with the relevant national legislation and international agreements.

Droppers

(Ph. Eur. text 2.1.1)

The term 'drops' means standard drops delivered from a standard dropper as described below.

Standard droppers (Figure 2.1.1.-1) are constructed of practically colourless glass. The lower extremity has a circular orifice in a flat surface at right angles to the axis.

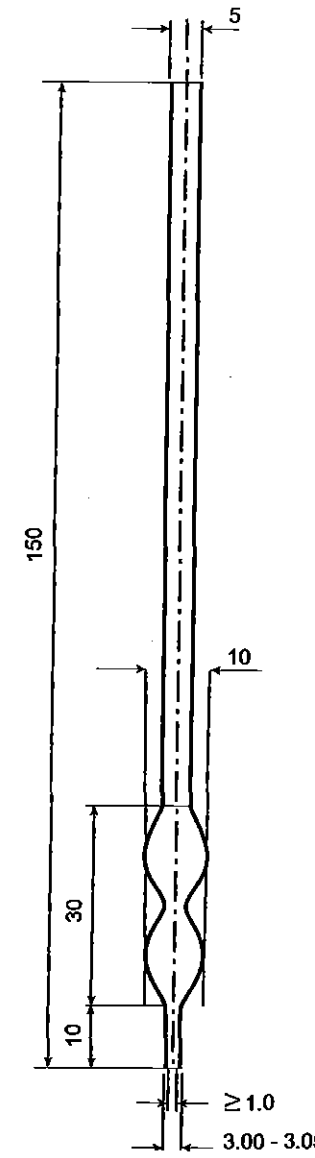

Figure 2.1.1.-1. – *Standard dropper*
Dimensions in millimetres

Other droppers may be used provided they comply with the following test.

20 drops of *water R* at 20 ± 1 °C flowing freely from the dropper held in the vertical position at a constant rate of 1 drop per second weighs 1000 ± 50 mg.

The dropper must be carefully cleaned before use. Carry out 3 determinations on any given dropper. No result may deviate by more than 5 per cent from the mean of the 3 determinations.

Acacia See *Acacia (0307)*.

Acacia Solution

Dissolve 100 g of *acacia R* in 1000 mL of *water R*. Stir with a mechanical stirrer for 2 h. Centrifuge at about 2000 *g* for 30 min to obtain a clear solution.

Storage: in polyethylene containers of about 250 mL capacity at a temperature of 0 °C to −20 °C.

Acebutolol Hydrochloride *(34381-68-5)*

See *Acebutolol hydrochloride (0871)*.

Acetal Acetaldehyde diethyl acetal; 1,1-Diethoxyethane; $C_6H_{14}O_2$ = 118.2 *(105-57-7)*

Clear, colourless, volatile liquid, miscible with water and with ethanol (96 per cent).

d_{20}^{20}: about 0.824.

n_D^{20}: about 1.382.

bp: about 103 °C.

Acetaldehyde Ethanal; C_2H_4O = 44.1 (*75-07-0*)

Clear, colourless flammable liquid, miscible with water and with ethanol (96 per cent).

d_{20}^{20}: about 0.788.

n_D^{20}: about 1.332.

bp: about 21 °C.

Acetaldehyde Ammonia Trimer Trihydrate 2,4,6-Trimethylhexahydro-1,3,5-triazine trihydrate; $C_6H_{15}N_3,3H_2O$ = 183.3 (*58052-80-5*)

mp: 95 °C to 97 °C.

Content: minimum 95.0 per cent.

Colourless or white or pale yellow crystals or powder

Assay. Dissolve 0.900 g in *water R* and dilute to 50.0 mL with the same solvent. Titrate with *1 M hydrochloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *1 M hydrochloric acid* is equivalent to 61.08 mg of $C_6H_{15}N_3,3H_2O$.

Acetamide C_2H_5NO = 59.07 (*60-35-5*)

mp: about 78°.

General reagent grade of commerce.

Acetic Acid

Content: 290 g/L to 310 g/L of $C_2H_4O_2$ (M_r 60.1).

Dilute 30 g of *glacial acetic acid R* to 100 mL with *water R*.

Acetic Acid (6 per cent) Acetic acid, dilute R1

Content: 57.5 g/L to 62.5 g/L (M_r 60.1).

Dilute 6 g of *glacial acetic acid R* to 100 mL with *water R*.

Acetic Acid, Anhydrous Anhydrous glacial acetic acid; $C_2H_4O_2$ = 60.1 (*64-19-7*)

Content: minimum 99.6 per cent *m/m* of $C_2H_4O_2$.

Colourless liquid or white or almost white, shining, fern-like crystals, miscible with or very soluble in water, in ethanol (96 per cent), in glycerol (85 per cent), and in most fatty and essential oils.

d_{20}^{20}: 1.052 to 1.053.

bp: 117 °C to 119 °C.

A 100 g/L solution is strongly acid (*2.2.4*).

A 5 g/L solution neutralised with *dilute ammonia R2* gives reaction (b) of acetates (*2.3.1*).

Freezing point (*2.2.18*): minimum 15.8 °C.

Water (*2.5.12*): maximum 0.4 per cent. If the water content is more than 0.4 per cent it may be adjusted by adding the calculated amount of *acetic anhydride R*.

Storage: protected from light.

Acetic Acid, Dilute

Content: 115 g/L to 125 g/L of $C_2H_4O_2$ (M_r 60.1).

Dilute 12 g of *glacial acetic acid R* to 100 mL with *water R*.

Acetic Acid, Glacial $C_2H_4O_2$ = 60.1 (*64-19-7*)

See *Acetic acid, glacial (0590)*.

Solutions of molarity *x*M should be prepared by diluting 57*x* mL (60*x* g) of glacial acetic acid to 1000 mL with *water*.

Acetic Anhydride $C_4H_6O_3$ = 102.1 (*108-24-7*)

Content: minimum 97.0 per cent *m/m* of $C_4H_6O_3$.

Clear, colourless liquid.

bp: 136 °C to 142 °C.

Assay. Dissolve 2.00 g in 50.0 mL of *1 M sodium hydroxide* in a ground-glass-stoppered flask and boil under a reflux condenser for 1 h. Titrate with *1 M hydrochloric acid*, using 0.5 mL of *phenolphthalein solution R* as indicator. Calculate the number of millilitres of *1 M sodium hydroxide* required for 1 g (n_1). Dissolve 2.00 g in 20 mL of *cyclohexane R* in a ground-glass-stoppered flask, cool in ice and add a cold mixture of 10 mL of *aniline R* and 20 mL of *cyclohexane R*. Boil the mixture under a reflux condenser for 1 h, add 50.0 mL of *1 M sodium hydroxide* and shake vigorously. Titrate with *1 M hydrochloric acid*, using 0.5 mL of *phenolphthalein solution R* as indicator. Calculate the number of millilitres of *1 M sodium hydroxide* required for 1 g (n_2). Calculate the percentage of $C_4H_6O_3$ from the following expression:

$$10.2(n_1 - n_2)$$

Acetic Anhydride Solution R1

Dissolve 25.0 mL of *acetic anhydride R* in *anhydrous pyridine R* and dilute to 100.0 mL with the same solvent.

Storage: protected from light and air.

Acetic Anhydride–Dioxan Solution

Add 1 mL of *acetic anhydride* to 50 mL of *1,4-dioxan*.

Acetic Anhydride–Sulfuric Acid Solution

Carefully mix 5 mL of *acetic anhydride R* with 5 mL of *sulfuric acid R*. Add dropwise and with cooling to 50 mL of *anhydrous ethanol R*.

Prepare immediately before use.

Acetone Propan-2-one; (*67-64-1*)

See *Acetone (0872)*.

Acetonitrile Methyl cyanide; Ethanenitrile; C_2H_3N = 41.05 (*75-05-8*)

Clear, colourless liquid, miscible with water, with acetone and with methanol.

d_{20}^{20}: about 0.78.

n_D^{20}: about 1.344.

A 100 g/L solution is neutral to litmus paper.

Distillation range (*2.2.11*). Not less than 95 per cent distils between 80 °C and 82 °C.

Acetonitrile used in spectrophotometry complies with the following additional test.

Absorbance (*2.2.25*): maximum 0.01 from 255 nm to 420 nm, determined using *water R* as compensation liquid.

Acetonitrile for Chromatography

See *Acetonitrile R*.

Acetonitrile used in chromatography complies with the following additional tests.

Absorbance (*2.2.25*): maximum 0.01 at 240 nm and higher wavelengths, determined using *water R* as compensation liquid.

Content (*2.2.28*): minimum 99.8 per cent.

Acetonitrile R1

Complies with the requirements prescribed for *acetonitrile R* and with the following additional requirements.

Content: minimum 99.9 per cent.

Absorbance (*2.2.25*): maximum 0.10, determined at 200 nm using *water R* as the compensation liquid.

Acetoxyvalerenic Acid (2*E*)-3-[(1*RS*,4*S*,7*R*,7a*R*)-1-(Acetyloxy)-3,7-dimethyl-2,4,5,6,7,7a-hexahydro-1*H*-inden-4-yl]-2-methylprop-2-enoic acid; $C_{17}H_{24}O_4$ = 292.4 (*81397-67-3*)

Colourless or pale yellow viscous oil.

Absorbance (*2.2.25*). A solution in *methanol R* shows an absorption maximum at about 216 nm.

Acetyl Chloride C_2H_3ClO = 78.5 (*75-36-5*)

Clear, colourless liquid, flammable, decomposes in contact with water and with ethanol (96 per cent), miscible with ethylene chloride.

d_{20}^{20}: about 1.10.

Distillation range (*2.2.11*). Not less than 95 per cent distils between 49 °C and 53 °C.

Acetylacetamide 3-Oxobutanamide; $C_4H_7NO_2$ = 101.1 (*5977-14-0*)

mp: 53 °C to 56 °C.

Acetylacetone 2,4-Pentanedione; $C_5H_8O_2$ = 100.1 (*123-54-6*)

Colourless or slightly yellow, easily flammable liquid, freely soluble in water, miscible with acetone, with ethanol (96 per cent) and with glacial acetic acid.

n_D^{20}: 1.452 to 1.453.

bp: 138 °C to 140 °C.

Acetylacetone Reagent R1

To 100 mL of *ammonium acetate solution R* add 0.2 mL of *acetylacetone R*.

Acetylacetone Reagent R2

Dissolve 0.2 mL of *acetylacetone R*, 3 mL of *glacial acetic acid R* and 25 g of *ammonium acetate R* in *water R* and dilute to 100 mL with the same solvent.

4-Acetylbiphenyl 4-Phenylacetophenone; $C_{14}H_{12}O$ = 196.2 (*51-42-3*)

mp: about 117°.

General reagent grade of commerce.

***N*-Acetyl-ε-caprolactam** *N*-Acetylhexane-6-lactam; $C_8H_{13}NO_2$ = 155.2 (*1888-91-1*)

Colourless liquid, miscible with anhydrous ethanol.

d_{20}^{20}: about 1.100.

n_D^{20}: about 1.489.

bp: about 135 °C.

Acetylcholine Chloride $C_7H_{16}ClNO_2$ = 181.7 (*60-31-1*)

Crystalline powder, very soluble in cold water and in ethanol (96 per cent). It decomposes in hot water and in alkalis.

Storage: at −20 °C.

***N*-Acetyl-L-cysteine** $C_5H_9NO_3S$ = 163.2 (*616-91-1*)

$[\alpha]_D^{20}$: about +4.6.

mp: about 110°.

General reagent grade of commerce.

Acetylene Ethyne; C_2H_2 = 26.04 (*74-86-2*)

Content: minimum 99.0 per cent *V/V*.

Acetyleugenol 2-Methoxy-4-(2-propenyl)phenylacetate; $C_{12}H_{14}O_3$ = 206.2 (*93-28-7*)

Yellow coloured, oily liquid, practically insoluble in water, freely soluble in ethanol (96 per cent).

n_D^{20}: about 1.521.

bp: 281 °C to 282 °C.

Acetyleugenol used in gas chromatography complies with the following additional test.

Assay. Gas chromatography (*2.2.28*) as prescribed in the monograph *Clove oil (1091)*.

Test solution. The substance to be examined.

Content: minimum 98.0 per cent, calculated by the normalisation procedure.

***N*-Acetylglucosamine** 2-(Acetylamino)-2-deoxy-D-glucopyranose; $C_8H_{15}NO_6$ = 221.2 (*7512-17-6*)

mp: about 202 °C.

Acetyl-11-keto-β-boswellic Acid 3α-(Acetyloxy)-11-oxours-12-en-24-oic acid; (4β)-3α-(Acetyloxy)-11-oxours-12-en-23-oic acid; $C_{32}H_{48}O_5$ = 512.7 (*67416-61-9*)

White or almost white powder, insoluble in water, soluble in acetone, in anhydrous ethanol and in methanol.

mp: 271 °C to 274 °C.*Acetyl-11-keto-β-boswellic acid used in liquid chromatography complies with the following additional test.*

Assay. Liquid chromatography (*2.2.29*) as prescribed in the monograph on *Indian frankincense (2310)*.

Content: minimum 90 per cent, calculated by the normalisation procedure.

N-(α)-Acetyl-L-lysine (2*S*)-2-Acetamido-6-aminohexanoic acid; $C_8H_{16}N_2O_3$ = 188.2 (*1946-82-3*)

N-(ε)-Acetyl-L-lysine (2*S*)-6-Acetamido-2-aminohexanoic acid; $C_8H_{16}N_2O_3$ = 188.2 (*692-04-6*)

***N*-Acetylneuraminic Acid** Sialic acid; $C_{11}H_{19}NO_9$ = 309.3 (*131-48-6*)

White or almost white acicular crystals, soluble in water and in methanol, slightly soluble in anhydrous ethanol, practically insoluble in acetone.

$[\alpha]_D^{20}$: about −36, determined on a 10 g/L solution.

mp: about 186 °C, with decomposition.

Acetylsalicylic Acid 2-(Acetyloxy)benzoic acid; $C_9H_8O_4$ = 180.2 (*50-78-2*)

White or almost white, crystalline powder or colourless crystals, slightly soluble in water, freely soluble in ethanol (96 per cent).

***N*-Acetyltryptophan** 2-Acetylamino-3-(indol-3-yl) propanoic acid; $C_{13}H_{14}N_2O_3$ = 246.3 (*1218-34-4*)

White or almost white powder or colourless crystals, slightly soluble in water. It dissolves in dilute solutions of alkali hydroxides.

mp: about 205 °C.

Assay. Liquid chromatography (*2.2.29*) as prescribed in the monograph *Tryptophan (1272)*.

Test solution. Dissolve 10.0 mg in a mixture of 10 volumes of *acetonitrile R* and 90 volumes of *water R* and dilute to 100.0 mL with the same mixture of solvents.

Content: minimum 99.0 per cent, calculated by the normalisation procedure.

Acetyltyrosine Ethyl Ester Ethyl *N*-acetyl-L-tyrosinate; $C_{13}H_{17}NO_4,H_2O$ = 269.3 (*36546-50-6*)

White or almost white, crystalline powder suitable for the assay of chymotrypsin.

$[\alpha]_D^{20}$: + 21 to + 25, determined on a 10 g/L solution in *ethanol (96 per cent) R*.

$A_{1\,cm}^{1\%}$: 60 to 68, determined at 278 nm in *ethanol (96 per cent) R*.

Acetyltyrosine Ethyl Ester, 0.2M 0.2M Ethyl acetyltyrosinate

Dissolve 0.54 g of *acetyltyrosine ethyl ester R* in *ethanol (96 per cent) R* and dilute to 10.0 mL with the same solvent.

α1-Acid-glycoprotein Silica Gel for Chiral Separation
A very finely divided silica gel for chromatography consisting of spherical particles coated with α1-acid glycoprotein.

Acid Blue 83 Brilliant blue; Coomassie brilliant blue R 250; $C_{45}H_{44}N_3NaO_7S_2$ = 826 (*6104-59-2*)
Colour Index No. 42660
Brown powder insoluble in cold water, slightly soluble in boiling water and in anhydrous ethanol, soluble in sulfuric acid, glacial acetic acid and in dilute solutions of alkali hydroxides.

Acid Blue 90 Coomassie brilliant blue G; $C_{47}H_{48}N_3NaO_7S_2$ = 854 (*6104-58-1*)
Colour Index No. 42655
A dark brown powder, with a violet sheen and some particles having a metallic lustre, soluble in water and in anhydrous ethanol.
$A_{1\,cm}^{1\%}$: greater than 500, determined at 577 nm in a 0.01 g/L solution in buffer solution pH 7.0 and calculated with reference to the dried substance.
Loss on drying (*2.2.32*): maximum 5.0 per cent, determined on 0.500 g by drying in an oven at 105 °C.

Acid Blue 92 Coomassie blue; Anazolene sodium; Trisodium 8-hydroxy-4′-(phenylamino)azonaphthalene-3,5′,6-trisulfonate; $C_{26}H_{16}N_3Na_3O_{10}S_3$ = 696 (*3861-73-2*)
Colour Index No. 13390
Dark blue crystals, soluble in water, in acetone and in ethylene glycol monoethylether, slightly soluble in ethanol (96 per cent).

Acid Blue 92 Solution Coomassie blue solution
Dissolve 0.5 g of *acid blue 92 R* in a mixture of 10 mL of *glacial acetic acid R*, 45 mL of *ethanol (96 per cent) R* and 45 mL of *water R*.

Acid Blue 93 Methyl blue; Poirrier blue; $C_{37}H_{27}N_3Na_2O_9S_3$ = 800 (*28983-56-4*)
Colour Index No. 42780
Mixture of triphenylrosaniline di- and trisulfonate and of triphenylpararosaniline.
Dark blue powder.
Colour change: pH 9.4 to pH 14.0.

Acid Blue 93 Solution
Dissolve 0.2 g of *acid blue 93 R* in *water R* and dilute to 100 mL with the same solvent.

Acrylamide Propenamide; C_3H_5NO = 71.1 (*79-06-1*)
Colourless or white flakes or a white or almost white, crystalline powder, very soluble in water and in methanol, freely soluble in anhydrous ethanol.
mp: about 84 °C.

Acrylamide/bisacrylamide (29:1) Solution, 30 per cent
Prepare a solution containing 290 g of *acrylamide R* and 10 g of *methylenebisacrylamide R* per litre of *water R*. Filter.

Acrylamide/bisacrylamide (36.5:1) Solution, 30 per cent
Prepare a solution containing 292 g of *acrylamide R* and 8 g of *methylenebisacrylamide R* per litre of *water R*. Filter.

Acrylic Acid Prop-2-enoic acid; Vinylformic acid; $C_3H_4O_2$ = 72.1 (*79-10-7*)
Content: minimum 99 per cent.
It is stabilised with 0.02 per cent of hydroquinone monomethyl ether.
Corrosive liquid, miscible with water and ethanol (96 per cent). It polymerises readily in the presence of oxygen.
d_{20}^{20}: about 1.05.
n_D^{20}: about 1.421.
bp: about 141 °C.
mp: 12 °C to 15 °C.

Actein (23*R*,24*R*,25*S*,26*S*)-3β-(β-D-Xylopyranosyloxy)-16β,23:23,26:24,25-triepoxy-26-hydroxy-9,19-cyclolanostan-12β-yl acetate; $C_{37}H_{56}O_{11}$ = 677 (*18642-44-9*)

Acteoside 2-(3,4-Dihydroxyphenyl)ethyl 3-*O*-(6-deoxy-α-L-mannopyranosyl)-4-*O*-[(2*E*)-3-(3,4-dihydroxyphenyl)prop-2-enoyl]-β-D-glucopyranoside; Verbascoside; $C_{29}H_{36}O_{15}$ = 624.6 (*61276-17-3*)
Light yellowish powder, freely soluble in water and in methanol.
mp: about 140 °C, with decomposition.

Adamantane Tricyclo[3.3.1.1^{3,7}]decane; $C_{10}H_{16}$ = 136.2 (*281-23-2*)
mp: about 270 °C.

Adenine (*73-24-5*)
See *Adenine (0800)*.

Adenosine 6-Amino-9-β-D-ribofuranosyl-9*H*-purine; $C_{10}H_{13}N_5O_4$ = 267.2 (*58-61-7*)
White or almost white, crystalline powder, slightly soluble in water, practically insoluble in acetone and in ethanol (96 per cent). It dissolves in dilute solutions of acids.
mp: about 234 °C.

Adipic Acid $C_6H_{10}O_4$ = 146.1 (*124-04-9*)
Prisms, freely soluble in methanol, soluble in acetone, practically insoluble in light petroleum.
mp: about 152 °C.

Adrenaline (1*R*)-1-(3,4-Dihydroxyphenyl)-2-(methylamino)ethanol; 4-[(1*R*)-1-hydroxy-2-(methylamino) ethyl]benzene-1,2-diol; $C_9H_{13}NO_3$ = 183.2 (*51-43-4*)
White or almost white powder, gradually becoming brown on exposure to light and air, very slightly soluble in water and in ethanol (96 per cent), insoluble in acetone. It dissolves in dilute solutions of mineral acids and alkali hydroxides.
mp: about 215 °C.

Adrenaline Acid Tartrate L-Epinephrine D-hydrogen tartrate; $C_{13}H_{19}NO_9$ = 333 (*51-42-3*)
General reagent grade of commerce.

(±) Adrenaline Hydrochloride DL-adrenaline hydrochloride; DL-epinephrine hydrochloride; $C_9H_{13}NO_3$, HCl = 220 (*329-63-5*)
General reagent grade of commerce.

Adrenalone Hydrochloride 1-(3,4-Dihydroxyphenyl)-2-(methylamino)ethanone hydrochloride; 3′,4′-Dihydroxy-2-(methylamino)acetophenone hydrochloride; $C_9H_{12}ClNO_3$ = 217.7 (*62-13-5*)
Pale yellow crystals, freely soluble in water, soluble in ethanol (96 per cent).
mp: about 244 °C.

Aescin Escin; (*6805-41-0*)
A mixture of related saponins obtained from the seeds of *Aesculus hippocastanum* L.
Fine, almost white or slightly reddish or yellowish, amorphous powder.
Chromatography. Thin-layer chromatography (*2.2.27*).

Test solution. Dissolve 10 mg of *aescin R* in *ethanol (70 per cent V/V) R* and dilute to 10 mL with the same solvent.

Plate: TLC silica gel plate R.

Mobile phase: the upper layer of a mixture of 10 volumes of *glacial acetic acid R*, 40 volumes of *water R* and 50 volumes of *butanol R*.

Application: 20 µL of the test solution as bands of 20 mm by 3 mm.

Development: over a path of 12 cm.

Drying: at 100-105 °C.

Detection: spray with about 10 mL of *anisaldehyde solution R* for a plate 200 mm square and heat again at 100-105 °C.

Results: the chromatogram shows a principal band with an R_F of about 0.4.

$C_{54}H_{84}O_{23},2H_2O$ = 1138

Aflatoxin B_1 (6a*R*,9a*S*)-4-Methoxy-2,3,6a,9a-tetrahydrocyclopenta[*c*]furo[3′,2′:4,5]furo[2,3-*h*][1]benzopyran-1,11-dione; $C_{17}H_{12}O_6$ = 312.3 (*1162-65-8*)

White or faint yellow crystals.

Agar (*9002-18-0*)

The dried extract from *Gelidium* sp. and other algae belonging to the class Rhodophyceae.

Microbiological reagent grade of commerce.

Agarose for Chromatography (*9012-36-6*)

Swollen beads 60-140 µm in diameter presented as a 4 per cent suspension in *water R*.

Used in size-exclusion chromatography for the separation of proteins with relative molecular masses of 6×10^4 to 20×10^6 and of polysaccharides with relative molecular masses of 3×10^3 to 5×10^6.

Agarose for Chromatography, Cross-linked (*61970-08-9*)

Prepared from agarose by reaction with 2,3-dibromopropanol in strongly alkaline conditions.

It occurs as swollen beads and is presented as a suspension.

Used in size-exclusion chromatography for the separation of proteins and of polysaccharides.

Agarose for Chromatography R1, Cross-linked (*65099-79-8*)

Prepared for agarose by reaction with 2,3-dibromopropanol in strongly alkaline conditions.

It occurs as swollen beads 60-140 µm in diameter and is presented as a 4 per cent suspension in *water R*.

Used in size-exclusion chromatography for the separation of proteins with relative molecular masses of 7×10^4 to 40×10^6 and of polysaccharides with relative molecular masses of 1×10^5 to 2×10^7.

Agarose for Electrophoresis (*9012-36-6*)

A neutral, linear polysaccharide, the main component of which is derived from agar.

White or almost white powder, practically insoluble in cold water, very slightly soluble in hot water.

Agarose-DEAE for Ion Exchange Chromatography (*57407-08-6*)

Cross-linked agarose substituted with diethylaminoethyl groups, presented as beads.

Agarose/Cross-linked Polyacrylamide

Agarose trapped within a cross-linked polyacrylamide network; it is used for the separation of globular proteins with relative molecular masses of 2×10^4 to 35×10^4.

Agnuside (1*RS*,4a*SR*,5*RS*,7a*RS*)-5-Hydroxy-7-[[(4-hydroxybenzoyl)oxy]methyl]-1,4a,5,7a-tetrahydrocyclopenta[*c*]pyran-1-yl β-D-glucopyranoside; $C_{22}H_{26}O_{11}$ = 466.4 (*11027-63-7*)

White or almost white crystals.

Air, Hydrocarbon-free

Complies with the requirements prescribed for the monograph *Medicinal air (1238)* with the following additional requirement.

Hydrocarbons: maximum 5 ppm *V/V*, calculated as CH_4.

Alanine L-Alanine; (*56-41-7*)

See *Alanine (0752)*.

β-Alanine (*107-95-9*)

See *3-aminopropionic acid R*.

Albumin, Bovine (*9048-46-8*)

Bovine serum albumin containing about 96 per cent of protein.

White to light brownish-yellow powder.

Water (*2.5.12*): maximum 3.0 per cent, determined on 0.800 g.

Albumin, Bovine R1 (*9048-46-8*)

Bovine serum albumin containing about 96 per cent of protein.

White or light brownish-yellow powder.

Albumin, Human

Human serum albumin containing not less than 96 per cent of albumin.

Albumin Solution, Human Albumin; (*9048-46-8*)

See *Human albumin solution (0255)*.

Albumin Solution R1, Human

Dilute *human albumin solution R* with a 9 g/L solution of *sodium chloride R* to a concentration of 1 g/L of protein. Adjust the pH to 3.5-4.5 with *glacial acetic acid R*.

Alcohol (*64-17-5*)

See *Ethanol (96 per cent) R*.

Alcohol, Aldehyde-free Ethanol (96%), aldehyde-free

Mix 1200 mL of *ethanol (96 per cent) R* with 5 mL of a 400 g/L solution of *silver nitrate R* and 10 mL of a cooled 500 g/L solution of *potassium hydroxide R*. Shake, allow to stand for a few days and filter. Distil the filtrate immediately before use.

Alcohol (*x*% v/v)

See *Ethanol (x per cent V/V) R*.

Aldehyde Dehydrogenase

Enzyme obtained from baker's yeast which oxidises acetaldehyde to acetic acid in the presence of nicotinamide-adenine dinucleotide, potassium salts and thiols, at pH 8.0.

Aldehyde Dehydrogenase Solution

Dissolve in *water R* a quantity of *aldehyde dehydrogenase R*, equivalent to 70 units and dilute to 10 mL with the same solvent. This solution is stable for 8 h at 4 °C.

Aldrin $C_{12}H_8Cl_6$ = 364.9 (*309-00-2*)

bp: about 145 °C.

mp: about 104 °C.

A suitable certified reference solution (10 ng/µL in cyclohexane) may be used.

Aleuritic Acid (9*RS*,10*SR*)-9,10,16-Trihydroxyhexadecanoic acid; $C_{16}H_{32}O_5$ = 304.4 (*533-87-9*)

White or almost white powder, greasy to the touch, soluble in methanol.

mp: about 101 °C.

Alizarin S Alizarin red S; $C_{14}H_7NaO_7S,H_2O$ = 360.3 (*130-22-3*)

Schultz No. 1145

Colour Index No. 58005

Orange-yellow powder, freely soluble in water and in ethanol (96 per cent).

Alizarin S Solution

A 1 g/L solution of *alizarin S R*.

Test for sensitivity. If alizarin S solution is used for the standardisation of *0.05 M barium perchlorate*, it shows a colour change from yellow to orange-red when it is tested according to the standardisation of *0.05 M barium perchlorate*.

Colour change: pH 3.7 (yellow) to pH 5.2 (violet).

Aloe Emodin $C_{15}H_{10}O_5$ = 270.2 (*481-72-1*)

1,8-Dihydroxy-3-(hydroxymethyl)anthracene-9,10-dione.

1,8-Dihydroxy-3-(hydroxymethyl)anthraquinone.

Alovudine 1-[(2*R*,4*S*,5*R*)-4-Fluoro-5-(hydroxymethyl) tetrahydrofuran-2-yl]-5-methylpyrimidine-2,4(1*H*,3*H*)-dione; Fluorodeoxythymidine; 3′-Deoxy-3′-fluorothymidine; $C_{10}H_{13}FN_2O_4$ = 244.2 (*25526-93-6*)

Content: minimum 95 per cent.

Colourless crystals.

Aluminium Al = 26.98 (*7429-90-5*)

White or almost white, malleable, flexible, bluish metal, available as bars, sheets, powder, strips or wire. In moist air an oxide film forms which protects the metal from corrosion.

Analytical grade.

Aluminium Chloride Aluminium chloride hexahydrate; $AlCl_3,6H_2O$ = 241.4 (*7784-13-6*)

Content: minimum 98.0 per cent of $AlCl_3,6H_2O$.

White or slightly yellowish, crystalline powder, hygroscopic, freely soluble in water and in ethanol (96 per cent).

Storage: in an airtight container.

Aluminium Chloride Reagent

Dissolve 2.0 g of *aluminium chloride R* in 100 mL of a 5 per cent *V/V* solution of *glacial acetic acid R* in *methanol R*.

Aluminium Chloride Solution

Dissolve 65.0 g of *aluminium chloride R* in *water R* and dilute to 100 mL with the same solvent. Add 0.5 g of *activated charcoal R*, stir for 10 min, filter and add to the filtrate, with continuous stirring, sufficient of a 10 g/L solution of *sodium hydroxide R* (about 60 mL) to adjust the pH to about 1.5.

Aluminium Hydroxide Gel $Al(OH)_3$+ aq (*21645-51-2*)

A hydrated grade of aluminium hydroxide of commerce.

Aluminium Nitrate Aluminium nitrate nonahydrate; $Al(NO_3)_3,9H_2O$ = 375.1 (*7784-27-2*)

Crystals, deliquescent, very soluble in water and ethanol (96 per cent), very slightly soluble in acetone.

Storage: in an airtight container.

Aluminium Oxide, Activated Acid Al_2O_3 = 102.1

An almost white, fine, granular powder, very hygroscopic, activated by heating at 200° to 250° for 3 hours.

Mean particle size, 50 to 200 µm.

Aluminium Oxide, Anhydrous (*1344-28-1*)

Aluminium oxide, consisting of γ-Al_2O_3, dehydrated and activated by heat treatment.

Particle size: 75 µm to 150 µm.

Al_2O_3 = 102.0

Aluminium Oxide, Basic

A basic grade of *anhydrous aluminium oxide R* suitable for column chromatography.

pH (*2.2.3*). Shake 1 g with 10 mL of *carbon dioxide-free water R* for 5 min. The pH of the suspension is 9 to 10.

When used in monographs other than those of the European Pharmacopoeia, the following test applies.

Activity Pack into a chromatographic tube (25 cm × 10 mm) sufficient of the basic aluminium oxide to form a column 50 mm deep. Apply to the column a mixture of 5 mL of sudan yellow solution and 5 mL of sudan red solution and elute with 20 mL of a mixture of 1 volume of benzene and 4 volumes of petroleum spirit (boiling range, 60° to 80°). Sudan red forms a zone about 10 mm in depth on the upper part of the column separated by a colourless zone from a yellow zone of sudan yellow below.

Aluminium Oxide for Chromatography, Deactivated

Aluminium oxide suitably deactivated for the separation and detection of traces of polar hydrocarbons, with porous layer open tubular (PLOT) design.

Aluminium Oxide, Deactivated

To a suitable basic alumina add 1.5 to 2% of *water*, mix well and allow to stand overnight in a stoppered bottle.
The product complies with the following test.

Prepare a column (20 cm × 10 mm) using the alumina and *hexane*. Add a solution of 0.25 g of *calciferol* in 10 mL of *hexane*. When the level of the solution falls just to the top of the column, begin eluting with a 17.5% v/v solution of *ether* in *hexane* adjusting the rate of flow, if necessary, to between 1 and 2 mL per minute. Collect 200 mL of eluate; no calciferol is present. Collect a further 100 mL of eluate; it contains not less than 95% of the calciferol used in the test, when determined by the Assay described under Calciferol Oral Solution.

Aluminium Oxide G

A fine, white, homogeneous powder of an average particle size between 10 and 40 µm containing about 10% w/w of calcium sulfate hemihydrate.

Content of calcium sulfate Carry out the test described under *silica gel G*.

Acidity or alkalinity pH of a suspension prepared by shaking 1 g with 10 mL of *carbon dioxide-free water*, about 7.5, Appendix V L.

Aluminium Oxide, Neutral See *Aluminium oxide, hydrated (0311)*.

Aluminium Potassium Sulfate Alum; Aluminium potassium sulphate; Aluminium potassium sulfate dodecahydrate; Aluminium potassium sulphate dodecahydrate; (*7784-24-9*)

See *Alum (0006)*.

Aluminium Sulfate Aluminium sulfate; $Al_2(SO_4)_3,16H_2O$ = 630.4 (*10043-01-3*)

Analytical reagent grade of commerce.

Aluminium Test Strip

Commercially available test strip for the determination of aluminium in aqueous solvents at a level below 5 ppm.

Allantoin $C_4H_6N_4O_3$ = 158.1 (*97-59-6*)

Allantoin of the British Pharmacopoeia

Amaranth S CI 16185; acid red 27; $C_{20}H_{11}N_2Na_3O_{10}S_3$ = 604 (*915-67-3*)

General reagent grade of commerce.

A deep brown or deep reddish brown, fine powder.

When used for the titration of iodine and iodides with potassium iodate, the colour changes from orange-red to yellow.

Amaranth Solution

A 0.2% w/v solution of *amaranth S*.

Americium-243 Spiking Solution

Contains 50 Bq/L ^{243}Am and a 134 mg/L solution of *lanthanum chloride heptahydrate R* in a 103 g/L solution of *hydrochloric acid R*.

Amido Black 10B Disodium 5-amino-4-hydroxy-6-[(4-nitrophenyl)azo]-3-(phenylazo)naphthalene-2,7-disulfonate; $C_{22}H_{14}N_6Na_2O_9S_2$ = 617 (*1064-48-8*)

Schultz No. 299

Colour Index No. 20470

Dark-brown to black powder, sparingly soluble in water, soluble in ethanol (96 per cent).

Amido Black 10B Solution

A 5 g/L solution of *amido black 10B R* in a mixture of 10 volumes of *acetic acid R* and 90 volumes of *methanol R*.

4-Aminoantipyrine 4-Amino-1,5-dimethyl-2-phenyl-1,2-dihydro-3*H*-pyrazol-3-one; $C_{11}H_{13}N_3O$ = 203.2 (*83-07-8*)

Light yellow needles or powder, sparingly soluble in water, freely soluble in ethanol (96 per cent).

mp: about 108 °C.

4-Aminoantipyrine Solution

A 1 g/L solution of *4-aminoantipyrine R* in *buffer solution pH 9.0 R*.

Aminoazobenzene α-Aminoazobenzene; 4-Aminoazobenzene; $C_{12}H_{11}N_3$ = 197.2 (*60-09-3*)

Colour Index No. 11000

Brownish-yellow needles with a bluish tinge, slightly soluble in water, freely soluble in ethanol (96 per cent).

mp: about 128 °C.

3-Aminobenzoic Acid $C_7H_7NO_2$ = 137.1 (*99-05-8*)

White or almost white crystals. An aqueous solution turns brown on standing in air.

mp: about 174 °C.

Storage: in an airtight container, protected from light.

4-Aminobenzoic Acid $C_7H_7NO_2$ = 137.1 (*150-13-0*)

White or almost white, crystalline powder, slightly soluble in water, freely soluble in ethanol (96 per cent), practically insoluble in light petroleum.

mp: about 187 °C.

Chromatography. Thin-layer chromatography (*2.2.27*) as prescribed in the monograph *Procaine hydrochloride (0050)*; the chromatogram shows only one principal spot.

Storage: protected from light.

4-Aminobenzoic Acid Solution

Dissolve 1 g of *4-aminobenzoic acid R* in a mixture of 18 mL of *anhydrous acetic acid R*, 20 mL of *water R* and 1 mL of *phosphoric acid R*. Immediately before use, mix 2 volumes of the solution with 3 volumes of *acetone R*.

(4-Aminobenzoyl)-L-glutamic Acid *N*-(4-Aminobenzoyl)-L-glutamic acid; $C_{12}H_{14}N_2O_5$ = 266.3 (*4271-30-1*)

White or almost white, crystalline powder.

mp: about 175 °C, with decomposition.

2-Aminobutan-1-ol Aminobutanol; $C_4H_{11}NO$ = 89.1 (*5856-63-3*)

Oily liquid, miscible with water, soluble in ethanol (96 per cent).

d_{20}^{20}: about 0.94.

n_D^{20}: about 1.453.

bp: about 180 °C.

4-Amino-*N*-butyric Acid 4-Aminobutanoic acid; $C_4H_9NO_2$ = 103.1 (*56-12-2*)

Leaflets from methanol and ether, needles from water and ethanol (96 per cent). Freely soluble in water, practically insoluble or slightly soluble in other solvents.

mp: about 202 °C (decreases on rapid heating).

2-Amino-5-chlorobenzophenone Aminochlorobenzophenone; $C_{13}H_{10}ClNO$ = 231.7 (*719-59-5*)

Yellow, crystalline powder, practically insoluble in water, freely soluble in acetone, soluble in ethanol (96 per cent).

mp: about 97 °C.

Content: minimum 95.0 per cent.

Storage: protected from light.

6-Aminohexanoic Acid 6-Aminocaproic acid; $C_6H_{13}NO_2$ = 131.2 (*60-32-2*)

Colourless crystals, freely soluble in water, sparingly soluble in methanol, practically insoluble in anhydrous ethanol.

mp: about 205 °C.

***p*-Aminohippuric Acid** Aminohippuric acid; $C_9H_{10}N_2O_3$ = 194.2 (*61-78-9*)

White or almost white powder, sparingly soluble in water, soluble in ethanol (96 per cent).

mp: about 200 °C.

Aminohippuric Acid Reagent

Dissolve 3 g of *phthalic acid R* and 0.3 g of *aminohippuric acid R* in *ethanol (96 per cent) R* and dilute to 100 mL with the same solvent.

4-Amino-3-hydroxynaphthalene-1-sulfonic Acid Aminohydroxynaphthalenesulfonic acid; $C_{10}H_9NO_4S$ = 239.3 (*116-63-2*)

White or grey needles, turning pink on exposure to light, especially when moist, practically insoluble in water and in ethanol (96 per cent), soluble in solutions of alkali hydroxides and in hot solutions of sodium metabisulfite.

Storage: protected from light.

Aminohydroxynaphthalenesulfonic Acid Solution Aminohydroxynaphthalenesulphonic acid solution

Mix 5.0 g of *anhydrous sodium sulfite R* with 94.3 g of *sodium hydrogensulfite R* and 0.7 g of *aminohydroxynaphthalenesulfonic acid R*. Dissolve 1.5 g of the mixture in *water R* and dilute to 10.0 mL with the same solvent. Prepare the solution daily.

Aminohydroxynaphthalenesulfonic Acid Solution, Strong

Aminohydroxynaphthalenesulphonic acid solution, strong.

Dissolve 0.25 g of *4-amino-3-hydroxynaphthalene-1-sulfonic acid* in 75 mL of a 15% w/v solution of *sodium metabisulfite*, warming to assist solution if necessary. Add 2.5 mL of a 20% w/v solution of *sodium sulfite*, mix and add sufficient of the sodium metabisulfite solution to produce 100 mL.

5-Aminoimidazole-4-carboxamide Hydrochloride $C_4H_6N_4O,HCl$ = 162.6 (*72-40-2*)

mp: about 251°, with decomposition.

General reagent grade of commerce.

***cis*-Aminoindanol** (1*S*,2*R*)-1-Amino-2,3-dihydro-1*H*-inden-2-ol; (−)-*cis*-1-Aminoindan-2-ol; $C_9H_{11}NO$ = 149.2 (*126456-43-7*)

Content: minimum 98.0 per cent (sum of enantiomers, determined by gas chromatography).

$[\alpha]_D^{20}$: −69 to −59, determined on a 2 g/L solution in *chloroform R*.

mp: 118 °C to 122 °C.

3-Aminomethylalizarin-N,N-diacetic Acid Aminomethylalizarindiacetic acid; $C_{19}H_{15}NO_8,2H_2O$ = 421.4 (*3952-78-1*)

Fine, pale brownish-yellow or orange-brown powder, practically insoluble in water, soluble in solutions of alkali hydroxides.

mp: about 185 °C.

Loss on drying (*2.2.32*): maximum 10.0 per cent, determined on 1.000 g.

Aminomethylalizarindiacetic Acid Reagent

Solution A. Dissolve 0.36 g of *cerous nitrate R* in *water R* and dilute to 50 mL with the same solvent.

Solution B. Suspend 0.7 g of *aminomethylalizarindiacetic acid R* in 50 mL of *water R*. Dissolve with the aid of about 0.25 mL of *concentrated ammonia R*, add 0.25 mL of *glacial acetic acid R* and dilute to 100 mL with *water R*.

Solution C. Dissolve 6 g of *sodium acetate R* in 50 mL of *water R*, add 11.5 mL of *glacial acetic acid R* and dilute to 100 mL with *water R*.

To 33 mL of *acetone R* add 6.8 mL of solution C, 1.0 mL of solution B and 1.0 mL of solution A and dilute to 50 mL with *water R*.

Test for sensitivity. To 1.0 mL of *fluoride standard solution (10 ppm F) R* add 19.0 mL of *water R* and 5.0 mL of the aminomethylalizarindiacetic acid reagent. After 20 min, the solution assumes a blue colour.

Storage: use within 5 days.

Aminomethylalizarindiacetic Acid Solution

Dissolve 0.192 g of *aminomethylalizarindiacetic acid R* in 6 mL of freshly prepared *1 M sodium hydroxide*. Add 750 mL of *water R*, 25 mL of *succinate buffer solution pH 4.6 R* and, dropwise, *0.5 M hydrochloric acid* until the colour changes from violet-red to yellow (pH 4.5 to 5). Add 100 mL of *acetone R* and dilute to 1000 mL with *water R*.

4-Aminomethylbenzoic Acid $C_8H_9NO_2$ = 151.2 (*56-91-7*)

3-(Aminomethyl)pyridine (3-Pyridylmethyl)amine; 3-picolylamine; $C_6H_8N_2$ = 108.1 (*3731-52-0*)

General reagent grade of commerce.

8-Aminonaphthalene-2-sulfonic Acid 8-Amino-2-naphthalenesulfonic acid; 1-naphthylamine-7-sulfonic acid; 8-aminonaphthalene-2-sulfonic acid; $C_{10}H_9NO_3S$ = 223.2 (*119-28-8*)

General reagent grade of commerce.

Aminonaphthalenesulfonic Acid Solution

Aminonaphthalenesulphonic acid solution.

Mix 0.5 g of *8-aminonaphthalene-2-sulfonic acid*, 30 mL of *glacial acetic acid* and 120 mL of *water* and heat with stirring until dissolved. Allow to cool and filter.

Use the solution within 3 weeks.

2-Amino-5-nitrobenzophenone Aminonitrobenzophenone; $C_{13}H_{10}N_2O_3$ = 242.2 (*1775-95-7*)

Yellow, crystalline powder, practically insoluble in water, soluble in tetrahydrofuran, slightly soluble in methanol.

mp: about 160 °C.

$A_{1\,cm}^{1\%}$: 690 to 720, determined at 233 nm using a 0.01 g/L solution in *methanol R*.

6-Aminopenicillanic Acid (2*S*,5*R*,6*R*)-6-Amino-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid; $C_8H_{12}N_2O_3S$ = 216.3 (*551-16-6*)

Appearance: white or almost white powder.

mp: about 205 °C, with decomposition.

Aminophenazone 4-(Dimethylamino)-1,5-dimethyl-2-phenyl-1,2-dihydro-3*H*-pyrazol-3-one; $C_{13}H_{17}N_3O$ = 231.3 (*58-15-1*)

White or almost white, crystalline powder or colourless crystals, soluble in water, freely soluble in ethanol (96 per cent).

mp: about 108 °C.

2-Aminophenol C_6H_7NO = 109.1 (*95-55-6*)

Pale yellowish-brown crystals which rapidly become brown, sparingly soluble in water, soluble in ethanol (96 per cent).

mp: about 172 °C.

Storage: in an airtight container, protected from light.

3-Aminophenol C_6H_7NO = 109.1 (*591-27-5*)

Pale yellowish-brown crystals, sparingly soluble in water.

mp: about 122 °C.

4-Aminophenol C_6H_7NO = 109.1 (*123-30-8*)

Content: minimum 95 per cent.

White or slightly coloured, crystalline powder, becoming coloured on exposure to air and light, sparingly soluble in water, soluble in anhydrous ethanol.

mp: about 186 °C, with decomposition.

Storage: protected from light.

Aminopolyether 4,7,13,16,21,24-hexaoxa-1,10-diazabicyclo[8,8,8]hexacosane; $C_{18}H_{36}N_2O_6$ = 376.5 (*23978-09-8*)

mp: 70 °C to 73 °C.

4-(4-Aminophenoxy)-N-methylpicolinamide 4-(4-Aminophenoxy)-*N*-methylpyridine-2-carboxamide; $C_{13}H_{13}N_3O_2$ = 243.3 (*284462-37-9*)

Content: minimum 99.0 per cent.

Light brown powder.

mp: 110 °C to 112 °C.

3-Aminopropionic Acid 3-Amino-1-propanol; $C_3H_7NO_2$ = 89.1 (*107-95-9*)

Content: minimum 99 per cent.

White or almost white, crystalline powder, freely soluble in water, slightly soluble in ethanol (96 per cent), practically insoluble in acetone.

mp: about 200 °C, with decomposition.

Aminopterine 4-Aminofolic acid; $C_{19}H_{20}N_8O_5$ = 440.4 (*54-62-6*)

Yellowish powder.

mp: about 230 °C.

Aminopyrazolone 4-Aminophenazone; (*83-07-8*)

See *4-aminoantipyrine R*.

Aminopyrazolone Solution See *4-aminoantipyrine solution R*.

3-Aminosalicylic Acid 3-Amino-2-hydroxybenzoic acid; $C_7H_7NO_3$ = 153.1 (*570-23-0*)

mp: about 240 °C.

Slightly soluble in water.

4-Aminosalicylic Acid 4-Amino-2-hydroxybenzoic acid; $C_7H_7NO_3$ = 153.1 (*65-49-6*)

White or almost white, bulky powder, slightly soluble in water, soluble in ethanol (96 per cent), in dilute nitric acid and in sodium hydroxide. It darkens on exposure to air and light.

mp: 135 °C to 145 °C.

Storage: at a temperature not exceeding 30 °C, in an airtight container, protected from light.

Ammonia

Content: 170 g/L to 180 g/L of NH_3 (M_r 17.03).

Dilute 67 g of *concentrated ammonia R* to 100 mL with *water R*.

d_{20}^{20}: 0.931 to 0.934.

When used in the test for iron, *ammonia R* complies with the following additional requirement. Evaporate 5 mL of ammonia to dryness on a water-bath, add 10 mL of *water R*, 2 mL of a 200 g/L solution of *citric acid monohydrate R* and 0.1 mL of *thioglycollic acid R*. Make alkaline by adding *ammonia R* and dilute to 20 mL with *water R*. No pink colour develops.

Storage: protected from atmospheric carbon dioxide, at a temperature below 20 °C.

For 18M and 13.5M ammonia use analytical reagent grade solutions of commerce containing 35% and 25% w/w of NH_3 and weighing 0.88 g and 0.91 g per mL, respectively.

Solutions of molarity *x*M should be prepared by diluting 75*x* mL of 13.5M ammonia or 56*x* mL of 18M ammonia to 1000 mL with *water*.

Ammonia, Chloride-free

13.5M *ammonia* that complies with the following test.

Chloride Evaporate 54 mL on a water bath almost to dryness and dilute to 15 mL with *water*. The solution complies with the *limit test for chlorides*, Appendix VII (1 ppm).

Ammonia, Concentrated See *Concentrated ammonia solution (0877)*.

Ammonia, Lead-free

Complies with the requirements prescribed for *dilute ammonia R1* with the following additional test: to 20 mL of lead-free ammonia, add 1 mL of *lead-free potassium cyanide solution R*, dilute to 50 mL with *water R* and add 0.10 mL of *sodium sulfide solution R*. The solution is not more intensely coloured than a reference solution prepared without sodium sulfide.

Ammonia, Methanolic

Solutions of the requisite molarity may be obtained by diluting 13.5M *ammonia* or 18M *ammonia* with *methanol* as directed under *ammonia*.

Ammonia R1, Concentrated

Content: minimum 30.0 per cent *m/m* of NH_3 (M_r 17.03).

A clear, colourless liquid.

d_{20}^{20}: less than 0.892.

Assay. Weigh accurately a ground-glass-stoppered flask containing 50.0 mL of *1 M hydrochloric acid*. Introduce 2 mL of *concentrated ammonia R1* and weigh again. Titrate the solution with *1 M sodium hydroxide*, using 0.5 mL of *methyl red mixed solution R* as indicator.

1 mL of *1 M hydrochloric acid* is equivalent to 17.03 mg of NH_3.

Storage: protected from atmospheric carbon dioxide, at a temperature below 20 °C.

Ammonia R1, Dilute

Content: 100 g/L to 104 g/L of NH_3 (M_r 17.03).

Dilute 41 g of *concentrated ammonia R* to 100 mL with *water R*.

Ammonia R2, Dilute

Content: 33 g/L to 35 g/L of NH_3 (M_r 17.03).

Dilute 14 g of *concentrated ammonia R* to 100 mL with *water R*.

Ammonia R3, Dilute

Content: 1.6 g/L to 1.8 g/L of NH_3 (M_r 17.03).

Dilute 0.7 g of *concentrated ammonia R* to 100 mL with *water R*.

Ammonia R4, Dilute

Content: 8.4 g/L to 8.6 g/L of NH_3 (M_r 17.03).

Dilute 3.5 g of *concentrated ammonia R* to 100 mL with *water R*.

Ammonium Acetate $C_2H_7NO_2$ = 77.1 (*631-61-8*)

Colourless crystals, very deliquescent, very soluble in water and in ethanol (96 per cent).

Storage: in an airtight container.

Ammonium Acetate Solution

Dissolve 150 g of *ammonium acetate R* in *water R*. Add 3 mL of *glacial acetic acid R* and dilute to 1000 mL with *water R*.

Storage: use within 1 week.

(1*R*)-(–)-Ammonium 10-Camphorsulfonate 10-Camphorsulfonic acid; 10-camphorsulphonic acid; Ammonium salt; (1R)-(–)-Ammonium 10-Camphorsulfonate; $C_{10}H_{19}NO_4S$ = 249.3

Content: minimum 97.0 per cent of (1*R*)-(–)-ammonium 10-camphorsulfonate.

$[\alpha]_D^{20}$: −18 ± 2, determined on a 50 g/L solution.

Ammonium Carbamate Carbamic acid ammonium salt; $CH_6N_2O_2$ = 78.1 (*1111-78-0*)

Ammonium Carbonate A mixture of varying proportions of ammonium hydrogen carbonate (NH_4HCO_3, M_r 79.1) and ammonium carbamate (NH_2COONH_4, M_r 78.1); (*506-87-6*)

White or almost white translucent mass, slowly soluble in about 4 parts of water. It is decomposed by boiling water. Ammonium carbonate liberates not less than 30 per cent *m/m* of NH_3 (M_r 17.03).

Assay. Dissolve 2.00 g in 25 mL of *water R*. Slowly add 50.0 mL of *1 M hydrochloric acid*, titrate with *1 M sodium hydroxide*, using 0.1 mL of *methyl orange solution R* as indicator.

1 mL of *1 M hydrochloric acid* is equivalent to 17.03 mg of NH_3.

Storage: at a temperature below 20 °C.

Ammonium Carbonate Solution

A 158 g/L solution of *ammonium carbonate R*.

Ammonium Carbonate Solution R1

Dissolve 20 g of *ammonium carbonate R* in 20 mL of *dilute ammonia R1* and dilute to 100 mL with *water R*.

Ammonium Carbonate Solution, Dilute

Dissolve 5 g of *ammonium carbonate* in a mixture of 7.5 mL of 5M *ammonia* and 50 mL of *water*, dilute to 100 mL with *water* and filter, if necessary.

Ammonium Cerium(IV) Nitrate Ammonium and cerium nitrate; $(NH_4)_2Ce(NO_3)_6$ = 548.2 (*16774-21-3*)

Orange-yellow, crystalline powder, or orange transparent crystals, soluble in water.

Ammonium Cerium(IV) Sulfate Ammonium and cerium sulfate; $(NH_4)_4Ce(SO_4)_4,2H_2O$ = 633 (*10378-47-9*)

Orange-yellow, crystalline powder or crystals, slowly soluble in water.

Ammonium Chloride (*12125-02-9*)

See *Ammonium chloride (0007)*.

Ammonium Chloride Solution

A 107 g/L solution of *ammonium chloride R*.

Ammonium Citrate Diammonium hydrogen citrate; $C_6H_{14}N_2O_7$ = 226.2 (*3012-65-5*)

White or almost white, crystalline powder or colourless crystals, freely soluble in water, slightly soluble in ethanol (96 per cent).

pH (*2.2.3*): about 4.3 for a 22.6 g/L solution.

Ammonium Citrate Solution

Dissolve, with cooling, 500 g of *citric acid* in a mixture of 200 mL of *water* and 200 mL of 13.5M *ammonia*. Filter and dilute to 1000 mL with *water*.

Ammonium Cobaltothiocyanate Solution

Dissolve 37.5 g of *cobalt(II) nitrate* and 150 g of *ammonium thiocyanate* in sufficient *water* to produce 1000 mL.

Use within 1 day of preparation.

Ammonium Dihydrogen Orthophosphate Ammonium dihydrogen phosphate; $(NH_4)H_2PO_4$ = 115.0 (*7722-76-1*)

White or almost white, crystalline powder or colourless crystals, freely soluble in water.

pH (*2.2.3*): about 4.2 for a 23 g/L solution.

Ammonium Formate CH_5NO_2 = 63.1 (*540-69-2*)

Deliquescent crystals or granules, very soluble in water, soluble in ethanol (96 per cent).

mp: 119 °C to 121 °C.

Storage: in an airtight container.

Ammonium Hexafluorogermanate(IV) $(NH_4)_2GeF_6$ = 222.7 (*16962-47-3*)

White or almost white crystals, freely soluble in water.

Ammonium Hydrogen Carbonate Ammonium bicarbonate; NH_4HCO_3 = 79.1 (*1066-33-7*)

Content: minimum 99 per cent.

Ammonium Iron(III) Citrate Ferric ammonium citrate; (*1185-57-5*)

General reagent grade (brown) of commerce.

Ammonium Iron(II) Sulfate Ferrous ammonium sulfate; $Fe(NH_4)_2(SO_4)_2,6H_2O$ = 392.2 (*7783-85-9*)

Pale bluish-green crystals or granules, freely soluble in water, practically insoluble in ethanol (96 per cent).

Storage: protected from light.

Ammonium Iron(III) Sulfate Ferric ammonium sulfate; $FeNH_4(SO_4)_2,12H_2O$ = 482.2 (*7783-83-7*)

Pale-violet crystals, efflorescent, very soluble in water, practically insoluble in ethanol (96 per cent).

Ammonium Iron(III) Sulfate Solution R1

Ammonium iron(III) sulphate solution R1

Dissolve 0.2 g of *ammonium iron(III) sulfate* in 50 mL of *water*, add 5 mL of *nitric acid* and dilute to 100 mL with *water*.

Ammonium Iron(III) Sulfate Solution R2 Ferric ammonium sulfate solution R2

A 100 g/L solution of *ferric ammonium sulfate R*. If necessary filter before use.

Ammonium Iron(III) Sulfate Solution R5 Ferric ammonium sulfate solution R5

Shake 30.0 g of *ferric ammonium sulfate R* with 40 mL of *nitric acid R* and dilute to 100 mL with *water R*. If the solution is turbid, centrifuge or filter it.

Storage: protected from light.

Ammonium Iron(III) Sulfate Solution R6 Ferric ammonium sulfate solution R6

Dissolve 20 g of *ferric ammonium sulfate R* in 75 mL of *water R*, add 10 mL of a 2.8 per cent *V/V* solution of *sulfuric acid R* and dilute to 100 mL with *water R*.

Ammonium Mercaptoacetate Solution

Add 300 mL of *water* to 50 mL of *mercaptoacetic acid*, neutralise with about 40 mL of 13.5M *ammonia* and dilute to 500 mL with *water*.

Ammonium Mercurithiocyanate Reagent

Dissolve 30 g of *ammonium thiocyanate* and 27 g of *mercury(II) chloride* in sufficient *water* to produce 1000 mL.

Ammonium Metavanadate Ammonium vanadate; NH_4VO_3 = 117.0 (*7803-55-6*)

White or slightly yellowish, crystalline powder, slightly soluble in water, soluble in *dilute ammonia R1*.

Ammonium Metavanadate Solution

Ammonium vanadate solution

Dissolve 1.2 g of *ammonium vanadate R* in 95 mL of *water R* and dilute to 100 mL with *sulfuric acid R*.

Ammonium Molybdate $(NH_4)_6Mo_7O_{24},4H_2O$ = 1236 (*12054-85-2*)

Colourless or slightly yellow or greenish crystals, soluble in water, practically insoluble in ethanol (96 per cent).

Ammonium Molybdate Reagent

Mix, in the given order, 1 volume of a 25 g/L solution of *ammonium molybdate R*, 1 volume of a 100 g/L solution of *ascorbic acid R* and 1 volume of *sulfuric acid R* (294.5 g/L H_2SO_4). Add 2 volumes of *water R*.

Storage: use within 1 day.

Ammonium Molybdate Reagent R1

Mix 10 mL of a 60 g/L solution of *disodium arsenate R*, 50 mL of *ammonium molybdate solution R*, 90 mL of *dilute sulfuric acid R* and dilute to 200 mL in *water R*.

Storage: in amber flasks at 37 °C for 24 h.

Ammonium Molybdate Reagent R2

Dissolve 50 g of *ammonium molybdate R* in 600 mL of *water R*. To 250 mL of cold *water R* add 150 mL of *sulfuric acid R* and cool. Mix the 2 solutions together. *Storage*: use within 1 day.

Ammonium Molybdate Solution

A 100 g/L solution of *ammonium molybdate R*.

Ammonium Molybdate Solution R2

Dissolve 5.0 g of *ammonium molybdate R* with heating in 30 mL of *water R*. Cool, adjust the pH to 7.0 with *dilute ammonia R2* and dilute to 50 mL with *water R*.

Ammonium Molybdate Solution R3

Solution A. Dissolve 5 g of *ammonium molybdate R* in 20 mL of *water R* with heating.

Solution B. Mix 150 mL of *ethanol (96 per cent) R* with 150 mL of *water R*. Add with cooling 100 mL of *sulfuric acid R*.

Immediately before use add 80 volumes of solution B to 20 volumes of solution A.

Ammonium Molybdate Solution R4

Dissolve 1.0 g of *ammonium molybdate R* in *water R* and dilute to 40 mL with the same solvent. Add 3 mL of *hydrochloric acid R* and 5 mL of *perchloric acid R* and dilute to 100 mL with *acetone R*.

Storage: protected from light; use within 1 month.

Ammonium Molybdate Solution R5

Dissolve 1.0 g of *ammonium molybdate R* in 40.0 mL of a 15 per cent *V/V* solution of *sulfuric acid R*. Prepare the solution daily.

Ammonium Molybdate Solution R6

Slowly add 10 mL of *sulfuric acid R* to about 40 mL of *water R*. Mix and allow to cool. Dilute to 100 mL with *water R* and mix. Add 2.5 g of *ammonium molybdate R* and 1 g of *cerium sulfate R*, and shake for 15 min to dissolve.

Ammonium Molybdate-Sulfuric Acid Solution

Dissolve 10 g of *ammonium molybdate* in sufficient *water* to produce 100 mL and add the solution slowly to 250 mL of cold 10M *sulfuric acid*.

Store in a plastic bottle protected from light.

Ammonium Nitrate NH_4NO_3 = 80.0 (*6484-52-2*)

White or almost white, crystalline powder or colourless crystals, hygroscopic, very soluble in water, freely soluble in methanol, soluble in ethanol (96 per cent).

Storage: in an airtight container.

Ammonium Nitrate R1

Complies with the requirements prescribed for *ammonium nitrate R* with the following additional requirements.

Acidity. The solution of the substance is slightly acid (*2.2.4*).

Chlorides (*2.4.4*): maximum 100 ppm, determined on 0.50 g.

Sulfates (*2.4.13*): maximum 150 ppm, determined on 1.0 g.

Sulfated ash (*2.4.14*): maximum 0.05 per cent, determined on 1.0 g.

Ammonium Oxalate $C_2H_8N_2O_4,H_2O$ = 142.1 (*6009-70-7*)

Colourless crystals, soluble in water.

Ammonium Oxalate Solution

A 40 g/L solution of *ammonium oxalate R*.

Ammonium Persulfate Ammonium Peroxodisulfate; Ammonium Peroxodisulphate; Ammonium Persulphate; $(NH_4)_2S_2O_8$ = 228.2 (*7727-54-0*)

White or almost white, crystalline powder or granular crystals, freely soluble in water.

Ammonium Polysulfide Solution Ammonium polysulphide solution

Dissolve a sufficient quantity of *precipitated sulfur* to produce a deep orange solution in a solution prepared in the following manner. Immediately before use saturate 120 mL of 6M *ammonia* with *hydrogen sulfide* and add 80 mL of 6M *ammonia*.

Ammonium Pyrrolidinedithiocarbamate Ammonium tetramethylenedithiocarbamate; $C_5H_{12}N_2S_2$ = 164.3 (*5108-96-3*)

White or pale yellow, crystalline powder, sparingly soluble in water, very slightly soluble in ethanol (96 per cent).

Storage: in a bottle containing a piece of ammonium carbonate in a muslin bag.

Ammonium Pyrrolidinedithiocarbamate Solution

A 1.0% w/v solution of *ammonium pyrrolidinedithiocarbamate* that has been washed immediately before use with three 25-mL quantities of *4-methylpentan-2-one*.

Ammonium Reineckate Ammonium tetrathiocyanatodiamminochromate(III) monohydrate; $NH_4[Cr(NCS)_4(NH_3)_2],H_2O$ = 354.4 (*13573-16-5*)

Red powder or crystals, sparingly soluble in cold water, soluble in hot water and in ethanol (96 per cent).

Ammonium Reineckate Solution

A 10 g/L solution of *ammonium reineckate R*. Prepare immediately before use.

Ammonium Sulfamate Ammonium sulphamate; $NH_2SO_3NH_4$ = 114.1 (*7773-06-0*)

White or almost white, crystalline powder or colourless crystals, hygroscopic, very soluble in water, slightly soluble in ethanol (96 per cent).

mp: about 130 °C.

Storage: in an airtight container.

Ammonium Sulfate Ammonium sulphate; $(NH_4)_2SO_4$ = 132.1 (*7783-20-2*)

Colourless crystals or white or almost white granules, very soluble in water, practically insoluble in acetone and in ethanol (96 per cent).

pH (*2.2.3*): 4.5 to 6.0 for a 50 g/L solution in *carbon dioxide-free water R*.

Sulfated ash (*2.4.14*): maximum 0.1 per cent.

Ammonium Sulfide Solution Ammonium sulphide solution

Saturate 120 mL of *dilute ammonia R1* with *hydrogen sulfide R* and add 80 mL of *dilute ammonia R1*. Prepare immediately before use.

Ammonium Thiocyanate NH_4SCN = 76.1 (*1762-95-4*)

Colourless crystals, deliquescent, very soluble in water, soluble in ethanol (96 per cent).

Storage: in an airtight container.

Ammonium Thiocyanate Solution

A 76 g/L solution of *ammonium thiocyanate R*.

Amoxicillin Trihydrate See *Amoxicillin trihydrate (0260)*.

Amyl Acetate $C_7H_{14}O_2$ = 130.2

Consists principally of 3-methylbutyl acetate with a small proportion of 2-methylbutyl acetate.

bp: about 140°.

Analytical reagent grade of commerce.

A colourless liquid; weight per mL, about 0.87 g.

Amyl Alcohol Isoamyl alcohol; $C_5H_{12}O$ = 88.1 (*123-51-3*)

Colourless liquid, slightly soluble in water, miscible with ethanol (96 per cent).

bp: about 130 °C.

α-Amylase 1,4-α-D-glucane-glucanohydrolase (EC 3.2.1.1)

White or light brown powder.

α-Amylase Solution

A solution of *α-amylase R* with an activity of 800 FAU/g.

β-Amyrin Olean-12-en-3β-ol; $C_{30}H_{50}O$ = 426.7 (*559-70-6*)

White or almost white powder.

mp: 187 °C to 190 °C.

L-Analyl-L-proline $C_8H_{14}N_2O_3$ = 186.2 (*13485-59-1*)

General reagent grade of commerce.

Andrographolide (3*E*,4*S*)-3-[2-[(1*R*,4a*S*,5*R*,6*R*,8a*S*)-6-Hydroxy-5-(hydroxymethyl)-5,8a-dimethyl-2-methylenedecahydronaphthalen-1-yl]ethylidene]-4-hydroxydihydrofuran-2(3*H*)-one; $C_{20}H_{30}O_5$ = 350.4 (*5508-58-7*)

Anethole 1-Methoxy-4-(propen-1-yl)benzene; $C_{10}H_{12}O$ = 148.2 (*4180-23-8*)

White or almost white, crystalline mass up to 20 °C to 21 °C, liquid above 23 °C, practically insoluble in water, freely soluble in anhydrous ethanol, soluble in ethyl acetate and in light petroleum.

n_D^{25}: about 1.56.

bp: about 230 °C.

Anethole used in gas chromatography complies with the following additional test.

Assay. Gas chromatography (*2.2.28*) as prescribed in the monograph *Anise oil (0804)*.

Test solution. The substance to be examined.

Content: minimum 99.0 per cent of *trans*-anethole (retention time: about 41 min), calculated by the normalisation procedure.

cis-Anethole (*Z*)-1-Methoxy-4-prop-1-enylbenzene; $C_{10}H_{12}O$ = 148.2

n_D^{25}: about 1.56.

bp: about 230°.

General reagent grade of commerce.

A white crystalline mass at 20°; liquid above 23°.

Cis-anethole used in gas chromatography complies with the following test.

Assay Examine by *gas chromatography*, Appendix III B, under the conditions described in the test for Chromatographic profile in the monograph for Anise Oil using the reagent being examined as solution (1).

The area of the principal peak is not less than 92.0% of the total area of the peaks.

Anhydrous Colloidal Silica (*7631-86-9*)

See *Anhydrous colloidal silica (0434)*.

Anhydrous Trisodium Orthophosphate Tribasic Sodium Phosphate; Na_3PO_4 = 163.94 (*7601-54-9*)

mp: about 75°

General reagent grade of commerce.

Aniline Benzeneamine; C_6H_7N = 93.1 (*62-53-3*)

Colourless or slightly yellowish liquid, soluble in water, miscible with ethanol (96 per cent).

d_{20}^{20}: about 1.02.

bp: 183 °C to 186 °C.

Storage: protected from light.

Aniline Hydrochloride Benzenamine hydrochloride; C_6H_8ClN = 129.6 (*142-04-1*)

Crystals. It darkens on exposure to air and light.

mp: about 198 °C.

Storage: protected from light.

Content: minimum 97.0 per cent.

Aniline Hydrochloride Solution

Dissolve 2 g of *aniline hydrochloride* in a mixture of 65 mL of *ethanol (96%)* and 35 mL of *water* and add 2 mL of *hydrochloric acid*.

Use within one day of preparation.

Anion Exchange Resin

Resin in chlorinated form with a quaternary ammonium funtionalised latex cross-linked with divinylbenzene.

Wash the resin with *1 M sodium hydroxide* on a sintered-glass filter (40) (*2.1.2*) until the washings are free from chloride, then wash with *water R* until the washings are neutral. Suspend in freshly prepared *ammonium-free water R* and protect from atmospheric carbon dioxide.

Anion Exchange Resin, Strongly Basic

Gel-type resin in hydroxide form containing quaternary ammonium groups [$CH_2N^+(CH_3)_3$, type 1] attached to a polymer lattice consisting of polystyrene cross-linked with 8 per cent of divinylbenzene.

Brown transparent beads.

Particle size: 0.2 mm to 1.0 mm.

Moisture content: about 50 per cent.

Total exchange capacity: minimum 1.2 meq/mL.

Anion Exchange Resin for Chromatography, Strongly Basic

Resin with a quaternary ammonium functionalised latex cross-linked with divinylbenzene.

Anion-exchange Resin for Chromatography, Strongly Basic R1

Non-porous resin agglomerated with a 100 nm alkyl quaternary ammonium functionalised latex.

Anion-exchange Resin for Chromatography, Strongly Basic R2

Resin agglomerated with a quaternary ammonium functionalised latex cross-linked with ethylvinylbenzene-divinylbenzene.

Anion Exchange Resin R1

Resin containing quaternary ammonium groups [$CH_2N^+(CH_3)_3$] attached to a lattice consisting of methacrylate.

Anion Exchange Resin R2

Conjugate of homogeneous 10 µm hydrophilic polyether particles, and a quaternary ammonium salt, providing a matrix suitable for strong anion-exchange chromatography of proteins.

Anion Exchange Resin R3

Resin with quaternary ammonium groups attached to a lattice of ethylvinylbenzene crosslinked with 55 per cent of divinylbenzene.

Anion Exchange Resin, Weak

Resin with diethylaminoethyl groups attached to a lattice consisting of poly(methyl methacrylate).

Anisaldehyde 4-Methoxybenzaldehyde; $C_8H_8O_2$ = 136.1 (*123-11-5*)

Oily liquid, very slightly soluble in water, miscible with ethanol (96 per cent).

bp: about 248 °C.

Anisaldehyde used in gas chromatography complies with the following additional test.

Assay. Gas chromatography (*2.2.28*) as prescribed in the monograph *Anise oil (0804)*.

Test solution. The substance to be examined.

Content: minimum 99.0 per cent, calculated by the normalisation procedure.

Anisaldehyde Solution

Mix in the following order, 0.5 mL of *anisaldehyde R*, 10 mL of *glacial acetic acid R*, 85 mL of *methanol R* and 5 mL of *sulfuric acid R*.

Anisaldehyde Solution R1

To 10 mL of *anisaldehyde R* add 90 mL of *ethanol (96 per cent) R*, mix, add 10 mL of *sulfuric acid R* and mix again.

Anisaldehyde Solution R2

To 170 mL of cold *methanol R* add 20 mL of *glacial acetic acid R* and 10 mL of *sulfuric acid R*. Mix well. Cool to room temperature and add 1.0 mL of *anisaldehyde R*.

Anise Ketone 1-(4-Methoxyphenyl)propan-2-one; $C_{10}H_{12}O_2$ = 164.2 (*122-84-9*)

***p*-Anisidine** 4-Methoxyaniline; C_7H_9NO = 123.2 (*104-94-9*)

White or almost white crystals, sparingly soluble in water, soluble in anhydrous ethanol.

Content: minimum 97.0 per cent.

Caution: skin irritant, sensitiser.

Storage: protected from light, at 0 °C to 4 °C.

On storage, *p*-anisidine tends to darken as a result of oxidation. A discoloured reagent can be reduced and decolorised in the following way: dissolve 20 g of *p-anisidine R* in 500 mL of *water R* at 75 °C. Add 1 g of *sodium sulfite heptahydrate R* and 10 g of *activated charcoal R* and stir for 5 min. Filter, cool the filtrate to about 0 °C and allow to stand at this temperature for at least 4 h. Filter, wash the crystals with a small quantity of *water R* at about 0 °C and dry the crystals *in vacuo* (*2.2.32*).

Anolyte for Isoelectric Focusing pH 3 to 5

0.1M glutamic acid, 0.5M phosphoric acid. Dissolve 14.71 g of *glutamic acid* in *water*, add 33 mL of *orthophosphoric acid* and dilute to 1000 mL with *water*.

Anthracene $C_{14}H_{10}$ = 178.2 (*120-12-7*)

White or almost white, crystalline powder, practically insoluble in water, slightly soluble in chloroform.

mp: about 218 °C.

Anthranilic Acid 2-Aminobenzoic acid; $C_7H_7NO_2$ = 137.1 (*118-92-3*)

A white or pale-yellow, crystalline powder, sparingly soluble in cold water, freely soluble in hot water, in ethanol (96 per cent) and in glycerol. Solutions in ethanol (96 per cent) or in ether and, particularly, in glycerol show a violet fluorescence.

mp: about 145 °C.

Anthrone 9(10*H*)-Anthracenone; $C_{14}H_{10}O$ = 194.2 (*90-44-8*)

Pale yellow, crystalline powder.

mp: about 155 °C. When used in an assay for glucose or dextrans, complies with the following test.

Sensitivity to glucose Add, carefully, 6 mL of a 0.2% w/v solution in a mixture of 19 mL of sulfuric acid and 1 mL of water to 3 mL of a solution containing 5 µg of D-glucose per mL and heat in a water bath for 5 minutes. The solution is a darker green than a solution prepared in the same manner but omitting the glucose.

Anthrone Reagent

A 0.2% w/v solution of *anthrone* in *nitrogen-free sulfuric acid*. The solution should be allowed to stand for 4 hours before use and should be discarded after 7 days.

Antimony Potassium Tartrate Antimony potassium oxide (+)-tartrate; $C_8H_4K_2O_{12}Sb_2,3H_2O$ = 668 (*28300-74-5*)

White or almost white, granular powder or colourless, transparent crystals, soluble in water and in glycerol, freely soluble in boiling water, practically insoluble in ethanol (96 per cent). The aqueous solution is slightly acid.

Antimony Trichloride $SbCl_3$ = 228.1 (*10025-91-9*)

Colourless crystals or a transparent crystalline mass, hygroscopic, freely soluble in anhydrous ethanol. Antimony trichloride is hydrolysed by water.

Storage: in an airtight container, protected from moisture.

Antimony Trichloride in Dichloroethane Solution

Prepare a 22.0% w/v solution of *antimony trichloride* in dry *1,2-dichloroethane* that has been purified by passing it down a column of *silica gel* and allow the solution to stand for 24 hours. To 100 mL add 2.5 mL of *acetyl chloride* and allow to stand for a further 24 hours before use.

Antimony Trichloride Solution

Rapidly wash 30 g of *antimony trichloride R* with two quantities, each of 15 mL, of *ethanol-free chloroform R*, drain off the washings, and dissolve the washed crystals immediately in 100 mL of *ethanol-free chloroform R*, warming slightly.

Storage: over a few grams of *anhydrous sodium sulfate R*.

Antithrombin III ATIII; (*90170-80-2*)

Antithrombin III is purified from human plasma by heparin agarose chromatography and should have a specific activity of at least 6 IU/mg.

Antithrombin III Solution R1

Reconstitute *antithrombin III R* as directed by the manufacturer and dilute with *tris(hydroxymethyl)aminomethane sodium chloride buffer solution pH 7.4 R* to 1 IU/mL.

Antithrombin III Solution R2

Reconstitute *antithrombin III R* as directed by the manufacturer and dilute with *tris(hydroxymethyl)aminomethane sodium chloride buffer solution pH 7.4 R* to 0.5 IU/mL.

Antithrombin III Solution R3

Reconstitute *antithrombin III R* as directed by the manufacturer and dilute to 0.3 IU/mL with *phosphate buffer solution pH 6.5 R*.

Antithrombin III Solution R4

Reconstitute *antithrombin III R* as directed by the manufacturer and dilute to 0.1 IU/mL with *tris(hydroxymethyl)aminomethane-EDTA buffer solution pH 8.4 R*.

Antithrombin III Solution R5

Reconstitute *antithrombin III R* as directed by the manufacturer and dilute to 0.125 IU/mL with

tris(hydroxymethyl)aminomethane-EDTA buffer solution pH 8.4 R1.

Antithrombin III Solution R6

Reconstitute *antithrombin III R* as directed by the manufacturer and dilute to 1.0 IU/mL with *tris(hydroxymethyl)aminomethane-EDTA buffer solution pH 8.4 R1.*

Apigenin 4′,5,7-Trihydroxyflavone; $C_{15}H_{10}O_5$ = 270.2 (*520-36-5*)

Light yellowish powder, practically insoluble in water, sparingly soluble in ethanol (96 per cent).

mp: about 310 °C, with decomposition.

Chromatography. Thin-layer chromatography (*2.2.27*) as prescribed in the monograph *Roman chamomile flower (0380)*: apply 10 µL of a 0.25 g/L solution in *methanol R*; the chromatogram shows in the upper third a principal zone of yellowish-green fluorescence.

Apigenin-7-glucoside Apigetrin; 7-(β-D-Glucopyranosyloxy)-5-hydroxy-2-(4-hydroxyphenyl)-4*H*-1-benzopyran-4-one; $C_{21}H_{20}O_{10}$ = 432.4 (*578-74-5*)

Light yellowish powder, practically insoluble in water, sparingly soluble in ethanol (96 per cent).

mp: 198 °C to 201 °C.

Chromatography. Thin-layer chromatography (*2.2.27*) as prescribed in the monograph *Roman chamomile flower (0380)*: apply 10 µL of a 0.25 g/L solution in *methanol R*; the chromatogram shows in the middle third a principal zone of yellowish fluorescence.

Apigenin-7-glucoside used in liquid chromatography complies with the following additional test.

Assay. Liquid chromatography (*2.2.29*) as prescribed in the monograph *Matricaria flower (0404)*.

Test solution. Dissolve 10.0 mg in *methanol R* and dilute to 100.0 mL with the same solvent.

Content: minimum 95.0 per cent, calculated by the normalisation procedure.

Apiole Apiol; apioline; parsley apiole; 5-allyl-4,7-dimethoxy-1,3-benzodioxol; $C_{12}H_{14}O_4$ = 222.24 (*484-31-1*)

General reagent grade of commerce.

Aprotinin (*9087-70-1*)

See *Aprotinin (0580)*.

L-Arabinose Arabinose; $C_5H_{10}O_5$ = 150.1 (*87-72-9*)

White or almost white, crystalline powder, freely soluble in water.

$[\alpha]_D^{20}$: + 103 to + 105, determined on a 50 g/L solution in *water R* containing about 0.05 per cent of NH_3.

Arachidic Alcohol Eicosan-1-ol; Arachidyl alcohol; Arachidyl alcohol; $C_{20}H_{42}O$ = 298.5 (*629-96-9*)

mp: about 65 °C.

Content: minimum 96 per cent of $C_{20}H_{42}O$.

Arachis Oil

Of the British Pharmacopoeia.

Arbutin Arbutoside; 4-Hydroxyphenyl-β-D-glucopyranoside; $C_{12}H_{16}O_7$ = 272.3 (*497-76-7*)

Fine, white or almost white, shiny needles, freely soluble in water, very soluble in hot water, soluble in ethanol (96 per cent).

Chromatography. Thin-layer chromatography (*2.2.27*) as prescribed in the monograph *Bearberry leaf (1054)*; the chromatogram shows only one principal spot.

Arginine L-Arginine; (*74-79-3*)

See *Arginine (0806)*.

Argon Ar = 39.95 (*7440-37-1*)

Content: minimum 99.995 per cent *V/V*.

Carbon monoxide (*2.5.25, Method I*): maximum 0.6 ppm *V/V*; after passage of 10 L of *argon R* at a flow rate of 4 L/h, not more than 0.05 mL of *0.002 M sodium thiosulfate* is required for the titration.

Argon R1 Ar = 39.95 (*7440-37-1*)

Content: minimum 99.99990 per cent *V/V*.

Argon for Chromatography Ar = 39.95 (*7440-37-1*)

Content: minimum 99.95 per cent *V/V*.

Aromadendrene (1*R*,2*S*,4*R*,8*R*,11*R*)-3,3,11-Trimethyl-7-methylenetricyclo- [6.3.0.$0^{2,4}$]undecane; $C_{15}H_{24}$ = 204.4 (*489-39-4*)

Clear, almost colourless liquid.

d_4^{20}: about 0.911.

n_D^{20}: about 1.497.

$[\alpha]_D^{20}$: about + 12.

bp: about 263 °C.

Aromadendrene used in gas chromatography complies with the following additional test.

Assay. Gas chromatography (*2.2.28*) as prescribed in the monograph on *Tea tree oil (1837)*.

Content: minimum 92 per cent, calculated by the normalisation procedure.

Arsenazo III 3,6-Bis[(2-arsonophenyl)diazenyl]-4,5-dihydroxynaphthalene-2,7-disulfonic acid; $C_{22}H_{18}As_2N_4O_{14}S_2$ = 776 (*1668-00-4*)

Brown powder.

Arsenious Trioxide Arsenic trioxide; As_2O_3 = 197.8 (*1327-53-3*)

Crystalline powder or a white or almost white mass, slightly soluble in water, soluble in boiling water.

Arsenite Solution

Dissolve 0.50 g of *arsenious trioxide* in 5 mL of 2M *sodium hydroxide* solution, add 2.0 g of *sodium hydrogen carbonate* and dilute to 100 mL with *water*.

L-Ascorbic Acid Ascorbic acid; (*50-81-7*)

See *Ascorbic acid (0253)*.

Ascorbic Acid Solution

Dissolve 50 mg in 0.5 mL of *water R* and dilute to 50 mL with *dimethylformamide R*.

Asiaticoside *O*-6-Deoxy-α-L-mannopyranosyl-(1→4)-*O*-β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl 2α,3β,23-trihydroxy-4α-urs-12-en-28-oate; $C_{48}H_{78}O_{19}$ = 959 (*16830-15-2*)

White or almost white powder, hygroscopic, soluble in methanol, slightly soluble in anhydrous ethanol, insoluble in acetonitrile.

mp: about 232 °C, with decomposition.

Water (*2.5.12*): 6.0 per cent.

Asiaticoside used in liquid chromatography complies with the following additional test.

Assay. Liquid chromatography (*2.2.29*) as prescribed in the monograph *Centella (1498)*.

Content: minimum 97.0 per cent, calculated by the normalisation procedure.

Storage: protected from humidity.

Asparagine $C_4H_8N_2O_3$ = 132.12 (*70-47-3*)

Aspartic Acid (*56-84-8*)

See *Aspartic acid (0797)*.

D-Aspartic Acid $C_4H_7NO_4$ = 133.1 (*1783-96-6*)

L-Aspartyl-L-phenylalanine Aspartame; $C_{13}H_{16}N_2O_5$ = 280.3 (*13433-09-5*)

White or almost white powder.

mp: about 210 °C, with decomposition.

Astragaloside IV (20*R*,24*S*)-20,24-Epoxy-16β,25-dihydroxy-3β-(β-D-xylopyranosyloxy)-9,19-cyclolanostan-6α-yl β-D-glucopyranoside; $C_{41}H_{68}O_{14}$ = 785 (*84687-43-4*)

Atenolol $C_{14}H_{22}N_2O_3$ = 266.3 (*56715-13-0*)

General reagent grade of commerce.

Atropine Sulfate Atropine sulphate; (*5908-99-6*)

See *Atropine sulfate (0068)*.

Aucubin [1*S*,4a*R*,5*S*,7a*S*)-5-Hydroxy-7-(hydroxymethyl)-1,4a,5,7a-tetrahydrocyclopenta[*c*]pyran-1-yl β-D-glucopyranoside; $C_{15}H_{22}O_9$ = 346.3 (*479-98-1*)

Crystals, soluble in water, in ethanol (96 per cent) and in methanol, practically insoluble in light petroleum.

mp: about 181 °C.

Azadirachtin $C_{35}H_{44}O_{16}$ = 720.7 (*11141-17-6*)

mp: about 165°.

General reagent grade of commerce.

Azobenzene $C_{12}H_{10}N_2$ = 182.2 (*103-33-3*)

mp: about 69°.

Use a grade of commerce suitable for chromatographic standardisations.

Azomethine H Monosodium salt hydrate; $C_{17}H_{12}NNaO_8S_2$ = 445.4 (*5941-07-1*)

Azomethine H Solution

Dissolve 0.45 g of *azomethine H R* and 1 g of *ascorbic acid R* with gentle heating in *water R* and dilute to 100 mL with the same solvent.

Baicalin $C_{21}H_{18}O_{11}$ = 446.4 (*21967-41-9*)

5,6-Dihydroxy-4-oxo-2-phenyl-4*H*-1-benzopyran-7-yl-β-D-glucopyranosiduronic acid.

Barbaloin Aloin; 1,8-Dihydroxy-3-hydroxymethyl-10-β-D-glucopyranosyl-10*H*-anthracen-9-one; $C_{21}H_{22}O_9$, H_2O = 436.4 (*1415-73-2*)

Yellow to dark-yellow, crystalline powder, or yellow needles, darkening on exposure to air and light, sparingly soluble in water and in ethanol (96 per cent), soluble in acetone, in ammonia and in solutions of alkali hydroxides.

$A^{1\%}_{1\,cm}$: about 192 at 269 nm, about 226 at 296.5 nm, about 259 at 354 nm, determined on a solution in *methanol R* and calculated with reference to the anhydrous substance.

Chromatography. Thin-layer chromatography (*2.2.27*) as prescribed in the monograph *Frangula bark (0025)*; the chromatogram shows only one principal spot.

Barbitone Sodium Barbital sodium; $C_8H_{11}N_2NaO_3$ = 206.2 (*144-02-5*)

Content: minimum 98.0 per cent.

A white or almost white, crystalline powder or colourless crystals, freely soluble in water, slightly soluble in ethanol (96 per cent).

Barbitone Barbital; (*57-44-3*)

See *Barbital (0170)*.

Barbituric Acid 2,4,6-Trihydroxypyrimidine; $C_4H_4N_2O_3$ = 128.1 (*67-52-7*)

White or almost white powder, slightly soluble in water, freely soluble in boiling water and in dilute acids.

mp: about 253 °C.

Barium Acetate Barium diacetate; $C_4H_6BaO_4$ = 255.4 (*543-80-6*)

White or almost white powder, soluble in water.

d^{20}_{20}: 2.47.

Barium Carbonate $BaCO_3$ = 197.3 (*513-77-9*)

White or almost white powder or friable masses, practically insoluble in water.

Barium Chloride Barium dichloride; $BaCl_2,2H_2O$ = 244.3 (*10326-27-9*)

Colourless crystals, freely soluble in water, slightly soluble in ethanol (96 per cent).

Barium Chloride Solution

A 10.0% w/v solution of *barium chloride*.

Barium Chloride Solution R1

A 61 g/L solution of *barium chloride R*.

Barium Chloride Solution R2

A 36.5 g/L solution of *barium chloride R*.

Barium Hydroxide Barium dihydroxide; $Ba(OH)_2,8H_2O$ = 315.5 (*12230-71-6*)

Colourless crystals, soluble in water.

Barium Hydroxide Solution

A 47.3 g/L solution of *barium hydroxide R*.

Barium Nitrate $Ba(NO_3)_2$ = 261.3 (*10022-31-8*)

Crystals or crystalline powder, freely soluble in water, very slightly soluble in ethanol (96 per cent) and in acetone.

mp: about 590 °C.

Barium Sulfate (*7727-43-7*)

See *Barium sulfate (0010)*.

Benzaldehyde C_7H_6O = 106.1 (*100-52-7*)

Colourless or slightly yellow liquid, slightly soluble in water, miscible with ethanol (96 per cent).

d^{20}_{20}: about 1.05.

n^{20}_D: about 1.545.

Distillation range (*2.2.11*). Not less than 95 per cent distils between 177 °C and 180 °C.

Storage: protected from light.

Benzalkonium Chloride

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Benzalkonium Chloride Solution

Of the British Pharmacopoeia.

Benzalphthalide 3-Benzylidenephthalide; $C_{15}H_{10}O_2$ = 222.24 (*575-61-1*)

Light yellow powder or crystals.

Analytical reagent grade of commerce.

Benzene C_6H_6 = 78.1 (*71-43-2*)

Clear, colourless, flammable liquid, practically insoluble in water, miscible with ethanol (96 per cent).

bp: about 80 °C.

Where benzene is used to prepare a reference solution, for safety reasons, the pure reagent may be replaced by a commercially available reference material containing a certified amount of benzene.

Where benzene is used to prepare a reference solution, for safety reasons, the pure reagent may be replaced by a commercially available reference material containing a certified amount of benzene.

Benzene-1,2,4-triol Hydroxyhydroquinone; Hydroxyquinol; $C_6H_6O_3$ = 126.1 (*533-73-3*)

Freely soluble in water, in ethanol (96 per cent) and in ethyl acetate.

mp: about 140 °C.

Benzethonium Chloride Benzyldimethyl[2-[2-[4-(1,1,3,3-tetramethylbutyl)phenoxy]ethoxy]ethyl]ammonium chloride; $C_{27}H_{42}ClNO_2$ = 448.1 (*121-54-0*)

Fine, white or almost white powder or colourless crystals, soluble in water and in ethanol (96 per cent).

mp: about 163 °C.

Storage: protected from light.

Benzidine Biphenyl-4,4′-diamine; $C_{12}H_{12}N_2$ = 184.2 (*92-87-5*)

Content: minimum 95 per cent.

White or slightly yellowish or reddish powder, darkening on exposure to air and light.

mp: about 120 °C.

Storage: protected from light.

Benzil Diphenylethanedione; $C_{14}H_{10}O_2$ = 210.2 (*134-81-6*)

Yellow, crystalline powder, practically insoluble in water, soluble in ethanol (96 per cent), ethyl acetate and toluene.

mp: 95 °C.

Benzocaine $C_9H_{11}NO_2$ = 165.2 (*94-09-7*)

See *Benzocaine (0011)*.

Benzohydrazide Benzoyldiazane; $C_7H_8N_2O$ = 136.2 (*613-94-5*)

Benzoic Acid (*65-85-0*)

See *Benzoic acid (0066)*.

Benzoin α-Hydroxy-α-phenylacetophenone; $C_{14}H_{12}O_2$ = 212.3 (*579-44-2*)

Slightly yellowish crystals, very slightly soluble in water, freely soluble in acetone, soluble in hot ethanol (96 per cent).

mp: about 137 °C.

Benzophenone Diphenylmethanone; $C_{13}H_{10}O$ = 182.2 (*119-61-9*)

Prismatic crystals, practically insoluble in water, freely soluble in ethanol (96 per cent).

mp: about 48 °C.

1,4-Benzoquinone Cyclohexa-2,5-diene-1,4-dione; $C_6H_4O_2$ = 108.1 (*106-51-4*)

Content: minimum 98.0 per cent.

Benzoylarginine Ethyl Ester Hydrochloride *N*-Benzoyl-L-arginine ethyl ester hydrochloride; Ethyl (*S*)-2-benzamido-5-guanidinovalerate hydrochloride; $C_{15}H_{23}ClN_4O_3$ = 342.8 (*2645-08-1*)

White or almost white, crystalline powder, very soluble in water and in anhydrous ethanol.

$[\alpha]_D^{20}$: −15 to −18, determined on a 10 g/L solution.

mp: about 129 °C.

$A_{1\,cm}^{1\%}$: 310 to 340, determined at 227 nm using a 0.01 g/L solution.

Benzoyl Chloride C_7H_5ClO = 140.6 (*98-88-4*)

Colourless, lachrymatory liquid, decomposed by water and by ethanol (96 per cent).

d_{20}^{20}: about 1.21.

bp: about 197 °C.

Benzoylecgonine Hydrate $C_{16}H_{19}NO_4,H_2O$ = 307.3 (*519-09-5*)

General reagent grade of commerce.

Benzoyl Peroxide $C_{14}H_{10}O_4$ = 242.2 (*94-36-0*)

mp: after drying, about 104°.

White or almost white granules.

General reagent grade of commerce.

For safety Benzoyl Peroxide should be kept moistened with about 23% w/w of water.

***N*-Benzoyl-L-prolyl-L-phenylalanyl-L-arginine 4-Nitroanilide Acetate** $C_{35}H_{42}N_8O_8$ = 703

3-Benzoylpropionic Acid 4-Oxo-4-phenylbutanoic acid; $C_{10}H_{10}O_3$ = 178.2 (*2051-95-8*)

mp: about 118 °C.

2-Benzoylpyridine Benzoylpyridine; $C_{12}H_9NO$ = 183.2 (*91-02-1*)

Colourless crystals, soluble in ethanol (96 per cent).

mp: about 43 °C.

Benzyl Alcohol (*100-51-6*)

See *Benzyl alcohol (0256)*.

Benzyl Benzoate (*120-51-4*)

See *Benzyl benzoate (0705)*.

Chromatography. Thin-layer chromatography (*2.2.27*) as prescribed in the monograph *Peru balsam (0754)*: apply 20 µL of a 0.3 per cent *V/V* solution in *ethyl acetate R*; after spraying and heating, the chromatogram shows a principal band with an R_F of about 0.8.

Benzyl Cinnamate Benzyl 3-phenylprop-2-enoate; $C_{16}H_{14}O_2$ = 238.3 (*103-41-3*)

Colourless or yellowish crystals, practically insoluble in water, soluble in ethanol (96 per cent).

mp: about 39 °C.

Chromatography. Thin-layer chromatography (*2.2.27*) as prescribed in the monograph *Peru balsam (0754)*: apply 20 µL of a 3 g/L solution in *ethyl acetate R*; after spraying and heating, the chromatogram shows a principal band with an R_F of about 0.6.

Benzyl Cyanide Phenylacetonitrile; C_8H_7N = 117.2 (*140-29-4*)

Content: minimum 95.0 per cent.

Clear, colourless or light yellow liquid.

n_D^{20}: about 1.523.

bp: about 233 °C.

Benzyl Ether Dibenzyl ether; $C_{14}H_{14}O$ = 198.3 (*103-50-4*)

Clear, colourless liquid, practically insoluble in water, miscible with acetone and with anhydrous ethanol.

d_{20}^{20}: about 1.043.

n_D^{20}: about 1.562.

bp: about 296 °C, with decomposition.

Benzylpenicillin Sodium (*69-57-8*)

See *Benzylpenicillin sodium (0114)*.

2-Benzylpyridine $C_{12}H_{11}N$ = 169.2 (*101-82-6*)

Content: minimum 98.0 per cent.

Yellow liquid.

mp: 13 °C to 16 °C.

4-Benzylpyridine $C_{12}H_{11}N$ = 169.2 (*2116-65-6*)

Content: minimum 98.0 per cent.

Yellow liquid.

mp: 72 °C to 78 °C.

Benzyltrimethylammonium Chloride
Trimethylphenylmethanaminium chloride; $C_{10}H_{16}ClN$ = 185.7 (*56-93-9*)

White or almost white powder, soluble in water.

mp: about 230 °C, with decomposition.

Berberine Chloride 9,10-Dimethoxy-5,6-dihydrobenzo[*g*]-1,3-benzodioxolo[5,6-*a*]quinolizinium chloride; $C_{20}H_{18}ClNO_4,2H_2O$ = 407.8 (*5956-60-5*)

Yellow crystals, slightly soluble in water, practically insoluble in ethanol (96 per cent).

mp: 204 °C to 206 °C.

Berberine chloride used in liquid chromatography complies with the following additional test.

Assay. Liquid chromatography (*2.2.29*) as prescribed in the monograph *Goldenseal rhizome (1831)*.

Content: minimum 95 per cent, calculated by the normalisation procedure.

Bergapten 5-Methoxypsoralen; $C_{12}H_8O_4$ = 216.2 (*484-20-8*)

Colourless crystals, practically insoluble in water, sparingly soluble in ethanol (96 per cent) and slightly soluble in glacial acetic acid.

mp: about 188 °C.

Beta-cyclodextrin Derivative of Silica Gel for Chiral Separation

Substituted beta-cyclodextrin coated on a very finely divided silica gel for chiral separation.

β-Cyclodextrin Derivative of Silica Gel for Chiral Separation See *Beta-cyclodextrin derivative of silica gel for chiral separation R*.

Betamethasone 9α-Fluoro-11β,17α,21-trihydroxy-16β-methylpregna-1,4-diene-3,20-dione; $C_{22}H_{29}FO_5$ = 392.5 (*378-44-9*)

mp: about 236°.

General reagent grade of commerce.

Betulin Lup-20(39)-ene-3β,28-diol; $C_{30}H_{50}O_2$ = 442.7 (*473-98-3*)

White or almost white, crystalline powder.

mp: 248 °C to 251 °C.

Bibenzyl 1,2-Diphenylethane; $C_{14}H_{14}$ = 182.3 (*103-29-7*)

White or almost white, crystalline powder, practically insoluble in water, very soluble in methylene chloride, freely soluble in acetone, soluble in ethanol (96 per cent).

mp: 50 °C to 53 °C.

Biphenyl $C_{12}H_{10}$ = 154.2 (*92-52-4*)

mp: 68 °C to 70 °C.

Biphenyl-4-ol 4-Phenylphenol; $C_{12}H_{10}O$ = 170.2 (*90-43-7*)

mp: 164° to 167°.

White or almost white, crystalline powder, practically insoluble in water.

(-)-α-Bisabolol Levomenol; $C_{15}H_{26}O$ = 222.4 (*23089-26-1*)

Colourless, viscous liquid with a slight, characteristic odour, practically insoluble in water, freely soluble in ethanol (96 per cent), in methanol, in toluene, in fatty oils and in essential oils.

d_{20}^{20}: 0.925 to 0.935.

n_D^{20}: 1.492 to 1.500.

$[\alpha]_D^{20}$: −54.5 to −58.0, determined on a 50 g/L solution in *ethanol (96 per cent) R*.

(−)-α-Bisabolol used for gas chromatography complies with the following additional test.

Assay. Gas chromatography (*2.2.28*) as prescribed in the monograph *Matricaria oil (1836)*.

Test solution. A 4 g/L solution in *cyclohexane R*.

Content: minimum 95.0 per cent, calculated by the normalisation procedure.

1,3-bis(2-Acetyl-3-hydroxyphenoxy)-2-propanol $C_{19}H_{20}O_7$ = 360.4 g/mol (*16150-44-0*)

Analytical reagent grade of commerce.

Bisbenzimide 4-[5-[5-(4-Methylpiperazin-1-yl) benzimidazol-2-yl]benzimidazol-2-yl]phenol trihydrochloride pentahydrate; $C_{25}H_{27}Cl_3N_6O,5H_2O$ = 624 (*23491-44-3*)

Bisbenzimide Stock Solution

Dissolve 5 mg of *bisbenzimide R* in *water R* and dilute to 100 mL with the same solvent.

Storage: in the dark.

Bisbenzimide Working Solution

Immediately before use, dilute 100 µL of *bisbenzimide stock solution R* to 100 mL with *phosphate buffered saline pH 7.4 R*.

Bis(diphenylmethyl) Ether $C_{26}H_{22}O$ = 350.5 (*574-42-5*)

[Oxybis(methanetriyl)]tetrakisbenzene. 1,1′,1″,1‴-(Oxymethylidyne)tetrakisbenzene.

Bismuth Nitrate Pentahydrate $Bi(NO_3)_3,5H_2O$ = 485.1 (*10035-06-0*)

mp: about 30 °C.

Bismuth Oxycarbonate Bismuth subcarbonate; (*5892-10-4*)

A basic salt corresponding approximately to the formula $(BiO)_2CO_3,½H_2O$, containing the equivalent of about 91% of Bi_2O_3.

Bismuth Oxynitrate Bismuth subnitrate; $4BiNO_3(OH)_2$, $BiO(OH)$ = 1462 (*1304-85-4*)

White or almost white powder, practically insoluble in water.

Bismuth Oxynitrate R1 Bismuth subnitrate R1

Content: 71.5 per cent to 74.0 per cent of bismuth (Bi), and 14.5 per cent to 16.5 per cent of nitrate, calculated as nitrogen pentoxide (N_2O_5).

Bismuth Oxynitrate Solution
Bismuth subnitrate solution

Dissolve 5 g of *bismuth subnitrate R1* in a mixture of 8.4 mL of *nitric acid R* and 50 mL of *water R* and dilute to 250 mL with *water R*. Filter if necessary.

Acidity. To 10 mL add 0.05 mL of *methyl orange solution R*. 5.0 mL to 6.25 mL of *1 M sodium hydroxide* is required to change the colour of the indicator.

Bismuth Subcarbonate See *Bismuth oxycarbonate*.

***N,O*-Bis(trimethylsilyl)acetamide** $C_8H_{21}NOSi_2$ = 203.4 (*10416-59-8*)

Colourless liquid.

d_{20}^{20}: about 0.83.

Bis(trimethylsilyl)trifluoroacetamide
N,O-bis(Trimethylsilyl)trifluoroacetamide;
$C_8H_{18}F_3NOSi_2$ = 257.4 (*25561-30-2*)

Colourless liquid.

d_{20}^{20}: about 0.97.

n_D^{20}: about 1.38.

bp_{12mm}: about 40 °C

Biuret $C_2H_5N_3O_2$ = 103.1 (*108-19-0*)

White or almost white crystals, hygroscopic, soluble in water, sparingly soluble in ethanol (96 per cent).

mp: 188 °C to 190 °C, with decomposition.

Storage: in an airtight container.

Biuret Reagent

Dissolve 1.5 g of *copper sulfate pentahydrate R* and 6.0 g of *sodium potassium tartrate R* in 500 mL of *water R*.
Add 300 mL of a carbonate-free 100 g/L solution of *sodium hydroxide R*, dilute to 1000 mL with the same solution and mix.

Blocking Solution

A 10 per cent *V/V* solution of *acetic acid R*.

Blue Dextran 2000 (*9049-32-5*)

Prepared from dextran having an average relative molecular mass of 2×10^6 by introduction of a polycyclic chromophore that colours the substance blue. The degree of substitution is 0.017.

It is freeze-dried and dissolves rapidly and completely in water and aqueous saline solutions.

Absorbance (*2.2.25*). A 1 g/L solution in a *phosphate buffer solution pH 7.0 R* shows an absorption maximum at 280 nm.

Boldine 1,10-Dimethoxy-6aα-aporphine-2,9-diol;
$C_{19}H_{21}NO_4$ = 327.3 (*476-70-0*)

White or almost white crystalline powder, very slightly soluble in water, soluble in ethanol (96 per cent) and in dilute solutions of acids.

$[\alpha]_D^{25}$: about + 127, determined on a 1 g/L solution in *anhydrous ethanol R*.

mp: about 163 °C.

Borate Solution

Dissolve 9.55 g of *disodium tetraborate R* in *sulfuric acid R*, heating on a water-bath, and dilute to 1 L with the same acid.

Boric Acid (*10043-35-3*)

See *Boric acid (0001)*.

Boric Acid Solution

Dissolve 5 g of *boric acid* in a mixture of 20 mL of *water* and 20 mL of *absolute ethanol* and dilute to 250 mL with *absolute ethanol*.

Boric Acid Solution, Cold Saturated

To 3 g of *boric acid R* add 50 mL of *water R* and shake for 10 min. Place the solution for 2 h in the refrigerator.

D-Borneol Borneol; $C_{10}H_{18}O$ = 154.3 (*507-70-0*)

Colourless crystals, readily sublimes, practically insoluble in water, freely soluble in ethanol (96 per cent) and in light petroleum.

mp: about 208 °C.

Chromatography. Thin-layer chromatography (*2.2.27*), using *silica gel G R* as the coating substance. Apply to the plate 10 µL of a 1 g/L solution in *toluene R*. Develop over a path of 10 cm using *chloroform R*. Allow the plate to dry in air, spray with *anisaldehyde solution R*, using 10 mL for a plate 200 mm square, and heat at 100-105 °C for 10 min.
The chromatogram obtained shows only one principal spot.

D-Bornyl Acetate Bornyl acetate; $C_{12}H_{20}O_2$ = 196.3 (*5655-61-8*)

Colourless crystals or a colourless liquid, very slightly soluble in water, soluble in ethanol (96 per cent).

mp: about 28 °C.

Chromatography. Thin-layer chromatography (*2.2.27*), using *silica gel G R* as the coating substance. Apply to the plate 10 µL of a 2 g/L solution in *toluene R*. Develop over a path of 10 cm using *chloroform R*. Allow the plate to dry in air, spray with *anisaldehyde solution R*, using 10 mL for a plate 200 mm square, and heat at 100-105 °C for 10 min.
The chromatogram obtained shows only one principal spot.

Boron Trichloride BCl_3 = 117.2 (*10294-34-5*)

Colourless gas. Reacts violently with water. Available as solutions in suitable solvents (2-chloroethanol, methylene chloride, hexane, heptane, methanol).

n_D^{20}: about 1.420.

bp: about 12.6 °C.

Caution: toxic and corrosive.

Boron Trichloride-Methanol Solution

A 12 per cent *m/m* solution of *boron trichloride R* in *methanol R*.

Storage: protected from light at −20 °C, preferably in sealed tubes.

Boron Trifluoride BF_3 = 67.8 (*7637-07-2*)

Colourless gas.

Boron Trifluoride Solution Boron trifluoride-methanol solution

A 140 g/L solution of *boron trifluoride R* in *methanol R*.

Bovine Coagulation Factor Xa (*9002-05-5*)

An enzyme which converts prothrombin to thrombin.
The semi-purified preparation is obtained from liquid bovine plasma and it may be prepared by activation of the zymogen factor X with a suitable activator such as Russell's viper venom.

Storage: freeze-dried preparation at −20 °C and frozen solution at a temperature lower than −20 °C.

Bovine Factor Xa Solution

Reconstitute as directed by the manufacturer and dilute with *tris(hydroxymethyl)aminomethane sodium chloride buffer solution pH 7.4 R*.

Any change in the absorbance of the solution, measured at 405 nm (*2.2.25*) against *tris(hydroxymethyl)aminomethane sodium chloride buffer solution pH 7.4 R* and from which the blank absorbance has been substracted, is not more than 0.20 per minute.

Bovine Factor Xa Solution R1

Reconstitute as directed by the manufacturer and dilute to 1.4 nkat/mL with *tris(hydroxymethyl)aminomethane-EDTA buffer solution pH 8.4 R*.

Bovine Factor Xa Solution R2

Reconstitute as directed by the manufacturer and dilute with *tris(hydroxymethyl)aminomethane-EDTA buffer solution pH 8.4 R1* to obtain a solution that gives an absorbance between 0.65 and 1.25 at 405 nm when determining the blank amidolytic activity according to general chapter *2.7.5* using the end-point method.

Brilliant Blue (*6104-59-2*)

See *acid blue 83 R*.

Brilliant Green CI 42040; basic green 1; $C_{27}H_{34}N_2O_4S$ = 482.6 (*633-03-4*)

Technical grade of commerce.

Small, glistening golden crystals.

Bromelains (*37189-34-7*)

Concentrate of proteolytic enzymes derived from *Ananas comosus* Merr.

Dull-yellow powder.

Activity. 1 g liberates about 1.2 g of amino-nitrogen from a solution of *gelatin R* in 20 min at 45 °C and pH 4.5.

Bromelains Solution

A 10 g/L solution of *bromelains R* in a mixture of 1 volume of *phosphate buffer solution pH 5.5 R* and 9 volumes of a 9 g/L solution of *sodium chloride R*.

Bromine Br_2 = 159.8 (*7726-95-6*)

Brownish-red fuming liquid, slightly soluble in water, soluble in ethanol (96 per cent).

d_{20}^{20}: about 3.1.

To prepare 0.05M bromine dissolve 3 g of *potassium bromate* and 5 g of *potassium bromide* in sufficient *water* to produce 1000 mL. Weaker solutions should be prepared using proportionately lesser amounts of reagents or by appropriate dilution.

Bromine Solution

Dissolve 30 g of *bromine R* and 30 g of *potassium bromide R* in *water R* and dilute to 100 mL with the same solvent.

Bromine Solution, Acetic

Dissolve 100 g of *potassium acetate* in *glacial acetic acid* and add 4 mL of *bromine* and sufficient *glacial acetic acid* to produce 1000 mL.

Bromine Water

Shake 3 mL of *bromine R* with 100 mL of *water R* to saturation.

Storage: over an excess of *bromine R*, protected from light.

Bromine Water R1

Shake 0.5 mL of *bromine R* with 100 mL of *water R*.

Storage: protected from light; use within 1 week.

Bromobenzene C_6H_5Br = 157.0 (*108-86-1*)

n_D^{20}: about 1.56.

bp: about 156°.

General reagent grade of commerce.

A colourless to pale yellow liquid; weight per mL, about 1.50 g.

Bromocresol Green 3′,3″,5′,5″-Tetrabromo-*m*-cresolsulfonphthalein; 4,4′-(3*H*-2,1-Benzoxathiol-3-ylidene)bis(2,6-dibromo-3-methylphenol)-*S*,*S*-dioxide; $C_{21}H_{14}Br_4O_5S$ = 698 (*76-60-8*)

Brownish-white powder, slightly soluble in water, soluble in ethanol (96 per cent) and in dilute solutions of alkali hydroxides.

Bromocresol Green-Methyl Red Solution

Dissolve 0.15 g of *bromocresol green R* and 0.1 g of *methyl red R* in 180 mL of *anhydrous ethanol R* and dilute to 200 mL with *water R*.

Bromocresol Green Solution

Dissolve 50 mg of *bromocresol green R* in 0.72 mL of *0.1 M sodium hydroxide* and 20 mL of *ethanol (96 per cent) R* and dilute to 100 mL with *water R*.

Test for sensitivity. To 0.2 mL of the bromocresol green solution add 100 mL of *carbon dioxide-free water R*. The solution is blue. Not more than 0.2 mL of *0.02 M hydrochloric acid* is required to change the colour to green.

Colour change: pH 3.6 (yellow) to pH 5.2 (blue).

Bromocresol Purple 3′,3″-Dibromo-*o*-cresolsulfonphthalein; 4,4′-(3*H*-2,1-Benzoxathiol-3-ylidene) bis(2-bromo-6-methylphenol)-*S*,*S*-dioxide; $C_{21}H_{16}Br_2O_5S$ = 540.2 (*115-40-2*)

Pinkish powder, practically insoluble in water, soluble in ethanol (96 per cent) and in dilute solutions of alkali hydroxides.

Bromocresol Purple Solution

Dissolve 50 mg of *bromocresol purple R* in 0.92 mL of *0.1 M sodium hydroxide* and 20 mL of *ethanol (96 per cent) R* and dilute to 100 mL with *water R*.

Test for sensitivity. To 0.2 mL of the bromocresol purple solution add 100 mL of *carbon dioxide-free water R* and 0.05 mL of *0.02 M sodium hydroxide*. The solution is bluish-violet. Not more than 0.2 mL of *0.02 M hydrochloric acid* is required to change the colour to yellow.

Colour change: pH 5.2 (yellow) to pH 6.8 (bluish-violet).

5-Bromo-2′-deoxyuridine 5-Bromo-1-(2-deoxy-β-d-*erythro*-pentofuranosyl)-1*H*,3*H*-pyrimidine-2,4-dione; $C_9H_{11}BrN_2O_5$ = 307.1 (*59-14-3*)

mp: about 194 °C.

Chromatography. Thin-layer chromatography (*2.2.27*) as prescribed in the monograph *Idoxuridine (0669)*: apply 5 µL of a 0.25 g/L solution; the chromatogram shows only one principal spot.

Bromomethoxynaphthalene 2-Bromo-6-methoxynaphthalene; $C_{11}H_9BrO$ = 237.1 (*5111-65-9*)

mp: about 109 °C.

Bromophenol Blue 3′,3″,5′,5″-Tetrabromophenolsulfonphthalein; 4,4′-(3*H*-2,1-Benzoxathiol-3-ylidene)bis(2,6-dibromophenol) *S*,*S*-dioxide; $C_{19}H_{10}Br_4O_5S$ = 670 (*115-39-9*)

Light orange-yellow powder, very slightly soluble in water, slightly soluble in ethanol (96 per cent), freely soluble in solutions of alkali hydroxides.

Bromophenol Blue Solution

Dissolve 0.1 g of *bromophenol blue R* in 1.5 mL of *0.1 M sodium hydroxide* and 20 mL of *ethanol (96 per cent) R* and dilute to 100 mL with *water R*.

Test for sensitivity. To 0.05 mL of the bromophenol blue solution add 20 mL of *carbon dioxide-free water R* and 0.05 mL of *0.1 M hydrochloric acid*. The solution is yellow. Not more than 0.1 mL of *0.1 M sodium hydroxide* is required to change the colour to bluish-violet.

Colour change: pH 2.8 (yellow) to pH 4.4 (bluish-violet).

Bromophenol Blue Solution R1

Dissolve 50 mg of *bromophenol blue R* with gentle heating in 3.73 mL of *0.02 M sodium hydroxide* and dilute to 100 mL with *water R*.

Bromophenol Blue Solution R2

Dissolve with heating 0.2 g of *bromophenol blue R* in 3 mL of *0.1 M sodium hydroxide* and 10 mL of *ethanol (96 per cent) R*. After solution is effected, allow to cool and dilute to 100 mL with *ethanol (96 per cent) R*.

Bromophos $C_8H_8BrCl_2O_3PS$ = 366.0 (*2104-96-3*)

A suitable certified reference solution (10 ng/µL in iso-octane) may be used.

Bromophos-ethyl $C_{10}H_{12}BrCl_2O_3PS$ = 394.0 (*4824-78-6*)

A suitable certified reference solution (10 ng/µL in iso-octane) may be used.

Bromothymol Blue 3′,3″-Dibromothymolsulfonphthalein; 4,4′-(3*H*-2,1-Benzoxathiol-3-ylidene)bis(2-bromo-6-isopropyl-3-methylphenol) *S*,*S*-dioxide; $C_{27}H_{28}Br_2O_5S$ = 624 (*76-59-5*)

Reddish-pink or brownish powder, practically insoluble in water, soluble in ethanol (96 per cent) and in dilute solutions of alkali hydroxides.

Bromothymol Blue Solution R1

Dissolve 50 mg of *bromothymol blue R* in a mixture of 4 mL of *0.02 M sodium hydroxide* and 20 mL of *ethanol (96 per cent) R* and dilute to 100 mL with *water R*.

Test for sensitivity. To 0.3 mL of *bromothymol blue solution R1* add 100 mL of *carbon dioxide-free water R*. The solution is yellow. Not more than 0.1 mL of *0.02 M sodium hydroxide* is required to change the colour to blue.

Colour change: pH 5.8 (yellow) to pH 7.4 (blue).

Bromothymol Blue Solution R2

A 10 g/L solution of *bromothymol blue R* in *dimethylformamide R*.

Bromothymol Blue Solution R3

Warm 0.1 g of *bromothymol blue R* with 3.2 mL of *0.05 M sodium hydroxide* and 5 mL of *ethanol (90 per cent V/V) R*. After solution is effected, dilute to 250 mL with *ethanol (90 per cent V/V) R*.

Bromothymol Blue Solution R4

Dissolve 100 mg of *bromothymol blue R* in a mixture of equal volumes of *ethanol (96 per cent) R* and *water R* and dilute to 100 mL with the same mixture of solvents. Filter if necessary.

BRP Indicator Solution

Dissolve 0.1 g of *bromothymol blue R*, 20 mg of *methyl red R* and 0.2 g of *phenolphthalein R* in *ethanol (96 per cent) R* and dilute to 100 mL with the same solvent. Filter.

Brucine 10,11-Dimethoxystrychnine; $C_{23}H_{26}N_2O_4$ = 394.5 (*357-57-3*)

Colourless crystals, slightly soluble in water, freely soluble in ethanol (96 per cent).

mp: about 178 °C.

Butanal Butyraldehyde; C_4H_8O = 72.1 (*123-72-8*)

d_{20}^{20}: 0.806.

n_D^{20}: 1.380.

bp: 75 °C.

***i*-Butane** Isobutane; 2-Methylpropane; C_4H_{10} = 58.12 (*75-28-5*)

Content: minimum 99.0 per cent *V/V*.

***N*-Butane** Butane; C_4H_{10} = 58.12 (*106-97-8*)

Content: minimum 99.0 per cent *V/V*.

Butane-1,3-diol $C_4H_{10}O_2$ = 90.1 (*6290-03-5*)

n_D^{20}: about 1.440.

bp: about 203°.

General reagent grade of commerce.

Butane-1,4-diol $HO(CH_2)_4OH$ = 90.12 (*110-63-4*)

Butan-1-ol 1-Butanol; *N*-Butanol; *N*-Butyl alcohol; Butanol; $C_4H_{10}O$ = 74.1 (*71-36-3*)

Clear, colourless liquid, miscible with ethanol (96 per cent).

d_{20}^{20}: about 0.81.

bp: 116 °C to 119 °C.

Butan-2-ol *sec*-Butyl alcohol; 2-butanol; $C_4H_{10}O$ = 74.12 (*78-92-2*)

d_{20}^{20}: about 0.81.

bp: about 99°.

Analytical reagent grade of commerce.

A colourless liquid.

Butan-2-ol R1 sec-Butyl alcohol; 2-Butanol R1; $C_4H_{10}O$ = 74.1 (*78-92-2*)

Content: minimum 99.0 per cent.

Clear, colourless liquid, soluble in water, miscible with ethanol (96 per cent).

d_{20}^{20}: about 0.81.

Distillation range (*2.2.11*). Not less than 95 per cent distils between 99 °C and 100 °C.

Assay. Gas chromatography (*2.2.28*) as prescribed in the monograph *Isopropyl alcohol (0970)*.

Butan-2-one Methyl ethyl ketone; Ethyl methyl ketone; (*78-93-3*)

See *methyl ethyl ketone R*.

Butyl Acetate $C_6H_{12}O_2$ = 116.2 (*123-86-4*)

Clear, colourless liquid, flammable, slightly soluble in water, miscible with ethanol (96 per cent).

d_{20}^{20}: about 0.88.

n_D^{20}: about 1.395.

Distillation range (*2.2.11*). Not less than 95 per cent distils between 123 °C and 126 °C.

Butyl Acetate R1

Content: minimum 99.5 per cent, determined by gas chromatography.

Clear, colourless liquid, flammable, slightly soluble in water, miscible with ethanol (96 per cent).

d_{20}^{20}: about 0.883.

n_D^{20}: about 1.395.

Butanol: maximum 0.2 per cent, determined by gas chromatography.

n-Butyl formate: maximum 0.1 per cent, determined by gas chromatography.

n-Butyl propionate: maximum 0.1 per cent, determined by gas chromatography.

Water: maximum 0.1 per cent.

***N*-Butylamine** 1-Butanamine; Butylamine; $C_4H_{11}N$ = 73.1 (*109-73-9*)

Distil and use within one month.

Colourless liquid, miscible with water, with ethanol (96 per cent).

n_D^{20}: about 1.401.

bp: about 78 °C.

***tert*-Butylamine** (*75-64-9*)

See *1,1-dimethylethylamine R*.

4-(Butylamino)benzoic Acid $C_{11}H_{15}NO_2$ = 193.2 (*4740-24-3*)

White or almost white powder.

Content: 96.5 per cent to 103.5 per cent.

Butylated Hydroxyanisole 2-*tert*-Butyl-4-methoxyphenol; $C_{11}H_{16}O_2$ = 180.2 (*25013-16-5*)

mp: about 61°.

General reagent grade of commerce.

A white or almost white, crystalline powder.

Butylated Hydroxytoluene (*128-37-0*)

See *Butylhydroxytoluene R*.

Butylboronic Acid $C_4H_{11}BO_2$ = 101.9 (*4426-47-5*)

Content: minimum 98 per cent.

mp: 90 °C to 92 °C.

Butyl Chloride See *1-Chlorobutane*.

***tert*-Butylhydroperoxide**
1,1-Dimethylethylhydroperoxide; $C_4H_{10}O_2$ = 90.1 (*75-91-2*)

Flammable liquid, soluble in organic solvents.

d_{20}^{20}: 0.898.

n_D^{20}: 1.401.

bp: 35 °C.

Butyl 4-hydroxybenzoate (*94-26-8*)

See *Butyl parahydroxybenzoate R*.

Butylhydroxytoluene (*128-37-0*)

See *Butylhydroxytoluene (0581)*.

2-Butyloctanol (2*S*)-2-Butyloctan-1-ol; $C_{12}H_{26}O$ = 186.3 (*3913-02-8*)

Butyl Parahydroxybenzoate (*94-26-8*)

See *Butyl parahydroxybenzoate (0881)*.

***tert*-Butyl Methyl Ether** (*1634-04-4*)

See *1,1-dimethylethyl methyl ether R*.

Butyl Methacrylate Butyl 2-methylpropenoate; $C_8H_{14}O_2$ = 142.2 (*97-88-1*)

Clear, colourless solution.

d_4^{20}: about 0.894.

n_D^{20}: about 1.424.

bp: about 163 °C.

Butyric Acid *N*-Butyric acid; $C_4H_8O_2$ = 88.1 (*107-92-6*)

Content: minimum 99.0 per cent.

Oily liquid, miscible with water and with ethanol (96 per cent).

d_{20}^{20}: about 0.96.

n_D^{20}: about 1.398.

bp: about 163 °C.

Butyrolactone Dihydro-2(3*H*)-furanone; γ-Butyrolactone; $C_4H_6O_2$ = 86.1 (*96-48-0*)

Oily liquid, miscible with water, soluble in methanol.

n_D^{25}: about 1.435.

bp: about 204 °C.

Cadmium Cd = 112.4 (*7440-43-9*)

Silvery-white, lustrous metal, practically insoluble in water, freely soluble in nitric acid and in hot hydrochloric acid.

Cadmium Acetate $C_4H_6O_4Cd,2H_2O$ = 266.5 (*5743-04-4*)

Analytical reagent grade of commerce.

Cadmium and Ninhydrin Solution

Dissolve 50 mg of *cadmium acetate* in a mixture of 5 mL of *water* and 1 mL of *glacial acetic acid* and dilute with *butan-2-one* to 50 mL. Immediately before use add and dissolve sufficient *ninhydrin* to produce a solution containing 0.2% w/v.

Cadmium Iodide CdI_2 = 366.2 (*7790-80-9*)

Analytical reagent grade of commerce.

Cadmium Iodide Solution

A 5.0% w/v solution of *cadmium iodide*.

Cadmium Nitrate Tetrahydrate
$Cd(NO_3)_2,4H_2O$ = 308.5 (*10022-68-1*)

Hygroscopic orthorhombic crystals, very soluble in water, soluble in acetone and in ethanol (96 per cent).

mp: about 59.5 °C.

Caesium Chloride CsCl = 168.4 (*7647-17-8*)

White or almost white powder, very soluble in water, freely soluble in methanol, practically insoluble in acetone.

Caffeic Acid 3,4-Dihydroxycinnamic acid; $C_9H_8O_4$ = 180.2 (*331-39-5*)

White or almost white crystals or plates, freely soluble in hot water and in ethanol (96 per cent), sparingly soluble in cold water.

Absorbance (*2.2.25*). A freshly prepared solution at pH 7.6 shows 2 absorption maxima at about 288 nm and about 313 nm.

Caffeine (*58-08-2*)

See *Caffeine (0267)*.

Calciferol Ergocalciferol; vitamin D_2; $C_{28}H_{44}O$ = 396.7 (*50-14-6*)

$[\alpha]_D^{20}$: about +105 (4% w/v in ethanol).

mp: about 117°.

Crystalline reagent grade of commerce.

Colourless crystals or a white, crystalline powder.

Calcium Acetate Calcium diacetate; See Calcium acetate (2128); $C_4H_6CaO_4$ = 158.2 (*62-54-4*)

Calcium Acetate, Dried $C_4H_6O_4Ca$ = 158.2 (*62-54-4*)

General reagent grade of commerce.

Calcium bis(formyl homotaurine) Calcium bis(3-formamidopropane-1-sulfonate); $C_8H_{16}CaN_2O_8S_2$ = 372.4

White or almost white powder.

Content: minimum 80.0 per cent.

Calcium Carbonate (*471-34-1*)

See *Calcium carbonate (0014)*.

Calcium Carbonate R1

Complies with the requirements prescribed for *calcium carbonate R* with the following additional requirement.

Chlorides (*2.4.4*): maximum 50 ppm.

Calcium Chloride Calcium Chloride Dihydrate; (*10035-04-8*)

See *Calcium chloride (0015)*.

Calcium Chloride, Anhydrous $CaCl_2$ = 111.0 (*10043-52-4*)

Content: minimum 98.0 per cent (dried substance).

White or almost white granules, deliquescent, very soluble in water, freely soluble in ethanol (96 per cent) and in methanol.

Loss on drying (*2.2.32*): maximum 5.0 per cent, determined by drying in an oven at 200 ± 10 °C.

Storage: in an airtight container, protected from moisture.

Calcium Chloride R1 Calcium chloride tetrahydrate; $CaCl_2,4H_2O$ = 183.1

Iron: maximum 0.05 ppm.

Calcium Chloride Solution

A 73.5 g/L solution of *calcium chloride R*.

Calcium Chloride Solution, 0.01M

Dissolve 0.147 g of *calcium chloride R* in *water R* and dilute to 100.0 mL with the same solvent.

Calcium Chloride Solution, 0.02M

Dissolve 2.94 g of *calcium chloride R* in 900 mL of *water R*, adjust to pH 6.0 to 6.2 and dilute to 1000.0 mL with *water R*.

Storage: at 2 °C to 8 °C.

Calcium Chloride Solution, 0.025M

Dissolve 0.368 g of *calcium chloride R* in *water R* and dilute to 100.0 mL with the same solvent.

Calcium Hydroxide Calcium dihydroxide; $Ca(OH)_2$ = 74.1 (*1305-62-0*)

White or almost white powder, almost completely soluble in 600 parts of water.

Calcium Hydroxide Solution

A freshly prepared saturated solution.

Calcium Lactate Calcium lactate pentahydrate; (*41372-22-9*)

See *Calcium lactate pentahydrate (0468)*.

Calcium Phosphate Monobasic Monohydrate Calcium tetrahydrogen bisphosphate monohydrate; Phosphoric acid calcium salt (2:1) monohydrate; $CaH_4O_8P_2,H_2O$ = 252.1 (*10031-30-8*)

White or almost white, crystalline powder, soluble in water.

Calcium Sulfate Calcium sulphate hemihydrate; Calcium sulphate; Plaster of Paris; $CaSO_4,{}^1/_2H_2O$ = 145.1 (*10034-76-1*)

White or almost white powder, soluble in about 1500 parts of water, practically insoluble in ethanol (96 per cent). When mixed with half its mass of water it rapidly solidifies to a hard and porous mass.

Calcium Sulfate Solution Calcium sulphate solution

Shake 5 g of *calcium sulfate R* with 100 mL of *water R* for 1 h and filter.

Calconcarboxylic Acid Patton and Reeder's reagent; 2-Hydroxy-1-(2-hydroxy-4-sulfo-1-naphthylazo)naphthalene-3-carboxylic acid; Calconecarboxylic acid; $C_{21}H_{14}N_2O_7S$ = 438.4 (*3737-95-9*)

Brownish-black powder, slightly soluble in water, very slightly soluble in acetone and in ethanol (96 per cent), sparingly soluble in dilute solutions of sodium hydroxide.

Calconcarboxylic Acid Triturate

Calconecarboxylic acid triturate

Mix 1 part of *calconecarboxylic acid R* with 99 parts of *sodium chloride R*.

Test for sensitivity. Dissolve 50 mg of calconecarboxylic acid triturate in a mixture of 2 mL of *strong sodium hydroxide solution R* and 100 mL of *water R*. The solution is blue but becomes violet on addition of 1 mL of a 10 g/L solution of *magnesium sulfate R* and 0.1 mL of a 1.5 g/L solution of *calcium chloride R* and turns pure blue on addition of 0.15 mL of *0.01 M sodium edetate*.

Camphene 2,2-Dimethyl-3-methylenebicyclo[2.2.1]heptane; $C_{10}H_{16}$ = 136.2 (*79-92-5*)

Camphene used in gas chromatography complies with the following additional test.

Assay. Gas chromatography (*2.2.28*) as prescribed in the monograph *Rosemary Oil (1846)*.

Content: minimum 90 per cent, calculated by the normalisation procedure.

Camphor (*76-22-2*)

See *Camphor, racemic (0655)*.

Camphor used in gas chromatography complies with the following additional test.

Assay. Gas chromatography (*2.2.28*) as prescribed in the monograph *Lavender oil (1338)*.

Test solution. A 10 g/L solution of the substance to be examined in *hexane R*.

Content: minimum 95.0 per cent, calculated by the normalisation procedure.

(1S)-(+)-10-Camphorsulfonic Acid (1S)-(+)-10-Camphorsulphonic Acid; $C_{10}H_{16}O_4S$ = 232.3 (*3144-16-9*)

Prismatic crystals, hygroscopic, soluble in water.

Content: minimum 99.0 per cent of (1*S*)-(+)-10-camphorsulfonic acid.

$[\alpha]_D^{20}$: + 20 ± 1, determined on a 43 g/L solution.

mp: about 194 °C, with decomposition.

ΔA (*2.2.41*): 10.2 × 10^3 determined at 290.5 nm on a 1.0 g/L solution.

Capric Acid Decanoic acid; $C_{10}H_{20}O_2$ = 172.3 (*334-48-5*)

Crystalline solid, very slightly soluble in water, soluble in anhydrous ethanol.

bp: about 270 °C.

mp: about 31.4 °C.

Capric acid used in the assay of total fatty acids in Saw palmetto fruit (1848) complies with the following additional test.

Assay. Gas chromatography (*2.2.28*) as prescribed in the monograph *Saw palmetto fruit (1848)*.

Content: minimum 98 per cent, calculated by the normalisation procedure.

Caproic Acid Hexanoic acid; $C_6H_{12}O_2$ = 116.2 (*142-62-1*)

Oily liquid, sparingly soluble in water.

d_4^{20}: about 0.926.

n_D^{20}: about 1.417.

bp: about 205 °C.

Caproic acid used in the assay of total fatty acids in Saw palmetto fruit (1848) complies with the following additional test.

Assay. Gas chromatography (*2.2.28*) as prescribed in the monograph *Saw palmetto fruit (1848)*.

Content: minimum 98 per cent, calculated by the normalisation procedure.

ε-Caprolactam Hexane-6-lactam; $C_6H_{11}NO$ = 113.2 (*105-60-2*)

Hygroscopic flakes, freely soluble in water, in anhydrous ethanol and in methanol.

mp: about 70 °C.

Capryl Alcohol Decan-1-ol; Decanol; Capric alcohol; See *Decanol R*.

Capsaicin (*E*)-*N*-[(4-Hydroxy-3-methoxyphenyl)methyl]-8-methylnon-6-enamide; $C_{18}H_{27}NO_3$ = 305.4 (*404-86-4*)

White or almost white, crystalline powder, practically insoluble in water, freely soluble in anhydrous ethanol.

mp: about 65 °C.

Capsaicin used in the assay in Capsicum (1859) complies with the following additional test.

Assay. Liquid chromatography (*2.2.29*) as prescribed in the monograph *Capsicum (1859)*.

Content: minimum 95.0 per cent, calculated by the normalisation procedure.

Carbazole Dibenzopyrrole; $C_{12}H_9N$ = 167.2 (*86-74-8*)

Crystals, practically insoluble in water, freely soluble in acetone, slightly soluble in anhydrous ethanol.

mp: about 245 °C.

Carbomer (*9007-20-9*)

A cross-linked polymer of acrylic acid; it contains a large proportion (56 per cent to 68 per cent) of carboxylic acid (CO_2H) groups after drying at 80 °C for 1 h. Average relative molecular mass about 3×10^6.

pH (*2.2.3*): about 3 for a 10 g/L suspension.

Carbon Dioxide (*124-38-9*)

See *Carbon dioxide (0375)*.

Carbon Dioxide R1 CO_2 = 44.01 (*124-38-9*)

Content: minimum 99.995 per cent *V/V*.

Carbon monoxide: less than 5 ppm.

Oxygen: less than 25 ppm.

Nitric oxide: less than 1 ppm.

Carbon Dioxide R2 CO_2 = 44.01 (*124-38-9*)

Content: minimum 99 per cent *V/V*.

Carbon Disulfide Carbon disulphide; CS_2 = 76.1 (*75-15-0*)

Colourless or yellowish, flammable liquid, practically insoluble in water, miscible with anhydrous ethanol.

d_{20}^{20}: about 1.26.

bp: 46 °C to 47 °C.

Carbon for Chromatography, Graphitised

Carbon chains having a length greater than C_9 .

Particle size: 400 µm to 850 µm.

Relative density: 0.72.

Surface area: 10 m^2/g.

Do not use at a temperature higher than 400 °C.

Carbon for Chromatography, Graphitised R1

Porous spherical carbon particles comprised of flat sheets of hexagonally arranged carbon atoms.

Particle size: 5 µm to 7 µm.

Pore volume: 0.7 cm^3/g.

Carbon Monoxide CO = 28.01 (*630-08-0*)

Content: minimum 99.97 per cent *V/V*.

Carbon Monoxide R1 CO = 28.01 (*630-08-0*)

Content: minimum 99 per cent *V/V*.

Carbon Tetrachloride Tetrachloromethane; CCl_4 = 153.8 (*56-23-5*)

Clear, colourless liquid, practically insoluble in water, miscible with ethanol (96 per cent).

d_{20}^{20}: 1.595 to 1.598.

bp: 76 °C to 77 °C.

Carbophenothion *O,O*-Diethyl *S*-[[(4-chlorophenyl)thio] methyl]-phosphorodithioate; $C_{11}H_{16}ClO_2PS_3$ = 342.9 (*786-19-6*)

Yellowish liquid, practically insoluble in water, miscible with organic solvents.

d_4^{25}: about 1.27.

For the monograph *Wool Fat (0134)*, a suitable certified reference solution (10 ng/µL in iso-octane) may be used.

5-Carboxyuracil 2,4-Dioxo-1,2,3,4-tetrahydropyrimidine-5-carboxylic acid; Uracil-5-carboxylic acid; $C_5H_4N_2O_4$ = 156.1 (*23945-44-0*)

mp: about 283 °C.

Car-3-ene 3,7,7-Trimethylbicyclo[4.1.0]hept-3-ene; 4,7,7-Trimethyl-3-norcarene; $C_{10}H_{16}$ = 136.2 (*498-15-7*)

Liquid with a pungent odour, slightly soluble in water, soluble in organic solvents.

d_{20}^{20}: about 0.864.

n_D^{20}: 1.473 to 1.474.

$[\alpha]_D^{20}$: + 15 to + 17.

bp: 170 °C to 172 °C.

Car-3-ene used in gas chromatography complies with the following additional test.

Assay. Gas chromatography (*2.2.28*) as prescribed in the monograph *Nutmeg oil (1552)*.

Content: minimum 95.0 per cent, calculated by the normalisation procedure.

Carminic Acid 7-α-D-Glucopyranosyl-3,5,6,8-tetrahydroxy-1-methyl-9,10-dioxo-9,10-dihydroanthracene-2-carboxylic acid; $C_{22}H_{20}O_{13}$ = 492.4 (*1260-17-9*)

Dark red powder, very slightly soluble in water, soluble in dimethyl sulfoxide, very slightly soluble in ethanol (96 per cent).

Carob Bean Gum Locust bean gum

The ground endosperm of the fruit kernels of *Ceratonia siliqua* L. Taub.

White or almost white powder containing 70 per cent to 80 per cent of a water-soluble gum consisting mainly of galactomannoglycone.

Carvacrol 5-Isopropyl-2-methylphenol; $C_{10}H_{14}O$ = 150.2 (*499-75-2*)

Brownish liquid, practically insoluble in water, very soluble in ethanol (96 per cent).

d_{20}^{20}: about 0.975.

n_D^{20}: about 1.523.

bp: about 237 °C.

Carvacrol used in gas chromatography complies with the following additional test.

Assay. Gas chromatography (*2.2.28*) as prescribed in the monograph *Peppermint oil (0405)*.

Test solution. Dissolve 0.1 g in about 10 mL of *acetone R*.

Content: minimum 95.0 per cent, calculated by the normalisation procedure.

Carveol *p*-Mentha-1(6),8-dien-2-ol; 2-Methyl-5-(1-methylethenyl)cyclohex-2-enol; $C_{10}H_{16}O$ = 152.2 (*99-48-9*)

The substance contains a variable content of *trans*- and *cis*-carveol.

Carveol used in gas chromatography complies with the following additional test.

Assay. Gas chromatography (*2.2.28*) as prescribed in the test for chromatographic profile in the monograph *Caraway oil (1817)*.

Content: minimum 97 per cent, calculated by the normalisation procedure.

Carvone (+)-*p*-Mentha-6,8-dien-2-one; (5*S*)-2-Methyl-5-(1-methylethenyl)-cyclohex-2-enone; $C_{10}H_{14}O$ = 150.2 (*2244-16-8*)

Liquid, practically insoluble in water, miscible with ethanol (96 per cent).

d_{20}^{20}: about 0.965

n_D^{20}: about 1.500.

$[\alpha]_D^{20}$: about + 61.

bp: about 230 °C.

Carvone used in gas chromatography complies with the following additional test.

Assay. Gas chromatography (*2.2.28*) as prescribed in the monograph *Peppermint oil (0405)* using the substance to be examined as the test solution.

Content: minimum 98.0 per cent, calculated by the normalisation procedure.

Carvone R1

Complies with the requirements prescribed for *carvone R* with the following additional requirement.

Assay. Gas chromatography (*2.2.28*) as prescribed in the test for chiral purity in the monograph *Caraway oil (1817)*.

Content: minimum 98 per cent.

(-)-Carvone (−)-*p*-Mentha-1(6),8-dien-2-one; (5*R*)-2-Methyl-5-(1-methylethenyl)cyclohex-2-enone; $C_{10}H_{14}O$ = 150.2 (*6485-40-1*)

Liquid.

d_{20}^{20}: about 0.965.

n_D^{20}: about 1.4988.

$[\alpha]_D^{20}$: about −62.

bp: about 230 °C.

Assay. Gas chromatography (*2.2.28*) as prescribed in the test for chiral purity in the monograph *Caraway oil (1817)*.

Content: minimum 99 per cent.

β-Caryophyllene (*E*)-(1*R*,9*S*)-4,11,11-Trimethyl-8-methylenebicyclo[7.2.0]undec-4-ene; $C_{15}H_{24}$ = 204.4 (*87-44-5*)

Oily liquid, practically insoluble in water, miscible with ethanol (96 per cent).

β-Caryophyllene used in gas chromatography complies with the following additional test.

Assay. Gas chromatography (*2.2.28*) as prescribed in the monograph *Clove oil (1091)*.

Test solution. The substance to be examined.

Content: minimum 90.0 per cent, calculated by the normalisation procedure.

Caryophyllene Oxide (-)-β-Caryophyllene epoxide; (1*R*,4*R*,6*R*,10*S*)-4,12,12-Trimethyl-9-methylene-5-oxatricyclo[8.2.0.$0^{4,6}$]dodecane; $C_{15}H_{24}O$ = 220.4 (*1139-30-6*)

Colourless, fine crystals with lumps.

mp: 62 °C to 63 °C.

Caryophyllene oxide used in gas chromatography complies with the following additional test.

Assay. Gas chromatography (*2.2.28*) as prescribed in the monograph *Turpentine oil, Pinus pinaster type (1627)*.

Content: minimum 99.0 per cent, calculated by the normalisation procedure.

Casein (*9000-71-9*)

Mixture of related phosphoproteins obtained from milk.

White or almost white, amorphous powder or granules, very slightly soluble in water and in non-polar organic solvents. It dissolves in concentrated hydrochloric acid giving a pale-violet solution. It forms salts with acids and bases.
Its isoelectric point is at about pH 4.7. Alkaline solutions are laevorotatory.

Casein Substrate, Concentrated

Suspend a quantity of *casein EPBRP* equivalent to 2.5 g in 5 mL of *water*, add 18 mL of 0.1M *sodium hydroxide* and stir for 1 minute. Add 60 mL of *water* and stir with a magnetic stirrer until the solution is practically clear. Adjust the pH of the solution to 8.0 with either 0.1M *sodium hydroxide* or 0.1M *hydrochloric acid* and add sufficient *water* to produce 100 mL.

Use on the day of preparation.

Casticin 5-Hydroxy-2-(3-hydroxy-4-methoxyphenyl)-3,6,7-trimethoxy-4*H*-1-benzopyran-4-one; $C_{19}H_{18}O_8$ = 374.3 (*479-91-4*)

Yellow crystals.

Catalpol (1a*S*,1b*S*,2*S*,5a*R*,6*S*,6a*S*)-6-Hydroxy-1a-(hydroxymethyl)-1a,1b,2,5a,6,6a-hexahydrooxireno[4,5]cyclopenta[1,2-*c*]pyran-2-yl β-D-glucopyranoside; $C_{15}H_{22}O_{10}$ = 362.3 (*2415-24-9*)

mp: 203 °C to 205 °C.

Catechin (+)-(2*R*,3*S*)-2-(3,4-Dihydroxyphenyl)-3,4-dihydro-2*H*-chromene-3,5,7-triol; Catechol; Cianidanol; Cyanidol; $C_{15}H_{14}O_6$,xH_2O = 290.3 for the anhydrous substance (*154-23-4*)

Catechol Pyrocatechol; $C_6H_6O_2$ = 110.1 (*120-80-9*)

Colourless or slightly yellow crystals, soluble in water, in acetone and in ethanol (96 per cent).

mp: about 102 °C.

Storage: protected from light.

Cathine Hydrochloride (1*S*,2*S*)-2-Amino-1-phenylpropan-1-ol hydrochloride; Norpseudoephedrine hydrochloride; $C_9H_{14}ClNO$ = 187.7 (*2153-98-2*)

White or almost white solid.

Content: minimum 95.0 per cent.

Catholyte for Isoelectric Focusing pH 3 to 5

(0.1M *β-Alanine*) Dissolve 8.9 g of alanine in sufficient *water* to produce 1000 mL.

Cation Exchange Resin

A resin in protonated form with sulfonic acid groups attached to a polymer lattice consisting of polystyrene cross-linked with 8 per cent of divinylbenzene. It is available as spherical beads.

Cation Exchange Resin R1

A resin in protonated form with sulfonic acid groups attached to a polymer lattice consisting of polystyrene cross-linked with 4 per cent of divinylbenzene. It is available as spherical beads.

Cation Exchange Resin R2

Resin containing strongly acidic propylenesulfonic acid groups.

Cation Exchange Resin, Strong

Strong cation-exchange resin in protonated form with sulfonic acid groups attached to a polymer lattice consisting of polystyrene cross-linked with divinylbenzene.

Cation-exchange Resin, Weak

Resin with a carboxylate functionalised latex cross-linked with ethylvinylbenzene-divinylbenzene.

Cation Exchange Resin (Calcium Form), Strong

Resin in calcium form with sulfonic acid groups attached to a polymer lattice consisting of polystyrene cross-linked with 8 per cent of divinylbenzene.

Cation Exchange Resin (Sodium Form), Strong

Resin in sodium form with sulfonic acid groups attached to a polymer lattice consisting of polystyrene cross-linked with divinylbenzene.

Cationic Resin, Weak

Polymethacrylic resin, slightly acid, with carboxyl groups present in a protonated form.

Particle size: 75 µm to 160 µm.

pH limits of use: 5 to 14.

Maximum temperature of use: 120 °C.

Cedarwood Oil

A grade of commerce for microscopy thickened as necessary in temperate or tropical climates.

Cellulose Cellulose for chromatography; (*9004-34-6*)

Fine, white or almost white, homogeneous powder with an average particle size less than 30 µm.

Preparation of a thin layer. Suspend 15 g in 100 mL of *water R* and homogenise in an electric mixer for 60 s. Coat carefully cleaned plates with a layer 0.1 mm thick using a spreading device. Allow to dry in air.

Cellulose F_{254} Cellulose for chromatography F_{254}

Microcrystalline cellulose F_{254}. A fine, white or almost white, homogeneous powder with an average particle size less than 30 µm, containing a fluorescent indicator having an optimal intensity at 254 nm.

Preparation of a thin layer. Suspend 25 g in 100 mL of *water R* and homogenise using an electric mixer for 60 s. Coat carefully cleaned plates with a layer 0.1 mm thick using a spreading device. Allow to dry in air.

Cellulose, Microcrystalline Cellulose for chromatography R1

Microcrystalline cellulose.

Preparation of a thin layer. Suspend 25 g in 90 mL of *water R* and homogenise in an electric mixer for 60 s. Coat carefully cleaned plates with a layer 0.1 mm thick using a spreading device. Allow to dry in air.

Cephaëline Dihydrochloride (*R*)-1-[(2*S*,3*R*,11b*S*)-3-Ethyl-1,3,4,6,7,11b-hexahydro-9,10-dimethoxy-2*H*- benzo[*a*] quinolizinylmethyl]-1,2,3,4-tetrahydro-7- methoxyisoquinolin-6-ol dihydrochloride heptahydrate; $C_{28}H_{40}Cl_2N_2O_4,7H_2O$ = 666 (*5884-43-5*)

$[\alpha]_D^{20}$: about +25 (2% w/v in *water*).

General reagent grade of commerce.

Cephalin Reagent

Solvents used to prepare this reagent should contain a suitable antioxidant such as *butylated hydroxyanisole* at a concentration of 0.002% w/v.

To 0.5 to 1 g of *acetone-dried ox brain* add 20 mL of *acetone* and leave for 2 hours. Centrifuge for 2 minutes at 500 g and decant the supernatant liquid. Dry the residue under reduced pressure and extract the dried material with 20 mL of *chloroform* for 2 hours, shaking the mixture frequently. After removal of the solid material by filtration or centrifugation, evaporate the chloroform from the extract under reduced pressure. Suspend the residue in 5 to 10 mL of *saline solution*. This stock emulsion may be stored frozen or freeze-dried for 3 months.

Cerium(III) Nitrate Cerium trinitrate hexahydrate; Cerous nitrate; $Ce(NO_3)_3,6H_2O$ = 434.3 (*10294-41-4*)

Colourless or pale yellow, crystalline powder, freely soluble in water and in ethanol (96 per cent).

Cerium(III) Nitrate Solution

Dissolve 0.22 g of *cerium(III) nitrate* in 50 mL of *water*, add 0.1 mL of *nitric acid* and 50 mg of *hydroxylamine hydrochloride* and dilute to 1000 mL with *water*.

Cerium(IV) Sulfate Ceric sulfate; Ceric sulphate; Cerium(IV) sulfate tetrahydrate; Cerium sulfate; $Ce(SO_4)_2,4H_2O$ = 404.3 (*10294-42-5*)

Yellow or orange-yellow, crystalline powder or crystals, very slightly soluble in water, slowly soluble in dilute acids.

Cetostearyl Alcohol (*67762-27-0*)

See *Cetostearyl alcohol (0702)*.

Cetrimide (*8044-71-1*)

See *Cetrimide (0378)*.

Cetirizine N-Oxide $C_{21}H_{25}ClN_2O_4$ = 404.9 (*1076199-80-8*)

Cetyl Alcohol Hexadecan-1-ol; $C_{16}H_{34}O$ = 242.4 (*36653-82-4*)

Content: minimum 95.0 per cent.

mp: about 48 °C.

Cetylpyridinium Chloride Monohydrate 1-Hexadecylpyridinium chloride monohydrate; $C_{21}H_{38}ClN$, H_2O = 358.0 (*6004-24-6*)

White or almost white powder, freely soluble in water and in ethanol (96 per cent).

mp: 80 °C to 83 °C.

Cetyltrimethylammonium Bromide Cetrimonium bromide; *N*-Hexadecyl-*N,N,N*-trimethylammonium bromide; $C_{19}H_{42}BrN$ = 364.5 (*57-09-0*)

White or almost white, crystalline powder, soluble in water, freely soluble in ethanol (96 per cent).

mp: about 240 °C.

Chamazulene 7-Ethyl-1,4-dimethylazulene; $C_{14}H_{16}$ = 184.3 (*529-05-5*)

Blue liquid, very slightly soluble in water, soluble in ethanol (96 per cent), miscible with fatty oils, with essential oils and with liquid paraffin, soluble with discolouration in phosphoric acid (85 per cent *m/m*) and sulfuric acid (50 per cent *V/V*).

Appearance of solution. 50 mg is soluble in 2.5 mL of *hexane R*. The blue solution is clear in a thin-layer obtained by tilting the test-tube.

Chamazulene used for gas chromatography complies with the following additional test.

Assay. Gas chromatography (*2.2.28*) as prescribed in the monograph *Matricaria oil (1836)*.

Test solution: a 4 g/L solution in *cyclohexane R*.

Content: minimum 95.0 per cent, calculated by the normalisation procedure.

Charcoal, Activated (*64365-11-3*)

See *Activated charcoal (0313)*.

Chloral Hydrate (*302-17-0*)

See *Choral hydrate (0265)*.

Chloral Hydrate Solution

A solution of 80 g in 20 mL of *water R*.

Chloramine Solution

A 20 g/L solution of *chloramine R*. Prepare immediately before use.

Chloramine Solution R1

A 0.1 g/L solution of *chloramine R*. Prepare immediately before use.

Chloramine Solution R2

A 0.2 g/L solution of *chloramine R*. Prepare immediately before use.

Chloramine T The sodium salt of *N*-chlorotoluene-*p*-sulfonamide; Chloramine; (*7080-50-4*)

See *Tosylchloramide sodium (0381)*.

Chlordane $C_{10}H_6Cl_8$ = 409.8 (*12789-03-6*)

bp: about 175 °C.

mp: about 106 °C.

A suitable certified reference solution of technical grade (10 ng/µL in iso-octane) may be used.

Chlordiazepoxide (*58-25-3*)

See *Chlordiazepoxide (0656)*.

Chlorfenvinphos $C_{12}H_{14}Cl_3O_4P$ = 359.6 (*470-90-6*)

A suitable certified reference solution (10 ng/µL in cyclohexane) may be used.

Chlorhexidine Acetate Chlorhexidine diacetate; $C_{22}H_{30}Cl_2N_{10},2C_2H_4O_2$ = 625.6 (*56-95-1*)

mp: about 154°.

General reagent grade of commerce.

Chlorhexidine Hydrochloride Chlorhexidine dihydrochloride; $C_{22}H_{30}Cl_2N_{10},2HCl$ (*3697-42-5*)

General reagent grade of commerce.

4′-Chloroacetanilide Chloroacetanilide; C_8H_8ClNO = 169.6 (*539-03-7*)

Content: minimum 95 per cent.

Crystalline powder, practically insoluble in water, soluble in ethanol (96 per cent).

mp: about 178 °C.

Chloroacetic Acid $C_2H_3ClO_2$ = 94.5 (*79-11-8*)

Colourless or white or almost white crystals, deliquescent, very soluble in water, soluble in ethanol (96 per cent).

Storage: in an airtight container.

3-Chloroaniline C_6H_6ClN = 127.6 (*108-42-9*)

n_D^{20}: about 1.594.

General reagent grade of commerce.

4-Chloroaniline Chloroaniline; C_6H_6ClN = 127.6 (*106-47-8*)

Crystals, soluble in hot water, freely soluble in ethanol (96 per cent).

mp: about 71 °C.

Chloroauric Acid Gold chloride; hydrogen tetrachloroaurate; $HAuCl_4$ + aq (*27988-77-8*)

General reagent grade of commerce.

Brown, deliquescent masses.

Chloroauric Acid Solution

General reagent grade of commerce containing about 2% w/v of $HAuCl_4,3H_2O$, or a 2.0% w/v solution of *chloroauric acid* in *water*.

Chlorobenzene C_6H_5Cl = 112.6 (*108-90-7*)

bp: about 131°.

Analytical reagent grade of commerce.

A colourless liquid.

4-Chlorobenzenesulfonamide 4-Chlorobenzenesulphonamide; $C_6H_6ClNO_2S$ = 191.6 (*98-64-6*)

White or almost white powder.

mp: about 145 °C.

2-Chlorobenzoic Acid $C_7H_5ClO_2$ = 156.6 (*118-91-2*)

Slightly soluble in water, soluble in hot water, very soluble in anhydrous ethanol.

bp: about 285 °C.

mp: about 140 °C.

4-Chlorobenzoic Acid $C_7H_5ClO_2$ = 156.57 (*74-11-3*)

mp: about 240°.

General reagent grade of commerce.

3-(4-Chlorobenzoyl)propionic Acid $C_{10}H_9ClNO_3$ = 212.6 (*3984-34-7*)

mp: about 129°.

General reagent grade of commerce.

4-Chlorobenzophenone $C_{13}H_9ClO$ = 216.66 (*134-85-0*)

bp: about 195°.

mp: about 75°.

***p*-Chlorobenzyhydrylpiperazine** 1-(4-Chlorobenzhydryl) piperazine; $C_{17}H_{19}ClN_2$ = 286.80 (*303-26-4*)

bp: about 180°.

mp: about 67°.

General reagent grade of commerce.

1-Chlorobutane Butyl chloride; C_4H_9Cl = 92.57 (*109-69-3*)

n_D^{20}: about 1.402.

bp: about 78°.

General reagent grade of commerce.

Weight per mL, about 0.886 g.

Chlorobutanol Anhydrous chlorbutol; 1,1,1-trichloro-2-methylpropan-2-ol; (*57-15-8*)

See *Chlorobutanol (0382)*.

4-Chloro-*o*-cresol 4-Chloro-2-methylphenol; C_7H_7ClO = 142.6 (*1570-64-5*)

mp: about 144°.

General reagent grade of commerce.

2-Chloro-2-deoxy-D-glucose $C_6H_{11}ClO_5$ = 198.6 (*14685-79-1*)

White or almost white crystalline, very hygroscopic powder, soluble in water and in dimethyl sulfoxide, practically insoluble in ethanol (96 per cent).

1-Chloro-2,4-dinitrobenzene $C_6H_3ClN_2O_4$ = 202.6 (*97-00-7*)

mp: about 51°.

Analytical reagent grade of commerce.

Pale yellow crystals or crystalline powder.

2-Chloroethanol Ethylene chlorohydrin; C_2H_5ClO = 80.5 (*107-07-3*)

Colourless liquid, soluble in ethanol (96 per cent).

d_{20}^{20}: about 1.197.

n_D^{20}: about 1.442.

bp: about 130 °C.

mp: about −89 °C.

2-Chloroethanol Solution

Dissolve 125 mg of *2-chloroethanol R* in *2-propanol R* and dilute to 50 mL with the same solvent. Dilute 5 mL of the solution to 50 mL with *2-propanol R*.

Chloroethylamine Hydrochloride 2-Chloroethanamine hydrochloride; $C_2H_7Cl_2N$ = 116.0 (*870-24-6*)

mp: about 145 °C.

(2-Chloroethyl)diethylamine Hydrochloride 2-Diethylaminoethyl chloride hydrochloride; $C_6H_{15}Cl_2N$ = 172.1 (*869-24-9*)

White or almost white, crystalline powder, very soluble in water and in methanol, freely soluble in methylene chloride, practically insoluble in hexane.

mp: about 211 °C.

Chloroform Trichloromethane; $CHCl_3$ = 119.4 (*67-66-3*)

Clear, colourless liquid, slightly soluble in water, miscible with ethanol (96 per cent).

d_{20}^{20}: 1.475 to 1.481.

bp: about 60 °C.

Ethanol: 0.4 per cent *m/m* to 1.0 per cent *m/m*.

Chloroform, Acidified

To 100 mL of *chloroform R* add 10 mL of *hydrochloric acid R*. Shake, allow to stand and separate the 2 layers.

Chloroform, Ethanol-free

Shake 200 mL of *chloroform R* with four quantities, each of 100 mL, of *water R*. Dry over 20 g of *anhydrous sodium sulfate R* for 24 h. Distil the filtrate over 10 g of *anhydrous sodium sulfate R*. Discard the first 20 mL of distillate. Prepare immediately before use.

Chloroform IR

Spectroscopic reagent grade of commerce.

Chloroform Stabilised with Amylene $CHCl_3$ = 119.4

Clear, colourless liquid, slightly soluble in water, miscible with ethanol (96%).

Water maximum 0.05%.

Residue on evaporation maximum 0.001%.

Minimum transmittance (*2.2.25*) using *water* as compensation liquid : 50% at 255 nm, 80% at 260 nm, 98% at 300 nm.

Content, minimum 99.8% of $CHCl_3$, determined by gas chromatography.

Chloroform Water

Shake 2.5 mL of *chloroform* with 900 mL of *water* until dissolved and dilute to 1000 mL with *water*.

Chlorogenic Acid (1*S*,3*R*,4*R*,5*R*)-3-[(3,4-Dihydroxycinnamoyl)oxy]-1,4,5-trihydroxycyclohexanecarboxylic acid; $C_{16}H_{18}O_9$ = 354.3 (*327-97-9*)

White or almost white, crystalline powder or needles, freely soluble in boiling water, in acetone and in ethanol (96 per cent).

$[\alpha]_D^{26}$: about −35.2.

mp: about 208 °C.

Chromatography. Thin-layer chromatography (*2.2.27*) as prescribed on Identification A in the monograph *Belladonna leaf dry extract, standardised (1294)*; the chromatogram shows only one principal zone.

Chlorogenic acid used in liquid chromatography complies with the following additional test.

Assay. Liquid chromatography (*2.2.29*) as prescribed in the monograph *Artichoke Leaf (1866)*.

Content: minimum 97.0 per cent.

5-Chloro-8-hydroxyquinoline 5-Chloroquinolin-8-ol; C_9H_6ClNO = 179.6 (*130-16-5*)

Sparingly soluble in cold dilute hydrochloric acid.

mp: about 123 °C.

Content: minimum 95.0 per cent.

3-Chloro-2-methylaniline 6-Chloro-2-toluidine; C_7H_8ClN = 141.6 (*87-60-5*)

Not miscible with water, slightly soluble in anhydrous ethanol.

d_{20}^{20}: about 1.171.

n_D^{20}: about 1.587.

bp: about 115 °C.

mp: about 2 °C.

2-Chloro-*N*-(2,6-dimethylphenyl)acetamide $C_{10}H_{12}ClNO$ = 197.7 (*1131-01-7*)

2-Chloronicotinic Acid 2-Chloropyridine-3-carboxylic acid; $C_6H_4ClNO_2$ = 157.6 (*2942-59-8*)

White or almost white powder.

mp: about 177 °C.

Content: minimum 95 per cent.

2-Chloro-4-nitroaniline $C_6H_5ClN_2O_2$ = 172.6 (*121-87-9*)

Yellow, crystalline powder, freely soluble in methanol.

mp: about 107 °C.

Storage: protected from light.

2-Chloro-5-Nitrobenzoioc Acid $C_7H_4ClNO_4$ = 201.6 (*2516-96-3*)

mp: 165 °C to 168 °C.

4-Chlorophenol Chlorophenol; C_6H_5ClO = 128.6 (*106-48-9*)

Colourless or almost colourless crystals, slightly soluble in water, very soluble in ethanol (96 per cent) and in solutions of alkali hydroxides.

mp: about 42 °C.

2-[2-(4-Chlorophenyl)acetyl]benzoic Acid $C_{15}H_{11}ClO_3$ = 274.7 (*53242-76-5*)

Chloroplatinic(IV) Acid Platinic chloride; Chloroplatinic acid; $H_2Cl_6Pt,6H_2O$ = 517.9 (*18497-13-7*)

Content: minimum 37.0 per cent *m/m* of platinum (A_r 195.1).

Brownish-red crystals or a crystalline mass, very soluble in water, soluble in ethanol (96 per cent).

Assay. Ignite 0.200 g to constant mass at 900 ± 50 °C and weigh the residue (platinum).

Storage: protected from light.

Chloroplatinic Acid Solution

A solution of *chloroplatinic(IV) acid* in *water*, containing the equivalent of 5.0% w/v of $H_2PtCl_6,6H_2O$.

3-Chloropropane-1,2-diol $C_3H_7ClO_2$ = 110.5 (*96-24-2*)

Colourless liquid, soluble in water and ethanol (96 per cent).

d_{20}^{20}: about 1.322.

n_D^{20}: about 1.480.

bp: about 213 °C.

1-Chloropropyl(dimethylamine) Hydrochloride 3-Dimethylaminopropyl chloride hydrochloride; $C_5H_{12}ClN$ = 158.1 (*5407-04-5*)

mp: about 142°.

General reagent grade of commerce.

4-Chlororesorcinol 4-Chlorobenzene-1,3-diol; 1,3-Dihydroxy-4-chlorobenzene; $C_6H_5ClO_2$ = 144.6 (*95-88-5*)

mp: 106 °C to 108 °C.

5-Chlorosalicylic Acid $C_7H_5ClO_3$ = 172.6 (*321-14-2*)

White or almost white, crystalline powder, soluble in methanol.

mp: about 173 °C.

4-Chlorosulfamoylbenzoic Acid $C_7H_6ClNO_4S$ = 235.7 (*1205-30-7*)

General reagent grade of commerce.

Chlorothiazide 6-Chloro-2*H*-1,2,4-benzothiadiazine-7-sulfonamide 1,1-dioxide; $C_7H_6ClN_3O_4S_2$ = 295.7 (*58-94-6*)

Content: minimum 98.0 per cent.

White or almost white, crystalline powder, very slightly soluble in water, sparingly soluble in acetone, slightly soluble in ethanol (96 per cent). It dissolves in dilute solutions of alkali hydroxides.

Chlorotriphenylmethane Triphenylchloromethane; triphenylmethyl chloride; $C_{19}H_{15}Cl$ = 278.8 (*76-83-5*)

mp: about 112°.

General reagent grade of commerce.

A pale yellow or buff, crystalline solid.

Chlorpyriphos $C_9H_{11}Cl_3NO_3PS$ = 350.6 (*2921-88-2*)

bp: about 200 °C.

mp: 42 °C to 44 °C.

A suitable certified reference solution (10 ng/µL in cyclohexane) may be used.

Chlorpyriphos-methyl $C_7H_7Cl_3NO_3PS$ = 322.5 (*5598-13-0*)

mp: 45 °C to 47 °C.

A suitable certified reference solution (10 ng/µL in cyclohexane) may be used.

Chlortetracycline Hydrochloride

See *Chlortetracycline hydrochloride (0173)*.

(5α)-Cholestane $C_{27}H_{48}$ = 372.7 (*481-21-0*)

Slightly soluble in anhydrous ethanol.

mp: about 81 °C.

Cholesterol (*57-88-5*)

See *Cholesterol (0993)*.

Cholesteryl Benzoate 5-Cholesten-3β-ol-3-benzoate; $C_{34}H_{50}O_2$ = 490.8 (*604-32-0*)

mp: about 150°.

General reagent grade of commerce.

Choline Chloride (2-Hydroxyethyl)trimethylammonium chloride; $C_5H_{14}ClNO$ = 139.6 (*67-48-1*)

Deliquescent crystals, very soluble in water and in ethanol (96 per cent).

Chromatography. Thin-layer chromatography (*2.2.27*) as prescribed in the monograph *Suxamethonium chloride (0248)*: apply 5 µL of a 0.2 g/L solution in *methanol R*; the chromatogram shows one principal spot.

Storage: in an airtight container.

Chondroitinase ABC

Pectin lyase-like enzyme secreted by *Flavobacterium heparinum*. Available in vials containing 5-10 units. It cleaves both glucuronate-containing disaccharides, e.g. chondroitin sulfate, and iduronate-containing disaccharides, e.g. dermatan sulfate.

Chondroitinase AC

Pectin lyase-like enzyme secreted by *Flavobacterium heparinum*. Available in vials containing 5-10 units. It cleaves only glucuronate-containing disaccharides, e.g. chondroitin sulfate.

Chrome Azurol S Trisodium 5-[(3-carboxylato- 5-methyl-4-oxocyclohexa-2,5-dien-1-ylidene)(2,6-dichloro-3-sulfonatophenyl)methyl]-2-hydroxy-3-methylbenzoate; Chromazurol S; $C_{23}H_{13}Cl_2Na_3O_9S$ = 605 (*1667-99-8*)

Schultz No. 841

Colour Index No. 43825

Brownish-black powder, soluble in water, slightly soluble in ethanol (96 per cent).

Chromic Acid Cleansing Mixture

See Chromic-sulfuric acid mixture.

Chromic-Sulfuric Acid Mixture

Chromic-sulphuric acid mixture

A saturated solution of *chromium(VI) oxide* in *sulfuric acid*.

Chromium(III) Acetylacetonate (*OC*-6-11)-Tris(2,4-pentanedionato-κ*O*,κ*O*′)chromium; $C_{15}H_{21}CrO_6$ = 349.3 (*21679-31-2*)

Chromium(VI) Oxide Chromium trioxide; CrO_3 = 100.0 (*1333-82-0*)

Dark brownish-red needles or granules, deliquescent, very soluble in water.

Storage: in an airtight glass container.

Chromium(III) Potassium Sulfate Chrome alum; Chromic potassium sulphate; Chromium(III) potassium sulphate; Chromic potassium sulfate; $CrK(SO_4)_2,12H_2O$ = 499.4 (*7788-99-0*)

Large, violet-red or black crystals, freely soluble in water, practically insoluble in ethanol (96 per cent).

Chromium(III) Trichloride Hexahydrate $[Cr(H_2O)_4Cl_2]Cl,2H_2O$ = 266.5 (*10060-12-5*)

Dark green crystalline powder, hygroscopic.

Storage: protected from humidity and oxidising agents.

Chromogenic Substrate R4

Dissolve D-phenylalanyl-L-pipecolyl-L-arginine-4-nitroanilide dihydrochloride in *water R* to give a 0.008 M solution. Dilute to 0.0025 M with *phosphate buffer solution pH 8.5 R* before use.

Chromogenic Substrate R5

Dissolve *N*-benzoyl-L-isoleucyl-L-glutamyl-glycyl-L-arginine-4-nitroanilide hydrochloride in *water R* to give a 0.003 M solution.

Chromophore Substrate R1 Chromogenic substrate R1

Dissolve *N*-α-benzyloxycarbonyl-D-arginyl-L-glycyl-L-arginine-4-nitroanilide dihydrochloride in *water R* to give a 0.003 M solution. Dilute in *tris(hydroxymethyl)aminomethane-EDTA buffer solution pH 8.4 R* to 0.0005 M before use.

Chromophore Substrate R2 Chromogenic substrate R2

Dissolve D-phenylalanyl-L-pipecolyl-L-arginine-4-nitroanilide dihydrochloride in *water R* to give a 0.003 M solution. Dilute before use in titrating in *tris(hydroxymethyl)aminomethane-EDTA buffer solution pH 8.4 R* to give a 0.0005 M solution.

Chromophore Substrate R3 Chromogenic substrate R3

Dissolve D-valyl-leucyl-lysyl-4-nitroanilide dihydrochloride in *water R* to give a 0.003 M solution.

Chromotrope IIB Chromotrope 2B; $C_{16}H_9N_3Na_2O_{10}S_2$ = 513.4 (*548-80-1*)

Schultz No. 67

Colour Index No. 16575

Reddish-brown powder, soluble in water giving a yellowish-red colour, practically insoluble in ethanol (96 per cent).

Chromotrope IIB Solution Chromotrope 2B solution

A 0.05 g/L solution of *chromotrope II B R* in *sulfuric acid R*.

Chromotropic Acid 4,5-Dihydroxy-2,7-naphthalenedisulfonic acid; $C_{10}H_8O_8S_2$ = 320.3 (*148-25-4*)

mp: about 300°.

General reagent grade of commerce.

Chromotropic Acid Sodium Salt Disodium 1,8-dihydroxynaphthalene-3,6-disulfonate dihydrate; $C_{10}H_6Na_2O_8S_2,2H_2O$ = 400.3 (*5808-22-0*)

Schultz No. 1136

A yellowish-white powder, soluble in water, practically insoluble in ethanol (96 per cent).

Chromotropic Acid, Sodium Salt Solution

Dissolve 0.60 g of *chromotropic acid, sodium salt R* in about 80 mL of *water R* and dilute to 100 mL with the same solvent. Use this solution within 24 h.

Chromotropic Acid Solution

Dissolve 0.5 g of *chromotropic acid* in sufficient *water* to produce 100 mL.

Use the solution within 24 hours.

Chromotropic Acid-Sulfuric Acid Solution Chromotropic Acid-Sulphuric Acid Solution

Dissolve 5 mg of *chromotropic acid, sodium salt R* in 10 mL of a mixture of 9 mL of *sulfuric acid R* and 4 mL of *water R*.

Chrysanthemin Cyanidin 3-*O*-glucoside chloride; Kuromanin chloride; 2-(3,4-Dihydroxyphenyl)-3-(β-D-glucopyranosyl)oxy-5,7-dihydroxy-1-benzopyrylium chloride; $C_{21}H_{21}ClO_{11}$ = 485.8 (*7084-24-4*)

Reddish-brown crystalline powder, soluble in water and in ethanol (96 per cent).

Absorbance (*2.2.25*). A 0.01 g/L solution in a mixture of 1 volume of *hydrochloric acid R* and 999 volumes of *methanol R* shows an absorption maximum at 528 nm.

α-Chymotrypsin for Peptide Mapping

α-Chymotrypsin of high purity, treated to eliminate tryptic activity.

Cimifugin (2*S*)-7-(Hydroxymethyl)-2-(1-hydroxy-1-methylethyl)-4-methoxy-2,3-dihydro-5*H*-furo[3,2-*g*][1]benzopyran-5-one; $C_{16}H_{18}O_6$ = 306.3 (*37921-38-3*)

Cinchonidine (*R*)-(Quinol-4-yl)[(2*S*,4*S*,5*R*)-5-vinylquinuclidin-2-yl]methanol; $C_{19}H_{22}N_2O$ = 294.4 (*485-71-2*)

White or almost white, crystalline powder, very slightly soluble in water and in light petroleum, soluble in ethanol (96 per cent).

$[\alpha]_D^{20}$: −105 to −110, determined on a 50 g/L solution in *ethanol (96 per cent) R*.

mp: about 208 °C, with decomposition.

Storage: protected from light.

Cinchonine (*S*)-(Quinol-4-yl)[(2*R*,4*S*,5*R*)-5-vinylquinuclidin-2-yl]methanol; $C_{19}H_{22}N_2O$ = 294.4 (*118-10-5*)

White or almost white, crystalline powder, very slightly soluble in water, sparingly soluble in ethanol (96 per cent) and in methanol.

$[\alpha]_D^{20}$: + 225 to + 230, determined on a 50 g/L solution in *ethanol (96 per cent) R*.

mp: about 263 °C.

Storage: protected from light.

Cineole 1,8-Cineole; Eucalyptol; 1,8-Epoxy-*p*-menthane; $C_{10}H_{18}O$ = 154.3 (*470-82-6*)

Colourless liquid, practically insoluble in water, miscible with anhydrous ethanol.

d_{20}^{20}: 0.922 to 0.927.

n_D^{20}: 1.456 to 1.459.

Freezing point (*2.2.18*): 0 °C to 1 °C.

Distillation range (*2.2.11*): 174 °C to 177 °C.

Phenol. Shake 1 g with 20 mL of *water R*. Allow to separate and add to 10 mL of the aqueous layer 0.1 mL of *ferric chloride solution R1*. No violet colour develops.

Turpentine oil. Dissolve 1 g in 5 mL of *ethanol (90 per cent V/V) R*. Add dropwise freshly prepared *bromine water R*. Not more than 0.5 mL is required to give a yellow colour lasting for 30 min.

Residue on evaporation: maximum 0.05 per cent.

To 10.0 mL add 25 mL of *water R*, evaporate on a water-bath and dry the residue to constant mass at 100-105 °C.

Cineole used in gas chromatography complies with the following additional test.

Assay. Gas chromatography (*2.2.28*) as prescribed in the monograph *Peppermint oil (0405)*.

Test solution. The substance to be examined.

Content: minimum 98.0 per cent, calculated by the normalisation procedure.

1,4-Cineole 1-Methyl-4-(1-methylethyl)-7-oxabicyclo [2.2.1]heptane; 1-Isopropyl-4-methyl-7-oxabicyclo[2.2.1] heptane; $C_{10}H_{18}O$ = 154.3 (*470-67-7*)

Colourless liquid.

d_4^{20}: about 0.900.

n_D^{20}: about 1.445.

bp: about 173 °C.

Cinnamaldehyde 3-Phenylpropenal; Cinnamic aldehyde; C_9H_8O = 132.2 (*104-55-2*)

Yellowish or greenish-yellow, oily liquid, slightly soluble in water, very soluble in ethanol (96 per cent).

n_D^{20}: about 1.620.

Storage: protected from light.

Cinnamamide (*E*)-3-Phenylprop-2-enamide; C_9H_9NO = 147.2 (*621-79-4*)

White or almost white powder.

mp: about 149 °C.

Cinnamic Acid *trans*-3-Phenylacrylic acid; (2E)-3-Phenylprop-2-enoic acid; *trans*-Cinnamic acid; $C_9H_8O_2$ = 148.2 (*140-10-3*)

Colourless crystals, very slightly soluble in water, freely soluble in ethanol (96 per cent).

mp: 133 °C.

***trans*-Cinnamic Aldehyde** (*E*)-3-Phenylprop-2-enal; C_9H_8O = 132.2 (*14371-10-9*)

trans-Cinnamic aldehyde used in gas chromatography complies with the following additional test.

Assay. Gas chromatography (*2.2.28*) as prescribed in the monograph *Cassia oil (1496)*.

Content: minimum 99.0 per cent, calculated by the normalisation procedure.

Cinnamyl Acetate 3-Phenylprop-2-en-1-yl acetate; $C_{11}H_{12}O_2$ = 176.2 (*103-54-8*)

n_D^{20}: about 1.542.

bp: about 262 °C.

Cinnamyl acetate used in gas chromatography complies with the following additional test.

Assay. Gas chromatography (*2.2.28*) as prescribed in the monograph *Cassia oil (1496)*.

Content: minimum 99.0 per cent, calculated by the normalisation procedure.

Citral 3,7-Dimethylocta-2,6-dienal; $C_{10}H_{16}O$ = 152.2 (*5392-40-5*)

Light yellow liquid, practically insoluble in water, miscible with ethanol (96 per cent) and with propylene glycol.

Chromatography. Thin-layer chromatography (*2.2.27*), using *silica gel* GF_{254} *R* as the coating substance: apply to the plate 10 µL of a 1 g/L solution in *toluene R*. Develop over a path of 15 cm using a mixture of 15 volumes of *ethyl acetate R* and 85 volumes of *toluene R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. The chromatogram shows only one principal spot.

Citral used in gas chromatography complies with the following additional test.

Assay. Gas chromatography (*2.2.28*) as prescribed in the monograph *Citronella oil (1609)*.

Content of citral (neral + geranial): minimum 95.0 per cent, calculated by the normalisation procedure.

Citrate Buffered Saline

Dissolve 11.76 g of *sodium citrate* in 1800 mL of water, adjust to pH 7.0 with 1M *hydrochloric acid* and add suffient *water* to produce 2000 mL.

Citrated Rabbit Plasma

Collect blood by intracardiac puncture from a rabbit kept fasting for 12 h, using a plastic syringe with a No. 1 needle containing a suitable volume of 38 g/L solution of *sodium citrate R* so that the final volume ratio of citrate solution to blood is 1: 9. Separate the plasma by centrifugation at 1500 *g* to 1800 *g* at 15 °C to 20 °C for 30 min.

Storage: at 0 °C to 6 °C; use within 4 h of collection.

Citric Acid Citric acid monohydrate; (*5949-29-1*)

See *Citric acid monohydrate (0456)*.

When used in the test for iron, it complies with the following additional requirement.

Dissolve 0.5 g in 10 mL of *water R*, add 0.1 mL of *thioglycollic acid R*, mix and make alkaline with *ammonia R*. Dilute to 20 mL with *water R*. No pink colour appears in the solution.

Citric Acid, Anhydrous (*77-92-9*)

See *Citric acid (0455)*.

Citric-Molybdic Acid Solution

Mix 54 g of *molybdenum(VI) oxide* with 200 mL of *water*, add 11 g of *sodium hydroxide* and heat, with stirring, until almost complete solution has been obtained. Dissolve 60 g of *citric acid* in 250 mL of *water* and add 140 mL of *hydrochloric acid*. Add the first solution to the second, stirring continuously, cool, filter if necessary, dilute to 1000 mL with *water* and add, dropwise, sufficient of a 1% w/v solution of *potassium bromate* to discharge the green colour.

Store in a well-closed container, protected from light.

Citronellal 3,7-Dimethyl-6-octenal; $C_{10}H_{18}O$ = 154.3 (*106-23-0*)

Very slightly soluble in water, soluble in ethanol (96 per cent).

d_{20}^{20}: 0.848 to 0.856.

n_D^{20}: about 1.446.

Citronellal used in gas chromatography complies with the following additional test.

Assay. Gas chromatography (*2.2.28*) as prescribed in the monograph *Citronella oil (1609)*.

Content: minimum 95.0 per cent, calculated by the normalisation procedure.

Citronellol 3,7-Dimethyloct-6-en-1-ol; $C_{10}H_{20}O$ = 156.3 (*106-22-9*)

Clear, colourless liquid, practically insoluble in water, miscible with ethanol (96 per cent).

d_{20}^{20}: 0.857.

n_D^{20}: 1.456.

bp: 220 °C to 222 °C.

Citronellol used in gas chromatography complies with the following additional test.

Assay. Gas chromatography (*2.2.28*) as prescribed in the monograph *Citronella oil (1609)*.

Content: minimum 95.0 per cent, calculated by the normalisation procedure.

Storage: in an airtight container, protected from light.

Citronellyl Acetate 3,7-Dimethyl-6-octen-1-yl acetate; $C_{12}H_{22}O_2$ = 198.3 (*150-84-5*)

d_{20}^{20}: 0.890.

n_D^{20}: 1.443.

bp: 229 °C.

Citronellyl acetate used in gas chromatography complies with the following additional test.

Assay. Gas chromatography (*2.2.28*) as prescribed in the monograph *Citronella oil (1609)*.

Content: minimum 95.0 per cent, calculated by the normalisation procedure.

Storage: in an airtight container, protected from light.

Citropten 5,7-Dimethoxycoumarin; $C_{11}H_{10}O_4$ = 206.2 (*487-06-9*)

Needle-shaped crystals, practically insoluble in water and in light petroleum, freely soluble in acetone and in ethanol (96 per cent).

mp: about 145 °C.

Chromatography. Thin-layer chromatography (*2.2.27*), using *silica gel* GF_{254} *R* as the coating substance: apply to the plate 10 µL of a 1 g/L solution in *toluene R*. Develop over a path of 15 cm using a mixture of 15 volumes of *ethyl acetate R* and 85 volumes of *toluene R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. The chromatogram obtained shows only one principal spot.

Clobetasol Propionate 21-Chloro-9-fluoro-11β,17-dihydroxy-16β-methylpregna-1,4-diene-3,20-dione 17-propionate; $C_{25}H_{32}ClFO_5$ = 467.0 (*25122-46-7*)

White or almost white crystalline powder, insoluble in water, soluble in ethanol (96 per cent) and in acetone.

$[\alpha]_D^{20}$: about + 104 (in dioxan).

mp: about 196 °C.

Coagulation Factor V Solution Clotting factor V solution

Coagulation factor V solution may be prepared by the following method or by any other method which excludes factor VIII.

Prepare the factor V reagent from fresh oxalated bovine plasma, by fractionation at 4 °C with a saturated solution of

ammonium sulfate R prepared at 4 °C. Separate the fraction which precipitates between 38 per cent and 50 per cent of saturation, which contains factor V without significant contamination with factor VIII. Remove the ammonium sulfate by dialysis and dilute the solution with a 9 g/L solution of *sodium chloride R* to give a solution containing between 10 per cent and 20 per cent of the quantity of factor V present in fresh human normal plasma.

Assay of factor V. Prepare two dilutions of the preparation of factor V in *imidazole buffer solution pH 7.3 R* containing 1 volume of the preparation in 10 volumes and in 20 volumes of the buffer solution respectively. Test each dilution as follows: mix 0.1 mL of *plasma substrate deficient in factor V R*, 0.1 mL of the solution to be examined, 0.1 mL of *thromboplastin R* and 0.1 mL of a 3.5 g/L solution of *calcium chloride R* and measure the coagulation times, i.e. the interval between the moment at which the calcium chloride solution is added and the first indication of the formation of fibrin, which may be observed visually or by means of a suitable apparatus.

In the same manner, determine the coagulation time (in duplicate) of four dilutions of human normal plasma in *imidazole buffer solution pH 7.3 R*, containing respectively, 1 volume in 10 (equivalent to 100 per cent of factor V), 1 volume in 50 (20 per cent), 1 volume in 100 (10 per cent), and 1 volume in 1000 (1 per cent). Using two-way logarithmic paper plot the average coagulation times for each dilution of human plasma against the equivalent percentage of factor V and read the percentage of factor V for the two dilutions of the factor V solution by interpolation. The mean of the two results gives the percentage of factor V in the solution to be examined.

Storage: in the frozen state at a temperature not higher than −20 °C.

Cobalt(II) Acetate Cobaltous acetate; $(CH_3CO_2)_2Co,4H_2O$ = 249.1 (*6147-53-1*)

General reagent grade of commerce.

Cobalt(II) Chloride Cobaltous chloride; Cobalt chloride; $CoCl_2,6H_2O$ = 237.9 (*7791-13-1*)

Red, crystalline powder or deep-red crystals, very soluble in water, soluble in ethanol (96 per cent).

Cobalt(II) Nitrate Cobaltous nitrate; Cobalt nitrate; $Co(NO_3)_2,6H_2O$ = 291.0 (*10026-22-9*)

Small garnet-red crystals, very soluble in water.

Codeine (*6059-47-8*)

See *Codeine monohydrate (0076)*.

Codeine Phosphate (*52-28-8*)

See *Codeine phosphate hemihydrate (0074)*.

2,4,6-Collidine 2,4,6-Trimethylpyridine; $C_8H_{11}N$ = 121.2 (*108-75-8*)

bp: about 170°.

General reagent grade of commerce.

A colourless to pale yellow liquid; weight per mL, about 0.92 g.

It may be stabilised by the addition of aluminium oxide.

Columbin (1*R*,4*R*,4a*R*,6a*R*,9*S*,10a*S*,10b*S*)-9-(3-Furanyl)-1,4,4a,5,6,6a,9,10,10a,10b-decahydro-4-hydroxy-4a,10a-dimethyl-1,4-etheno-3*H*,7*H*-benzo[1,2-c:3,4-c']dipyran-3,7-dione; $C_{20}H_{22}O_6$ = 358.4 (*546-97-4*)

Reagent grade of commerce.

Crystalline solid.

Congo Red Disodium (biphenyl-4,4′-diyl-bis-2,2′-azo) bis(1-aminonaphthalene-4-sulfonate); $C_{32}H_{22}N_6Na_2O_6S_2$ = 697 (*573-58-0*)

Schultz No. 360

Colour Index No. 22120

Brownish-red powder, soluble in water.

Congo Red Fibrin Fibrin congo red

Take 1.5 g of fibrin and leave overnight in 50 mL of a 20 g/L solution of *congo red R* in *ethanol (90 per cent V/V) R*. Filter, rinse the fibrin with *water R* and store under *ether R*.

Congo Red Paper

Immerse strips of filter paper for a few minutes in *congo red solution R*. Allow to dry.

Congo Red Solution

Dissolve 0.1 g of *congo red R* in a mixture of 20 mL of *ethanol (96 per cent) R* and *water R* and dilute to 100 mL with *water R*.

Test for sensitivity. To 0.2 mL of the congo red solution add 100 mL of *carbon dioxide-free water R* and 0.3 mL of *0.1 M hydrochloric acid*. The solution is blue. Not more than 0.3 mL of *0.1 M sodium hydroxide* is required to change the colour to pink.

Colour change: pH 3.0 (blue) to pH 5.0 (pink).

Convallatoxin 3β-[(6-Deoxy-α-L-mannopyranosyl)oxy]-5,14-dihydroxy-19-oxo-5β-card-20(22)-enolide; 5,14-Dihydroxy-19-oxo-3β-[(α-L-rhamnopyranosyl)oxy]-5β-card-20(22)-enolide; $C_{29}H_{42}O_{10}$ = 550.6 (*508-75-8*)

White or slightly yellow, crystalline powder, slightly soluble in water, soluble in ethanol, and in acetone, slightly soluble in ethyl acetate.

mp: 235-242 °C.

Coomassie Blue (*3861-73-2*)

See *acid blue 92 R*.

Coomassie Blue Solution See *acid blue 92 solution R*.

Coomassie Staining Solution

A 1.25 g/L solution of *acid blue 83 R* in a mixture consisting of 1 volume of *glacial acetic acid R*, 4 volumes of *methanol R* and 5 volumes of *water R*. Filter.

Coomassie Staining Solution R1

Dissolve 0.275 g of *acid blue 83 R* in 200 mL of *methanol R*. Stir until complete dissolution of the crystals (for about 2 h). Add 750 mL of *water R* and 50 mL of *glacial acetic acid R*. Stir overnight (for at least 16 h); filter.

Copper Cu = 63.55 (*7440-50-8*)

Cleaned foil, turnings, wire or powder of the pure metal of electrolytic grade.

Copper(II) Acetate Cupric acetate; Copper acetate; $C_4H_6CuO_4,H_2O$ = 199.7 (*6046-93-1*)

Blue-green crystals or powder, freely soluble in boiling water, soluble in water and in ethanol (96 per cent), slightly soluble in glycerol (85 per cent).

Copper Carbonate Approximately $CuCO_3,Cu(OH)_2,H_2O$ (*12069-69-1*)

General reagent grade of commerce.

Copper(II) Chloride Cupric chloride; $CuCl_2,2H_2O$ = 170.5 (*10125-13-0*)

Greenish-blue powder or crystals, deliquescent in moist air, efflorescent in dry air, freely soluble in water, in ethanol (96 per cent) and in methanol, sparingly soluble in acetone.

Storage: in an airtight container.

Copper Chloride-Pyridine Reagent

Dissolve 40 mg of *copper(II) chloride* in *pyridine*, warming until complete dissolution is effected, and cool. Add 1 mL of *carbon disulfide* and sufficient *pyridine* to produce 100 mL.

Copper Edetate Solution

To 2 mL of a 20 g/L solution of *copper acetate R* add 2 mL of *0.1 M sodium edetate* and dilute to 50 mL with *water R*.

Copper(II) Nitrate Chloride dinitrate trihydrate; Cupric nitrate; Copper nitrate; $Cu(NO_3)_2,3H_2O$ = 241.6 (*10031-43-3*)

Dark blue crystals, hygroscopic, very soluble in water giving a strongly acid reaction, freely soluble in ethanol (96 per cent) and in dilute nitric acid.

Storage: in an airtight container.

Copper Oxide Solution, Ammoniacal

Triturate 0.5 g of *copper carbonate* with 10 mL of *water* and gradually add 10 mL of 13.5M *ammonia*.

Copper(II) Sulfate Copper Sulfate; Copper sulphate; Copper(II) sulphate; Cupric sulfate; Cupric sulphate; Copper sulfate pentahydrate; $CuSO_4,5H_2O$ = 249.7 (*7758-99-8*)

Blue powder or deep-blue crystals, slowly efflorescent, very soluble in water, slightly soluble in ethanol (96 per cent).

Copper Sulfate, Anhydrous $CuSO_4$ = 159.6 (*7758-98-7*)

Greenish-grey powder, hygroscopic, freely soluble in water, slightly soluble in methanol and practically insoluble in ethanol (96 per cent).

Copper Sulfate-Pyridine Reagent

Copper sulphate-pyridine reagent

Dissolve 4 g of *copper(II) sulfate* in 90 mL of *water* and add 30 mL of *pyridine*.

Prepare immediately before use.

Copper Sulfate Solution Copper sulphate solution

A 125 g/L solution of *copper sulfate pentahydrate R*.

Copper Sulfate Solution R1

To 600 mL of *water R* slowly add 80 mL of *phosphoric acid R*. Dissolve with stirring 100 g of *anhydrous copper sulfate R* and dilute to 1 L with *water R*.

Copper Sulfate Solution, Weak

Copper sulphate solution, weak

A 10% w/v solution of *copper(II) sulfate*.

Copper Tetrammine, Ammoniacal Solution of

Dissolve 34.5 g of *copper sulfate pentahydrate R* in 100 mL of *water R* and, whilst stirring, add dropwise *concentrated ammonia R* until the precipitate which forms dissolves completely. Keeping the temperature below 20 °C, add dropwise with continuous shaking 30 mL of *strong sodium hydroxide solution R*. Filter through a sintered-glass filter (40) (*2.1.2*), wash with *water R* until the filtrate is clear and take up the precipitate with 200 mL of *concentrated ammonia R*. Filter through a sintered-glass filter (*2.1.2*) and repeat the filtration to reduce the residue to a minimum.

Corallin Sodium salt of rosolic acid; (*603-45-2*)

Colour Index No. 43811

General reagent grade of commerce.

Hard, dull red masses.

Corallin Solution, Alkaline

Dissolve 5 g of *corallin* in 100 mL of *ethanol (90%)*. Immediately before use add 1 mL of the solution to 20 mL of a 20% w/v solution of *sodium carbonate*.

Cortisone $C_{21}H_{28}O_5$ = 360.4 (*53-06-5*)

Content: minimum 95.0 per cent.

mp: 223-228 °C.

Cortisone Acetate (*50-04-4*)

See *Cortisone acetate (0321)*.

Corydaline (13*S*,13a*R*)-5,8,13,13a-Tetrahydro-2,3,9,10-tetramethoxy-13-methyl-6*H*-dibenzo[*a*,*g*]quinolizine; $C_{22}H_{27}NO_4$ = 369.4 (*518-69-4*)

Costunolide (3a*S*,6*E*,10*E*,11a*R*)-6,10-Dimethyl-3-methylene-3a,4,5,8,9,11a-hexahydrocyclodeca[*b*]furan-2(3*H*)-one; $C_{15}H_{20}O_2$ = 232.3 (*553-21-9*)

Coumaphos $C_{14}H_{16}ClO_5PS$ = 362.8 (*56-72-4*)

mp: 91 °C to 92 °C.

A suitable certified reference solution (10 ng/µL in iso-octane) may be used.

***o*-Coumaric Acid** (*E*)-2-Hydroxycinnamic acid; (2*E*)-3-(2-Hydroxyphenyl)prop-2-enoic acid; $C_9H_8O_3$ = 164.2 (*614-60-8*)

White or almost white powder.

mp: about 217 °C.

Coumarin 2*H*-Chromen-2-one; 2*H*-1-Benzopyran-2-one; $C_9H_6O_2$ = 146.1 (*91-64-5*)

Colourless, crystalline powder or orthorhombic or rectangular crystals, very soluble in boiling water, soluble in ethanol (96 per cent). It dissolves in solutions of alkali hydroxides.

mp: 68 °C to 70 °C.

Coumarin used in gas chromatography complies with the following additional test.

Assay. Gas chromatography (*2.2.28*) as prescribed in the monograph *Cassia oil (1496)*.

Content: minimum 98.0 per cent, calculated by the normalisation procedure.

Coumestrol $C_{15}H_8O_5$ = 268.22 (*479-13-0*)

General reagent grade of commerce.

***m*-Cresol** (*108-39-4*)

See *metacresol (2077)*.

***o*-Cresol** Cresol; C_7H_8O = 108.1 (*95-48-7*)

Crystals or a super-cooled liquid becoming dark on exposure to light and air, miscible with anhydrous ethanol, soluble in about 50 parts of water and soluble in solutions of alkali hydroxides.

d_{20}^{20}: about 1.05.

n_D^{20}: 1.540 to 1.550.

bp: about 190 °C.

Freezing point (*2.2.18*): minimum 30.5 °C.

Residue on evaporation: maximum 0.1 per cent *m/m*, determined by evaporating on a water-bath and drying in an oven at 100-105 °C.

Storage: protected from light, moisture and oxygen.

Distil before use.

***p*-Cresol** 4-Methylphenol; C_7H_8O = 108.1 (*106-44-5*)

Colourless or white or almost white crystals or crystalline mass.

d_{20}^{20}: about 1.02.

bp: about 202 °C.***m*-Cresol Purple**

m-Cresolsulfonphthalein; $C_{21}H_{18}O_5S$ = 382.44 (*2303-01-7*)

Olive-green, crystalline powder, slightly soluble in water, soluble in ethanol (96 per cent), in glacial acetic acid and in methanol.

***m*-Cresol Purple Solution**

Dissolve 0.1 g of *m-cresol purple R* in 13 mL of *0.01 M sodium hydroxide*, dilute to 100 mL with *water R* and mix.

Colour change: pH 1.2 (red) to pH 2.8 (yellow); pH 7.4 (yellow) to pH 9.0 (purple).

Cresol Red Cresolsulfonphthalein; 4,4′-(3*H*-2,1-Benzoxathiol-3-ylidene)bis-(2-methylphenol) *S*,*S*-dioxide; $C_{21}H_{18}O_5S$ = 382.4 (*1733-12-6*)

A reddish-brown crystalline powder, slightly soluble in water, soluble in ethanol (96 per cent) and in dilute solutions of alkali hydroxides.

Cresol Red Solution

Dissolve 0.1 g of *cresol red R* in a mixture of 2.65 mL of *0.1 M sodium hydroxide* and 20 mL of *ethanol (96 per cent) R* and dilute to 100 mL with *water R*.

Test for sensitivity. A mixture of 0.1 mL of the cresol red solution and 100 mL of *carbon dioxide-free water R* to which 0.15 mL of *0.02 M sodium hydroxide* has been added is purple-red. Not more than 0.15 mL of *0.02 M hydrochloric acid* is required to change the colour to yellow.

Colour change: pH 7.0 (yellow) to pH 8.6 (red).

Crystal Violet Basic violet 3; $C_{25}H_{30}ClN_3$ = 408.0 (*548-62-9*)

Schultz No. 78

Colour Index No. 42555

Dark-green powder or crystals, soluble in water and in ethanol (96 per cent).

Crystal Violet Solution

Dissolve 0.5 g of *crystal violet R* in *anhydrous acetic acid R* and dilute to 100 mL with the same solvent.

Test for sensitivity. To 50 mL of *anhydrous acetic acid R* add 0.1 mL of the crystal violet solution. On addition of 0.1 mL of *0.1 M perchloric acid* the bluish-purple solution turns bluish-green.

Cupri-citric Solution

Dissolve 25 g of *copper sulfate pentahydrate R*, 50 g of *citric acid monohydrate R* and 144 g of *anhydrous sodium carbonate R* in *water R* and dilute to 1000 mL with the same solvent.

Cupri-citric Solution R1

Dissolve 25 g of *copper sulfate pentahydrate R*, 50 g of *citric acid monohydrate R* and 144 g of *anhydrous sodium carbonate R* in *water R* and dilute to 1000 mL with the same solvent.

Adjust the solution so that it complies with the following requirements.

a) To 25.0 mL add 3 g of *potassium iodide R*. Add 25 mL of a 25 per cent *m/m* solution of *sulfuric acid R* with precaution and in small quantities. Titrate with *0.1 M sodium thiosulfate* using 0.5 mL of *starch solution R*, added towards the end of the titration, as indicator.

24.5 mL to 25.5 mL of *0.1 M sodium thiosulfate* is used in the titration.

b) Dilute 10.0 mL to 100.0 mL with *water R* and mix. To 10.0 mL of the solution, add 25.0 mL of *0.1 M hydrochloric acid* and heat for 1 h on a water-bath. Cool, adjust with *water R* to the initial volume and titrate with *0.1 M sodium hydroxide*, using 0.1 mL of *phenolphthalein solution R1* as indicator.

5.7 mL to 6.3 mL of *0.1 M sodium hydroxide* is used in the titration.

c) Dilute 10.0 mL to 100.0 mL with *water R* and mix. Titrate 10.0 mL of the solution with *0.1 M hydrochloric acid*, using 0.1 mL of *phenolphthalein solution R1* as indicator.

6.0 mL to 7.5 mL of *0.1 M hydrochloric acid* is used in the titration.

Cupriethylenediamine Hydroxide Solution (*14552-35-3*)

The molar ratio of ethylenediamine to copper is 2.00 ± 0.04.

This solution is commercially available.

Cupri-tartaric Solution

Solution A. Dissolve 34.6 g of *copper sulfate pentahydrate R* in *water R* and dilute to 500 mL with the same solvent.

Solution B. Dissolve 173 g of *sodium potassium tartrate R* and 50 g of *sodium hydroxide R* in 400 mL of *water R*. Heat to boiling, allow to cool and dilute to 500 mL with *carbon dioxide-free water R*.

Mix equal volumes of the 2 solutions immediately before use.

Cupri-tartaric Solution R1

Fehling's solution

Solution A Dissolve 34.6 g of *copper(II) sulfate* in a mixture of 0.5 mL of *sulfuric acid* and sufficient *water* to produce 500 mL.

Solution B Dissolve 176 g of *potassium sodium (+)-tartrate* and 77 g of *sodium hydroxide* in sufficient *water* to produce 500 mL.

Mix equal volumes of solutions A and B immediately before use.

Cupri-tartaric Solution R2 Dilute potassium cupri-tartrate solution

Add 1 mL of a solution containing 5 g/L of *copper sulfate pentahydrate R* and 10 g/L of *potassium tartrate R* to 50 mL of *sodium carbonate solution R1*. Prepare immediately before use.

Cupri-tartaric Solution R3

Prepare a solution containing 10 g/L of *copper sulfate pentahydrate R* and 20 g/L of *sodium tartrate R*. To 1.0 mL of the solution add 50 mL of *sodium carbonate solution R2*. Prepare immediately before use.

Cupri-tartaric Solution R4

Solution A. 150 g/L *copper sulfate pentahydrate R*.

Solution B. Dissolve 2.5 g of *anhydrous sodium carbonate R*, 2.5 g of *sodium potassium tartrate R*, 2.0 g of *sodium hydrogen carbonate R*, and 20.0 g of *anhydrous sodium sulfate R* in *water R* and dilute to 100 mL with the same solvent.

Mix 1 part of solution A with 25 parts of solution B immediately before use.

Curcumin 1,7-Bis(4-hydroxy-3-methoxyphenyl)hepta-1,6-diene-3,5-dione; $C_{21}H_{20}O_6$ = 368.4 (*458-37-7*)

Orange-brown, crystalline powder, practically insoluble in water, soluble in glacial acetic acid.

mp: about 183 °C.

Curcuminoids

A mixture of curcumin ($C_{21}H_{20}O_6$; M_r 368.4), demethoxycurcumin ($C_{20}H_{18}O_5$; M_r 338.4) and bis-demethoxycurcumin ($C_{19}H_{16}O_4$; M_r 308.3).

Cyanoacetic Acid Malonic acid mononitrile; $C_3H_3NO_2$ = 85.1 (*372-09-8*)

White or yellowish-white, hygroscopic crystals, very soluble in water.

Storage: in an airtight container.

Cyanocobalamin Coα-[α-(5,6-Dimethylbenzimidazolyl)]-Coβ-cyanocobamide; Vitamin B_{12}; (*68-19-9*)

See *Cyanocobalamin (0547)*.

Cyanogen Bromide Solution (*506-68-3*)

Add dropwise, with cooling *0.1 M ammonium thiocyanate* to *bromine water R* until the yellow colour disappears. Prepare immediately before use.

1-Cyanoguanidine Dicyandiamide; Cyanoguanidine; $C_2H_4N_4$ = 84.1 (*461-58-5*)

White or almost white, crystalline powder, sparingly soluble in water and in ethanol (96 per cent), practically insoluble in methylene chloride.

mp: about 210 °C.

Cyanopropyl(3)phenyl(3)methyl(94)polysiloxane

Polysiloxane substituted with 3 per cent of cyanopropyl groups, 3 per cent of phenyl groups and 94 per cent of methyl groups.

Cyanopropyl(7)phenyl(7)methyl(86)polysiloxane

Polysiloxane substituted with 7 per cent of cyanopropyl groups, 7 per cent of phenyl groups and 86 per cent of methyl groups.

Cyanopropyl(25)phenyl(25)methyl(50)polysiloxane

Polysiloxane substituted with 25 per cent of cyanopropyl groups, 25 per cent of phenyl groups and 50 per cent of methyl groups.

Cyanopropylpolysiloxane

Polysiloxane substituted with 100 per cent of cyanopropyl groups.

Cyasterone (2β,3β,5β,22*R*,24*S*,24^1*R*,25*S*)-24^1,26-Epoxy-2,3,14,20,22-pentahydroxystigmast-7-ene-6,26-dione; $C_{29}H_{44}O_8$ = 520.7 (*17086-76-9*)

Cyclobutane-1,1-dicarboxylic Acid $C_6H_{10}O_4$ = 144.1 (*5445-51-2*)

mp: about 160°.

General reagent grade of commerce.

α-Cyclodextrin Cyclohexakis-(1→4)-(α-D-glucopyranosyl); Cyclomaltohexaose; Alfadex; $C_{36}H_{60}O_{30}$ = 972 (*10016-20-3*)

β-Cyclodextrin (*7585-39-9*)

See *Betadex (1070)*.

γ-Cyclodextrin Gamma-cyclodextrin $C_{48}H_{80}O_{40}$ = 1297 (*17465-86-0*)

General reagent grade of commerce.

β-Cyclodextrin for Chiral Chromatography, Modified

30 per cent of 2,3-di-*O*-ethyl-6-*O*-*tert*-butyldimethylsilyl-β-cyclodextrin dissolved in polysiloxane substituted with 15 per cent of phenyl groups and 85 per cent of methyl groups.

β-Cyclodextrin for Chiral Chromatography, Modified R1

30 per cent of 2,3-di-*O*-acetyl-6-*O*-*tert*-butylsilyl-β-cyclodextrin dissolved in polysiloxane substituted with 15 per cent of phenyl groups and 85 per cent of methyl groups.

β-Cyclodextrin Hydroxypropyl Ether Derivative for Chiral Chromatography

Beta cyclodextrin, R,S-hydroxypropyl ether derivative, bonded to porous silica particles, 3 to 10 µm in diameter.

Cyclohexane C_6H_{12} = 84.2 (*110-82-7*)

Clear, colourless, flammable liquid, practically insoluble in water, miscible with organic solvents.

d_{20}^{20}: about 0.78.

bp: about 80.5 °C.

Cyclohexane used in spectrophotometry complies with the following additional test.

Absorbance (*2.2.25*): maximum 0.35 at 220 nm, 0.16 at 235 nm, 0.05 at 240 nm, 0.01 at 250 nm, determined using *water R* as compensation liquid.

Cyclohexane R1

Complies with the requirements prescribed for *cyclohexane R* with the following additional requirement.

The fluorescence, measured at 460 nm, under illumination with an excitant light beam at 365 nm, is not more intense than that of a solution containing 0.002 ppm of *quinine R* in *dilute sulfuric acid R1*.

Cyclohexylamine Cyclohexanamine; $C_6H_{13}N$ = 99.2 (*108-91-8*)

Colourless liquid, soluble in water, miscible with usual organic solvents.

n_D^{20}: about 1.460.

bp: 134 °C to 135 °C.

Cyclohexylenedinitrilotetra-acetic Acid (±)-trans-1,2-Diaminocyclohexane-N,N,N′,N′-tetra-acetic acid; $C_{14}H_{22}N_2O_8,H_2O$ = 364.4

White or almost white, crystalline powder.

mp: about 204 °C.

Cyclohexylmethanol Cyclohexylcarbinol; $C_7H_{14}O$ = 114.2 (*100-49-2*)

Liquid with a slight odour of camphor, soluble in ethanol (96 per cent).

n_D^{25}: about 1.464.

bp: about 185 °C.

3-Cyclohexylpropionic Acid $C_9H_{16}O_2$ = 156.2 (*701-97-3*)

Clear liquid.

d_{20}^{20}: about 0.998.

n_D^{20}: about 1.4648.

bp: about 130 °C.

Cyhalothrin $C_{23}H_{19}ClF_3NO_3$ = 449.9 (*91465-08-6*)

bp: 187 °C to 190 °C.

mp: about 49 °C.

A suitable certified reference solution (10 ng/µL in cyclohexane) may be used.

Cymarin 3β-[(2,6-Dideoxy-3-*O*-methyl-β-D-*ribo*-hexopyranosyl)oxy]-5β,14-dihydroxy-19-oxocard-20(22)-enolide; $C_{30}H_{44}O_9$ = 548.7 (*508-77-0*)

White or slightly yellow powder, slightly soluble in water, soluble in methanol.

mp: about 148 °C.

***p*-Cymene** 4-Isopropyltoluene; $C_{10}H_{14}$ = 134.2 (*99-87-6*)

Colourless liquid, practically insoluble in water, soluble in ethanol (96 per cent).

d_{20}^{20}: about 0.858.

n_D^{20}: about 1.4895.

bp: 175 °C to 178 °C.

p-Cymene used in gas chromatography complies with the following additional test.

Assay. Gas chromatography (*2.2.28*) as prescribed in the monograph *Peppermint oil (0405)*.

Test solution. The substance to be examined.

Content: minimum 96.0 per cent, calculated by the normalisation procedure.

Cynarin (1R,3R,4S,5R)-1,3-Bis[[3-(3,4- dihydroxyphenyl) propenoyl]oxy]-4,5- dihydroxycyclohexanecarboxylic acid; $C_{25}H_{24}O_{12}$ = 516.4 (*30964-13-7*)

White or almost white amorphous mass, odourless.

Cypermethrin $C_{22}H_{19}Cl_2NO_3$ = 416.3 (*52315-07-8*)

bp: 170 °C to 195 °C.

mp: 60 °C to 80 °C.

A suitable certified reference solution (10 ng/µL in cyclohexane) may be used.

L-Cysteine $C_3H_7NO_2S$ = 121.1 (*52-90-4*)

Powder, freely soluble in water, in ethanol (96 per cent) and in acetic acid, practically insoluble in acetone.

Cysteine Hydrochloride (*7048-04-6*)

See *Cysteine hydrochloride monohydrate (0895)*.

L-Cystine $C_6H_{12}N_2O_4S_2$ = 240.3 (*56-89-3*)

White or almost white, crystalline powder, practically insoluble in water and in ethanol (96 per cent). It dissolves in dilute solutions of alkali hydroxides.

$[\alpha]_D^{20}$: −218 to −224, determined in *1 M hydrochloric acid*.

mp: 250 °C, with decomposition.

Cytochrome c Ferricytochrome c (oxidized state); (*9007-43-6*)

Purity, minimum 95%.

Cytosine $C_4H_5N_3O$ = 111.1 (*71-30-7*)

Content: minimum 95.0 per cent.

Daidzein 7-Hydroxy-3-(4-hydroxyphenyl)-4*H*-1-benzopyran-4-one; $C_{15}H_{10}O_4$ = 254.2 (*486-66-8*)

Daidzin Daidzein-7-*O*-glucoside; 7-(β-D-Glucopyranosyloxy)-3-(4-hydroxyphenyl)-4*H*-1-benzopyran-4-one; $C_{21}H_{20}O_9$ = 416.4 (*552-66-9*)

***o,p'*-DDD** 1-(2-Chlorophenyl)-1-(4-chlorophenyl)-2,2-dichloroethane; $C_{14}H_{10}Cl_4$ = 320.0 (*53-19-0*)

A suitable certified reference solution (10 ng/µL in cyclohexane) may be used.

***p,p'*-DDD** 1,1-Bis(4-chlorophenyl)-2,2-dichloroethane; $C_{14}H_{10}Cl_4$ = 320.0 (*72-54-8*)

bp: about 193 °C.

mp: about 109 °C.

A suitable certified reference solution (10 ng/µL in cyclohexane) may be used.

***o,p'*-DDE** $C_{14}H_8Cl_4$ = 318.0 (*3424-82-6*)

1-(2-Chlorophenyl)-1-(4-chlorophenyl)-2,2-dichloroethylene.

A suitable certified reference solution (10 ng/µL in cyclohexane) may be used.

***p,p'*-DDE** 1,1-Bis(4-chlorophenyl)-2,2-dichloroethylene; $C_{14}H_8Cl_4$ = 318.0 (*72-55-9*)

bp: 316 °C to 317 °C.

mp: 88 °C to 89 °C.

A suitable certified reference solution (10 ng/µL in cyclohexane) may be used.

***o,p'*-DDT** 1-(2-Chlorophenyl)-1-(4-chlorophenyl)-2,2,2-trichloroethane; $C_{14}H_9Cl_5$ = 354.5 (*789-02-6*)

A suitable certified reference solution (10 ng/µL in cyclohexane) may be used.

***p,p'*-DDT** 1,1-Bis(4-chlorophenyl)-2,2,2-trichloroethane; $C_{14}H_9Cl_5$ = 354.5 (*50-29-3*)

bp: about 260 °C.

mp: 108 °C to 109 °C.

A suitable certified reference solution (10 ng/µL in cyclohexane) may be used.

Decanal Decyl aldehyde; $C_{10}H_{20}O$ = 156.3 (*112-31-2*)

Oily, colourless liquid, practically insoluble in water.

Decanal used in gas chromatography complies with the following additional test.

Assay. Gas chromatography (*2.2.28*) as prescribed in the monograph *Sweet orange oil (1811)*.

Content: minimum 97 per cent, calculated by the normalisation procedure.

***N*-Decane** Decane; $C_{10}H_{22}$ = 142.3 (*124-18-5*)

Colourless liquid, practically insoluble in water.

n_D^{20}: about 1.411.

bp: about 174 °C.

Decan-1-ol Capryl Alcohol; Capric alcohol; Decanol; $C_{10}H_{22}O$ = 158.3 (*112-30-1*)

Viscous liquid, solidifying at about 6 °C, practically insoluble in water, soluble in ethanol (96 per cent).

n_D^{20}: about 1.436.

bp: about 230 °C.

Decloxizine Hydrochloride 2-[2-(4-benzhydryl-1-piperazinyl)ethoxy]ethanol; $C_{21}H_{28}N_2O_2.2HCl$ = 413.39 (*3733-63-9*)

Analytical reagent grade of commerce.

Defluorohydroxy-PSMA-1007 (3*S*,10*S*,14*S*)-1-[4-[[(2*S*)-4-Carboxy-2-[(2*S*)-4-carboxy-2-(6-hydroxypyridin-3-amido) butanamido]butanamido]methyl]phenyl]-3-[(naphthalen-2-yl) methyl]-1,4,12-trioxo-2,5,11,13-tetraazahexadecane-10,14,16-tricarboxylic acid; $C_{49}H_{56}N_8O_{17}$ = 1029

White or almost white powder.

Defluorotrimethylaminium-PSMA-1007 Trifluoroacetate 5-[[(2*S*)-4-Carboxy-1-[[(2*S*)-4-carboxy-1-[[[4-[(3*S*,10*S*,14*S*)-10,14-dicarboxy-17-hydroxy-3-[(naphthalen-2-yl)methyl]-1,4,12,17-tetraoxo-2,5,11,13-tetraazaheptadecan-1-yl]phenyl]methyl]amino]-1-oxobutan-2-yl]amino]-1-oxobutan-2-yl]carbamoyl]-*N*,*N*,*N*-trimethylpyridin-2-aminium trifluoroacetate; $C_{54}H_{64}F_3N_9O_{18}$ = 1184 (*2226894-58-0*)

White or almost white powder.

Dehydrocostus Lactone (3a*S*,6a*R*,9a*R*,9b*S*)-3,6,9-Trismethylenedecahydroazuleno[4,5-*b*]furan-2(3*H*)-one; $C_{15}H_{18}O_2$ = 230.3 (*477-43-0*)

Deltamethrin $C_{22}H_{19}Br_2NO_3$ = 505.2 (*52918-63-5*)

bp: about 300 °C.

mp: about 98 °C.

A suitable certified reference solution (10 ng/µL in cyclohexane) may be used.

Demeclocycline Hydrochloride

See *Demeclocycline hydrochloride (0176)*.

Demethylflumazenil Ethyl 8-fluoro-6-oxo-5,6-dihydro-4*H*-imidazo[1,5-*a*][1,4]benzodiazepine-3-carboxylate; $C_{14}H_{12}FN_3O_3$ = 289.3 (*79089-72-8*)

Colourless needles, soluble in dimethyl sulfoxide and in hot methanol.

mp: about 288 °C.

14-Deoxy-11,12-didehydroandrographolide 3-[(1*E*)-2-[(1*R*,4a*S*,5*R*,6*R*,8a*R*)-6-Hydroxy-5-(hydroxymethyl)-5,8a-dimethyl-2-methylenedecahydronaphthalen-1-yl]ethenyl] furan-2(5*H*)-one; $C_{20}H_{28}O_4$ = 332.4 (*42895-58-9*)

2′-Deoxyuridine 1-(2-Deoxy-β-d-*erythro*-pentofuranosyl)-1*H*,3*H*-pyrimidine-2,4-dione; $C_9H_{12}N_2O_5$ = 228.2 (*951-78-0*)

mp: about 165 °C.

Chromatography. Thin-layer chromatography (*2.2.27*) as prescribed in the monograph *Idoxuridine (0669)*: apply 5 µL of a 0.25 g/L solution; the chromatogram shows only one principal spot.

4-Desoxypyridoxine Hydrochloride 5-(Hydroxymethyl)-2,4-dimethylpyridin-3-ol; 4-Deoxypyridoxine hydrochloride; $C_8H_{12}NO_2Cl$ = 189.6 (*148-51-6*)

Destaining Solution

A mixture consisting of 1 volume of *glacial acetic acid R*, 4 volumes of *methanol R* and 5 volumes of *water R*.

Desmethylmisonidazole (2*RS*)-3-(2-Nitro-1*H*-imidazol-1-yl)propane-1,2-diol; $C_6H_9N_3O_4$ = 187.2 (*13551-92-3*)

Content: minimum 95 per cent.

Yellow powder.

Deuterated Acetic Acid $C_2D_4O_2$; $C_2{}^2H_4O_2$ = 64.1 (*1186-52-3*)

Degree of deuteration: minimum 99.7 per cent.

d_{20}^{20}: about 1.12.

n_D^{20}: about 1.368.

bp: about 115 °C.

mp: about 16 °C.

Deuterated Acetone C_3D_6O; Deuteroacetone; $C_3{}^2H_6O$ = 64.1 (*666-52-4*)

Degree of deuteration: minimum 99.5 per cent.

Clear, colourless liquid, miscible with water, with dimethylformamide, with anhydrous ethanol and with methanol.

d_{20}^{20}: about 0.87.

n_D^{20}: about 1.357.

bp: about 55 °C.

Water and deuterium oxide. Not more than 0.1 per cent.

Deuterated Acetonitrile $C_2{}^2H_3N$ = 44.1 (*2206-26-0*)

Degree of deuteration: minimum 99.8 per cent.

Clear, colourless liquid, miscible with water, with acetone and with methanol.

d_{20}^{20}: about 0.78.

n_D^{20}: about 1.344.

Deuterated Dimethyl Sulfoxide C_2D_6OS; Deuterated dimethyl sulphoxide; ($2H_6$)-dimethyl sulphoxide; $C_2{}^2H_6OS$ = 84.2 (*2206-27-1*)

Degree of deuteration: minimum 99.8 per cent.

Very hygroscopic liquid, practically colourless, viscous, soluble in water, in acetone and in anhydrous ethanol.

d_{20}^{20}: about 1.18.

mp: about 20 °C.

Water and deuterium oxide: maximum 0.1 per cent.

Storage: in an airtight container.

Deuterated Methanol CD4O; C^2H_4O = 36.1 (*811-98-3*)

Degree of deuteration: minimum 99.8 per cent.

Clear, colourless liquid miscible with water, with ethanol (96 per cent) and with methylene chloride.

d_{20}^{20}: about 0.888.

n_D^{20}: about 1.326.

bp: 65.4 °C.

Deuterated Sodium Trimethylsilylpropionate C6H9D4NaO2Si; $C_6H_9{}^2H_4NaO_2Si$ = 172.3 (*24493-21-8*)

Degree of deuteration: minimum 98 per cent.

White or almost white powder.

Deuterium Chloride DCl; ^2HCl = 37.47 (*7698-05-7*)

Gas.

Degree of deuteration: minimum 99 per cent.

Caution: *toxic*.

Deuterium Chloride Solution

Dilute 1 mL of *deuterium chloride R* (38 per cent *m/m*) with 5 mL of *deuterium oxide R*.

Deuterium Oxide D2O; ^2H_2O = 20.03 (*7789-20-0*)

Degree of deuteration: minimum 99.7 per cent.

d_{20}^{20}: about 1.11.

n_D^{20}: about 1.328.

bp: about 101 °C.

Deuterium Oxide, Isotopically Pure

Deuterium oxide R1; ^2H_2O = 20.03 (*7789-20-0*)

Degree of deuteration: minimum 99.95 per cent.

Deuterochloroform (2H)-Chloroform; Chloroform-d; CDCl3; Deuterated chloroform; C^2HCl_3 = 120.4 (*865-49-6*)

Degree of deuteration: minimum 99.7 per cent.

Clear, colourless liquid, practically insoluble in water, miscible with acetone and with ethanol (96 per cent). It may be stabilised over silver foil.

d_{20}^{20}: about 1.51.

n_D^{20}: about 1.445.

bp: about 60 °C.

Water and deuterium oxide: maximum 0.05 per cent.

Devarda's Alloy (*8049-11-4*)

Copper, 50 parts; aluminium, 45 parts; zinc, 5 parts.

General reagent grade of commerce containing not more than 20 ppm of nitrogen as NH_4.

Developer Solution

Dilute 2.5 mL of a 20 g/L solution of *citric acid monohydrate R* and 0.27 mL of *formaldehyde R* to 500.0 mL with *water R*.

Dexamethasone $C_{22}H_{29}FO_5$ = 392.5 (*50-02-2*)

mp: about 263°.

General reagent grade of commerce.

Dextran for Chromatography R2, Cross-linked

Bead-form dextran with a fraction range suitable for the separation of peptides and proteins with relative molecular masses of 15 × 10^2 to 30 × 10^3. When dry, the beads have a diameter of 20-80 µm.

Dextran for Chromatography R3, Cross-linked

Bead-form dextran with a fraction range suitable for the separation of peptides and proteins with relative molecular masses of 4 × 10^3 to 15 × 10^4. When dry, the beads have a diameter of 40-120 µm.

3,3′-Diaminobenzidine Tetrahydrochloride 3,3′,4,4′-Biphenyl-tetramine; $C_{12}H_{18}Cl_4N_4$, $2H_2O$ = 396.1 (*7411-49-6*)

Almost white or slightly pink powder, soluble in water.

mp: about 280 °C, with decomposition.

1,2-Diamino-4,5-methylenedioxybenzene Dihydrochloride 2*H*-1,3-Benzodioxole-5,6-diamine dihydrochloride; $C_7H_{10}Cl_2N_2O_2$ = 225.1 (*81864-15-5*)

Content: minimum 99 per cent (HPLC).

Diammonium 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate) Diammonium 2,2′-azinobis(3-ethylbenzothiazoline-6-sulphonate); $C_{18}H_{24}N_6O_6S_4$ = 548.7 (*30931-67-0*)

Chromogenic substrate suitable for use in ELISA procedures.

Green tablets, freely soluble in water.

pH (*2.2.3*): 4.2 to 5.8 for a 0.1 g/L solution.

Diammonium Hydrogen Orthophosphate Ammonium phosphate; $(NH_4)_2HPO_4$ = 132.1 (*7783-28-0*)

White or almost white crystals or granules, hygroscopic, very soluble in water, practically insoluble in ethanol (96 per cent).

pH (*2.2.3*): about 8 for a 200 g/L solution.

Storage: in an airtight container.

Diatomaceous Filter-aid, Washed, Flux-calcined

To 500 g of flux-calcined, diatomaceous filter-aid (Celite 545 is suitable), add 2000 mL of *hydrochloric acid*, mix, allow to stand with occasional stirring for 12 hours, filter and wash the residue with *water* until the washings are neutral to *litmus paper*. Continue washing the residue on the filter paper, using 500 mL of *methanol* followed by 1000 mL of a mixture of equal volumes of *methanol* and *ether*. Finally dry the washed residue at 100° until the odour of solvent is no longer detectable. It should be stored in an airtight container.

Diatomaceous Support Diatomaceous earth; (*91053-39-3*)

White or almost white, fine granular powder, made up of siliceous frustules of fossil diatoms or of debris of fossil diatoms, practically insoluble in water and in ethanol (96 per cent).

The substance may be identified by microscopic examination with a magnification of × 500.

Diatomaceous Support, Acid-washed Diatomaceous earth for gas chromatography

White or almost white, fine granular powder, practically insoluble in water and in ethanol (96 per cent), made up of siliceous frustules of fossil diatoms or of debris of fossil diatoms. The substance may be identified by microscopic examination with a magnification of × 500. The substance is acid-washed, then water-washed until neutral.

Diatomaceous Support, Alkali-washed

Diatomaceous support that has been treated with potassium hydroxide solution to reduce peak-tailing of basic compounds.

Diatomaceous Support, Silanised Diatomaceous earth for gas chromatography, silanised

Diatomaceous earth for gas chromatography R silanised with dimethyldichlorosilane or other suitable silanising agents.

Diazinon $C_{12}H_{21}N_2O_3PS$ = 304.3 (*333-41-5*)

bp: about 306 °C.

A suitable certified reference solution (10 ng/µL in iso-octane) may be used.

Diazobenzenesulfonic Acid Solution

Diazobenzenesulphonic acid solution

Heat 0.2 g of *sulfanilic acid* with 20 mL of 1M *hydrochloric acid* until dissolved, cool to about 4° and add, dropwise, 2.2 mL of a 4% w/v solution of *sodium nitrite*, swirling continuously. Allow to stand in ice for 10 minutes and add 1 mL of a 5% w/v solution of *sulfamic acid*.

Diazobenzenesulfonic Acid Solution R1

Diazobenzenesulphonic acid solution R1

Dissolve 0.9 g of *sulfanilic acid R* in a mixture of 30 mL of *dilute hydrochloric acid R* and 70 mL of *water R*. To 3 mL of the solution add 3 mL of a 50 g/L solution of *sodium nitrite R*. Cool in an ice-bath for 5 min, add 12 mL of the sodium nitrite solution and cool again. Dilute to 100 mL with *water R* and keep the reagent in an ice-bath. Prepare extemporaneously but allow to stand for 15 min before use.

Dibenzosuberone Dibenzo[*a*,*d*]cyclohepta-1,4-dien-3-one; *H*-dibenzo[*a*,*d*]cyclohepten-5-one; $C_{15}H_{12}O$ = 208.3 (*1210-35-1*)

mp: about 34°.

General reagent grade of commerce.

Dibromomethane CH_2Br_2 = 173.8 (*74-95-3*)

Colourless liquid, slightly soluble in water.

bp: about 96 °C.

Di-*N*-butylamine *N*-Butylbutan-1-amine; Dibutylamine; $C_8H_{19}N$ = 129.3 (*111-92-2*)

Colourless liquid.

n_D^{20}: about 1.417.

bp: about 159 °C.

Dibutylammonium Phosphate for Ion-pairing

A colourless solution of 10 per cent to 15 per cent *V/V* of di-*n*-butylamine and 12 per cent to 17 per cent *V/V* of phosphoric acid in water, suitable for ion-pairing in liquid chromatography.

Dibutyl Ether $C_8H_{18}O$ = 130.2 (*142-96-1*)

Colourless, flammable liquid, practically insoluble in water, miscible with anhydrous ethanol.

d_{20}^{20}: about 0.77.

n_D^{20}: about 1.399.

Do not distil if the dibutyl ether does not comply with the test for peroxides.

Peroxides. Place 8 mL of *potassium iodide and starch solution R* in a 12 mL ground-glass-stoppered cylinder about 1.5 cm in diameter. Fill completely with the substance to be examined, shake vigorously and allow to stand protected from light for 30 min. No colour is produced.

The name and concentration of any added stabiliser are stated on the label.

Dibutyl Phthalate Di-*N*-butyl phthalate; $C_{16}H_{22}O_4$ = 278.3 (*84-74-2*)

Clear, colourless or faintly coloured, oily liquid, very slightly soluble in water, miscible with acetone and with ethanol (96 per cent).

d_{20}^{20}: 1.043 to 1.048.

n_D^{20}: 1.490 to 1.495.

Dicarboxidine Hydrochloride 4,4′-[(4,4′-Diaminobiphenyl-3,3′-diyl)dioxy]dibutanoic acid dihydrochloride; $C_{20}H_{26}Cl_2N_2O_6$ = 461.3 (*56455-90-4*)

Dichlofenthion $C_{10}H_{13}Cl_2O_3PS$ = 315.2 (*97-17-6*)

A suitable certified reference solution (10 ng/µL in cyclohexane) may be used.

Dichloroacetic Acid $C_2H_2Cl_2O_2$ = 128.9 (*79-43-6*)

Colourless liquid, miscible with water and ethanol (96 per cent).

d_{20}^{20}: about 1.566.

n_D^{20}: about 1.466.

bp: about 193 °C.

Dichloroacetic Acid Solution

Dilute 67 mL of *dichloroacetic acid R* to 300 mL with *water R* and neutralise to *blue litmus paper R* using *ammonia R*. Cool, add 33 mL of *dichloroacetic acid R* and dilute to 600 mL with *water R*.

3,5-Dichloroaniline 3,5-dichlorophenylamine; $C_6H_5Cl_2N$ = 162.0 (*626-43-7*)

mp: 46 °C to 52 °C.

1,2-Dichlorobenzene Dichlorobenzene; $C_6H_4Cl_2$ = 147.0 (*95-50-1*)

Colourless, oily liquid, practically insoluble in water, soluble in anhydrous ethanol.

d_{20}^{20}: about 1.31.

bp: about 180 °C.

2,4-Dichlorobenzoic Acid $C_7H_4Cl_2O_2$ = 191.0 (*50-84-0*)

Faintly beige powder.

mp: about 160 °C.

2,3-Dichloro-5,6-dicyanobenzoquinone 4,5-Dichloro-3,6-dioxo-cyclohexa-1,4-diene-1,2-dicarbonitrile; $C_8Cl_2N_2O_2$ = 227.0 (*84-58-2*)

Yellow or orange crystals, soluble in dioxan and in acetic acid, slightly soluble in methylene chloride. It decomposes in water.

mp: about 214 °C.

Storage: at a temperature of 2 °C to 8 °C.

(*S*)-3,5-Dichloro-2,6-dihydroxy-*N*-[(1-ethylpyrrolidin-2-yl)methyl]benzamide Hydrobromide $C_{14}H_{19}BrCl_2N_2O_3$ = 414.1 (*113310-88-6*)

White or almost white, crystalline powder.

$[\alpha]_D^{22}$: + 11.4, determined on a 15.0 g/L solution in *anhydrous ethanol R*.

mp: about 212 °C.

1,2-Dichloroethane Ethylene chloride; $C_2H_4Cl_2$ = 99.0 (*107-06-2*)

Clear, colourless liquid, soluble in about 120 parts of water and in 2 parts of ethanol (96 per cent).

d_{20}^{20}: about 1.25.

Distillation range (*2.2.11*). Not less than 95 per cent distils between 82 °C and 84 °C.

2,7-Dichlorofluorescein 2-(2,7-dichloro-6-hydroxy-3-oxo-3H-xanthen-9-yl)benzoic acid; Dichlorofluorescein; $C_{20}H_{10}Cl_2O_5$ = 401.2 (*76-54-0*)

Yellowish-brown or yellow-orange powder, slightly soluble in water, freely soluble in ethanol (96 per cent) and in dilute solutions of alkali hydroxides giving a solution showing a yellowish-green fluorescence.

5,7-Dichloro-8-hydroxyquinoline 5,7-Dichlorooxine; 5,7-Dichloroquinolin-8-ol; $C_9H_5Cl_2NO$ = 214.1 (*773-76-2*)

Yellow, crystalline powder, soluble in acetone, slightly soluble in ethanol (96 per cent).

mp: about 179 °C.

Content: minimum 95.0 per cent.

Dichloromethane Methylene chloride; CH_2Cl_2 = 84.9 (*75-09-2*)

Colourless liquid, sparingly soluble in water, miscible with ethanol (96 per cent).

bp: 39 °C to 42 °C.

Methylene chloride used in fluorimetry complies with the following additional test.

Fluorescence. Under irradiation at 365 nm, the fluorescence (*2.2.21*) measured at 460 nm in a 1 cm cell is not more intense than that of a solution containing 0.002 ppm of *quinine R* in *0.5 M sulfuric acid* measured in the same conditions.

Dichloromethane, Acidified Methylene chloride, acidified

To 100 mL of *methylene chloride R* add 10 mL of *hydrochloric acid R*, shake, allow to stand and separate the two layers. Use the lower layer.

Dichloromethane IR

Spectroscopic reagent grade of commerce.

Dichloromethane Reagent

Add 50 g of *sodium hydrogen carbonate* to 1000 mL of *dichloromethane*, allow to stand overnight and filter.

2,4-Dichloro-1-naphthol $C_{10}H_6Cl_2O$ = 213.1 (*2050-76-2*)

mp: about 107°.

General reagent grade of commerce.

2,6-Dichlorophenol $C_6H_4Cl_2O$ = 163.0 (*87-65-0*)

mp: 64 °C to 66 °C.

2,6-Dichlorophenolindophenol Sodium Salt The sodium derivative of 2,6-dichloro-*N*-(4-hydroxyphenyl)-1,4-benzoquinone monoimine; Tillman's reagent; Dichlorophenolindophenol, sodium salt; $C_{12}H_6Cl_2NNaO_2,2H_2O$ = 326.1 (*620-45-1*)

Dark-green powder, freely soluble in water and in anhydrous ethanol. The aqueous solution is dark blue; when acidified it becomes pink.

2,6-Dichlorophenolindophenol Solution

Warm 0.1 g of *2,6-dichlorophenolindophenol sodium salt* with 100 mL of *water* and filter.

Use within 3 days of preparation.

2,6-Dichlorophenolindophenol Solution, Double-strength Standard

Dissolve 0.1 g of *2,6-dichlorophenolindophenol sodium salt* in 100 mL of *water*, filter, standardise by the method described under *standard dichlorophenolindophenol solution* and dilute the solution with *water* so that 1 mL is equivalent to 0.2 mg of ascorbic acid.

Use within 3 days of preparation and standardise immediately before use.

Dichlorophenolindophenol Solution, Standard

Dissolve 50.0 mg of *dichlorophenolindophenol, sodium salt R* in 100.0 mL of *water R* and filter.

Assay. Dissolve 20.0 mg of *ascorbic acid R* in 10 mL of a freshly prepared 200 g/L solution of *metaphosphoric acid R* and dilute to 250.0 mL with *water R*. Titrate 5.0 mL rapidly with the dichloro-phenolindophenol standard solution, added from a microburette graduated in 0.01 mL, until the pink colour persists for 10 s, the titration occupying not more than 2 min. Dilute the dichlorophenolindophenol solution with *water R* to make 1 mL of the solution equivalent to 0.1 mg of ascorbic acid ($C_6H_8O_6$).

Storage: use within 3 days.

Standardise immediately before use.

2,6-Dichloroquinone-4-chloroimide

Dichloroquinonechlorimide; $C_6H_2Cl_3NO$ = 210.4 (*101-38-2*)

Pale yellow or greenish-yellow crystalline powder, practically insoluble in water, soluble in ethanol (96 per cent) and in dilute alkaline solutions.

mp: about 66 °C.

Dichlorvos 2,2-Dichlorovinyl dimethyl phosphate; $C_4H_7Cl_2O_4P$ = 221 (*62-73-7*)

Colourless or brownish-yellow liquid, soluble in water, miscible with most organic solvents.

n_D^{25}: about 1.452.

Di-2-cyanoethyl Ether (2-Cyanoethyl) ether; $C_6H_8N_2O$ = 124.1 (*1656-48-0*)

n_D^{20}: 1.4400.

bp: about 111°.

Chromatographic grade of commerce.

Dicyclohexyl Bicyclohexyl; $C_{12}H_{22}$ = 166.3 (*92-51-3*)

d_{20}^{20}: about 0.864.

bp: about 227 °C.

mp: about 4 °C.

Dicyclohexylamine *N,N*-Dicyclohexylamine; $C_{12}H_{23}N$ = 181.3 (*101-83-7*)

Colourless liquid, sparingly soluble in water, miscible with the usual organic solvents.

n_D^{20}: about 1.484.

bp: about 256 °C.

Freezing point (*2.2.18*): 0 °C to 1 °C.

1,3-Dicyclohexylurea Dicyclohexylurea; $C_{13}H_{24}N_2O$ = 224.4 (*2387-23-7*)

White or almost white, crystalline powder.

mp: about 232 °C.

Didocosahexaenoin Diglyceride of docosahexaenoic acid (C22:6); Glycerol didocosahexaenoate; (*all-Z*)-Docosahexaenoic acid, diester with propane-1,2,3-triol; $C_{47}H_{68}O_5$ = 713.0 (*88315-12-2*)

The reagent from Nu-Chek Prep, Inc. has been found suitable

Didodecyl 3,3′-thiodipropionate $C_{30}H_{58}O_4S$ = 514.8 (*123-28-4*)

White or almost white, crystalline powder, practically insoluble in water, freely soluble in acetone and in light petroleum, slightly soluble in ethanol (96 per cent).

mp: about 39 °C.

Dieldrin $C_{12}H_8Cl_6O$ = 380.9 (*60-57-1*)

bp: about 385 °C.

mp: about 176 °C.

A suitable certified reference solution (10 ng/µL in cyclohexane) may be used.

Diethanolamine 2,2′-Iminobisethanol; $C_4H_{11}NO_2$ = 105.1 (*111-42-2*)

Viscous, clear, slightly yellow liquid or deliquescent crystals melting at about 28 °C, very soluble in water, in acetone and in methanol.

d_{20}^{20}: about 1.09.

pH (*2.2.3*): 10.0 to 11.5 for a 50 g/L solution.

Diethanolamine used in the test for alkaline phosphatase complies with the following additional test.

Ethanolamine. Gas chromatography (*2.2.28*).

Internal standard solution. Dissolve 1.00 g of *3-aminopropanol R* in *acetone R* and dilute to 10.0 mL with the same solvent.

Test solution (a). Dissolve 5.00 g of the substance to be examined in *acetone R* and dilute to 10.0 mL with the same solvent.

Test solution (b). Dissolve 5.00 g of the substance to be examined in *acetone R*, add 1.0 mL of the internal standard solution and dilute to 10.0 mL with the same solvent.

Reference solutions. Dissolve 0.50 g of *ethanolamine R* in *acetone R* and dilute to 10.0 mL with the same solvent. To 0.5 mL, 1.0 mL and 2.0 mL of this solution, add 1.0 mL of the internal standard solution and dilute to 10.0 mL with *acetone R*.

Column:
— *size: l* = 1 m, Ø = 4 mm;
— *stationary phase: diphenylphenylene oxide polymer R* (180-250 µm).

Carrier gas: nitrogen for chromatography R.

Flow rate: 40 mL/min.

Temperature:

	Time (min)	Temperature (°C)
Column	0 → 3	125
	3 → 17.6	125 → 300
Injection port		250
Detector		280

Detection: flame-ionisation.

Injection: 1.0 µL.

Limit:
— *ethanolamine:* maximum 1.0 per cent.

2,5-Diethoxytetrahydrofuran Diethoxytetrahydrofuran, a mixture of the cis and trans Isomers; $C_8H_{16}O_3$ = 160.2 (*3320-90-9*)

Clear, colourless or slightly yellowish liquid, practically insoluble in water, soluble in ethanol (96 per cent) and in most other organic solvents.

d_{20}^{20}: about 0.98.

n_D^{20}: about 1.418.

Diethylamine $C_4H_{11}N$ = 73.1 (*109-89-7*)

Clear, colourless, flammable liquid, strongly alkaline, miscible with water and with ethanol (96 per cent).

d_{20}^{20}: about 0.71.

bp: about 55 °C.

Diethylamine R1 *N*-Ethylethanamine; $C_4H_{11}N$ = 73.1 (*109-89-7*)

Content: minimum 99.5 per cent.

Clear, colourless, flammable liquid, strongly alkaline, miscible with water and with ethanol (96 per cent).

d_{20}^{20}: about 0.71.

bp: about 55 °C.

Diethylaminoethyldextran

Anion-exchange resin presented as the hydrochloride.

Powder forming gels with water.

***N,N*-Diethylaniline** $C_{10}H_{15}N$ = 149.2 (*91-66-7*)

d_{20}^{20}: about 0.938.

bp: about 217 °C.

mp: about −38 °C.

Diethylene Glycol Digol; $C_4H_{10}O_3$ = 106.1 (*111-46-6*)

Content: minimum 99.5 per cent *m/m*.

Clear, colourless liquid, hygroscopic, miscible with water, with acetone and with ethanol (96 per cent).

d_{20}^{20}: about 1.118.

n_D^{20}: about 1.447.

bp: 244 °C to 246 °C.

Storage: in an airtight container.

N,N-Diethylethane-1,2-diamine
N,N-Diethylethylenediamine; $C_6H_{16}N_2$ = 116.2 (*100-36-7*)

Content: minimum 98.0 per cent.

Slightly oily liquid, colourless or slightly yellow, strong odour of ammonia, irritant to the skin, eyes and mucous membranes.

d_{20}^{20}: 0.827.

bp: 145 °C to 147 °C.

Water (*2.5.12*): maximum 1.0 per cent, determined on 0.500 g.

Di(2-ethylhexyl)Phthalate Dioctyl phthalate; $C_{24}H_{38}O_4$ = 390.5

Colourless, oily liquid, practically insoluble in water, soluble in organic solvents.

d_{20}^{20}: about 0.98.

n_D^{20}: about 1.486.

Viscosity (*2.2.9*): about 80 mPa·s.

Diethyl Phthalate $C_{12}H_{14}O_4$ = 222.2 (*84-66-2*)

bp: about 298°.

General reagent grade of commerce.

N,N-Diethyl-*p*-phenylenediamine Sulfate *N,N*-Diethyl-*p*-phenylenediamine sulphate; Diethylphenylenediamine sulphate; Diethylphenylenediamine sulfate; $C_{10}H_{18}N_2O_4S$ = 262.3 (*6283-63-2*)

White or slightly yellow powder, soluble in water.

mp: about 185 °C, with decomposition.

Storage: protected from light.

Diethylphenylenediamine Sulfate Solution
Diethylphenylenediamine sulphate solution

To 250 mL of *water R* add 2 mL of *sulfuric acid R* and 25 mL of *0.02 M sodium edetate*. Dissolve in this solution 1.1 g of *diethylphenylenediamine sulfate R* and dilute to 1000 mL with *water R*.

Do not use if the solution is not colourless.

Storage: protected from light and heat for 1 month.

Diethyl Sulfone 1-(Ethylsulfonyl)ethane; 1-(Ethanesulfonyl)ethane; $C_4H_{10}O_2S$ = 122.2 (*597-35-3*)

Content: minimum 97 per cent.

Crystalline powder.

mp: about 73 °C.

Diflubenzuron 1-(4-Chlorophenyl)-3-(2,6-difluorobenzoyl)urea; $C_{14}H_9ClF_2N_2O_2$ = 310.7 (*35367-38-5*)

Colourless or white or almost white crystals, practically insoluble in water, freely soluble in dimethyl sulfoxide, slightly soluble in acetone.

mp: 230 to 232 °C.

Digitonin 3β-[*O*-β-D-Glucopyranosyl-(1→3)-*O*-β-D-galactopyranosyl-(1→2)-*O*-[β-D-xylopyranosyl-(1→3)]-*O*-β-D-galactopyranosyl-(1→4)-*O*-β-D-galactopyranosyloxy]-(25*R*)-5α-spirostan-2α,15β-diol; $C_{56}H_{92}O_{29}$ = 1229 (*11024-24-1*)

Crystals, practically insoluble in water, sparingly soluble in anhydrous ethanol, slightly soluble in ethanol (96 per cent).

Digitoxin (*71-63-6*)

See *Digitoxin (0078)*.

Diglycine 2-[(2-Aminoacetyl)amino]acetic acid; Glycylglycine; $C_4H_8N_2O_3$ = 132.1 (*556-50-3*)

Digoxin See *Digoxin (0079)*.=

Digoxin Reagent

Add 98 mL of *glacial acetic acid* to 2 mL of *sulfuric acid* and add 0.1 mL of a 5% w/v solution of *anhydrous iron(III) chloride* in *glacial acetic acid*.

Dihydrocapsaicin *N*-[(4-Hydroxy-3-methoxyphenyl) methyl]-8-methylnonanamide; $C_{18}H_{29}NO_3$ = 307.4 (*19408-84-5*)

White or almost white, crystalline powder, practically insoluble in cold water, freely soluble in anhydrous ethanol.

10,11-Dihydrocarbamazepine 10,11-Dihydro-5*H*-dibenzo[*b,f*]azepine-5-carboxamide; $C_{15}H_{14}N_2O$ = 238.3 (*3564-73-6*)

mp: 205 °C to 210 °C.

Dihydrocarvone *p*-Menth-8-en-2-one; 2-Methyl-5-(1-methylethenyl)cyclohexanone; $C_{10}H_{16}O$ = 152.2 (*7764-50-3*)

Dihydrocarvone used in gas chromatography complies with the following additional test.

Assay. Gas chromatography (*2.2.28*) as prescribed in the test for chromatographic profile in the monograph *Caraway oil (1817)*.

Content calculated by the normalisation procedure:
— *major component (trans-dihydrocarvone)*: minimum 70 per cent;
— *sum of cis- and trans-dihydrocarvone*: minimum 98 per cent.

1,8-Dihydroxyanthraquinone Danthron; Dihydroxyanthraquinone; 1,8-Dihydroxyanthracene-9,10-dione; Dantron; $C_{14}H_8O_4$ = 240.2 (*117-10-2*)

Crystalline orange powder, practically insoluble in water, slightly soluble in ethanol (96 per cent), soluble in solutions of alkali hydroxides.

mp: about 195 °C.

2,4-Dihydroxybenzaldehyde β-Resorcylaldehyde; $C_7H_6O_3$ = 138.1 (*95-01-2*)

2,5-Dihydroxybenzoic Acid Gentisic acid; $C_7H_6O_4$ = 154.1 (*490-79-9*)

Light yellow crystals.

mp: about 200 °C.

5,7-Dihydroxy-4-methylcoumarin 5,7-Dihydroxy-4-methyl-2*H*-1-benzopyran-2-one; $C_{10}H_8O_4$ = 192.2 (*2107-76-8*)

Light yellowish powder, practically insoluble in water, sparingly soluble in ethanol (96 per cent).

mp: 295 °C to 303 °C.

1,3-Dihydroxynaphthalene Naphthalene-1,3-diol; $C_{10}H_8O_2$ = 160.2 (*132-86-5*)

Crystalline, generally brownish-violet powder, freely soluble in water and in ethanol (96 per cent).

mp: about 125 °C.

5,7-Di-iodo-8-hydroxyquinoline 5,7-Diiodoquinolin-8-ol; $C_9H_5I_2NO$ = 397.0 (*83-73-8*)

Yellowish-brown powder, sparingly soluble in acetone and in ethanol (96 per cent).

Content: minimum 95.0 per cent.

1,5-Di-iodopentane $C_5H_{10}I_2$ = 323.9 (*628-77-3*)

bp: about 101°.

General reagent grade of commerce.

A colourless liquid.

Di-isobutyl Ketone 2,6-Dimethyl-4-heptanone; $C_9H_{18}O$ = 142.2 (*108-83-8*)

Clear, colourless liquid, slightly soluble in water, miscible with most organic solvents.

n_D^{20}: about 1.414

bp: about 168 °C.

Di-isopropyl Ether Isopropyl ether; $C_6H_{14}O$ = 102.2 (*108-20-3*)

Clear, colourless liquid, very slightly soluble in water, miscible with ethanol (96 per cent).

d_{20}^{20}: 0.723 to 0.728.

bp: 67 °C to 69 °C.

Do not distil if the di-isopropyl ether does not comply with the test for peroxides.

Peroxides. Place 8 mL of *potassium iodide and starch solution R* in a 12 mL ground-glass-stoppered cylinder about 1.5 cm in diameter. Fill completely with the substance to be examined, shake vigorously and allow to stand protected from light for 30 min. No colour is produced.

The name and concentration of any added stabiliser are stated on the label.

Storage: protected from light.

Di-isopropyl Ether, Stabiliser-free

Di-isopropyl ether that is free from stabiliser.

Reagent grade of commerce.

Di-isopropylethylamine Ethyldi-isopropylamine; $C_8H_{20}N_2$ = 144.3 (*7087-68-5*)

n_D^{20}: about 1.414.

bp: about 127°.

General reagent grade of commerce.

***N,N*-Diisopropylethylamine** *N*-Ethyl-*N*-(propan-2-yl) propan-2-amine; *N*-Ethyldiisopropylamine; $C_8H_{19}N$ = 129.2 (*7087-68-5*)

Clear, colourless or light yellow liquid.

bp: 127 °C.

***N,N'*-Di-isopropylethylenediamine** *N,N'*-Bis(1-methylethyl)-1,2-ethanediamine; $C_8H_{20}N_2$ = 144.3 (*4013-94-9*)

Colourless or yellowish, corrosive, flammable, hygroscopic liquid.

d_{20}^{20}: about 0.798.

n_D^{20}: about 1.429.

bp: about 170 °C.

Dillapiole 6-Allyl-4,5-dimethoxy-1,3-benzodioxole; $C_{12}H_{14}O_4$ = 222.24 (*523-80-8*)

General reagent grade of commerce.

2,5-Dimethoxybenzaldehyde $C_9H_{10}O_3$ = 166.2 (*93-02-7*)

mp: about 50°.

General reagent grade of commerce.

4,4'-Dimethoxybenzophenone Bis(4-methoxyphenyl) methanone; $C_{15}H_{14}O_3$ = 242.3 (*90-96-0*)

White or almost white powder, practically insoluble in water and slightly soluble in ethanol (96 per cent).

mp: about 142 °C.

3,4-Dimethoxyphenethylamine $C_{10}H_{15}NO_2$ = 181.2 (*120-20-7*)

n_D^{20}: about 1.546.

An oily liquid; weight per mL, about 1.07 g.

General reagent grade of commerce.

3,4-Dimethoxy-L-phenylalanine (2*S*)-2-Amino-3-(3,4-dimethoxyphenyl)propanoic acid; $C_{11}H_{15}NO_4$ = 225.2 (*32161-30-1*)

Content: minimum 95 per cent.

White or almost white powder.

2,2-Dimethoxypropane Dimethoxypropane; $C_5H_{12}O_2$ = 104.1 (*77-76-9*)

Colourless liquid, decomposing on exposure to moist air or water.

d_{20}^{20}: about 0.847.

n_D^{20}: about 1.378.

bp: about 83 °C.

Dimethylacetamide *N,N*-Dimethylacetamide; C_4H_9NO = 87.1 (*127-19-5*)

Content: minimum 99.5 per cent.

Colourless liquid, miscible with water and with many organic solvents.

d_{20}^{20}: about 0.94.

n_D^{20}: about 1.437.

bp: about 165 °C.

Dimethylamine *N*-Methylmethanamine; C_2H_7N = 45.09 (*124-40-3*)

Content: minimum 98.0 per cent.

Colourless, flammable gas.

Dimethylamine Hydrochloride C_2H_8ClN = 81.6 (*506-59-2*)

mp: about 172°.

General reagent grade of commerce.

Dimethylamine Solution

A 400 g/L solution of *dimethylamine R*.

Clear, colourless solution.

Density: about 0.89.

bp: about 54 °C.

mp: about −37 °C.

4-Dimethylaminobenzaldehyde

Dimethylaminobenzaldehyde; $C_9H_{11}NO$ = 149.2 (*100-10-7*)

White or yellowish-white crystals, soluble in ethanol (96 per cent) and in dilute acids.

mp: about 74 °C.

Dimethylaminobenzaldehyde Reagent

Dissolve 0.5 g of *4-dimethylaminobenzaldehyde* in a cooled mixture of 53 mL of *sulfuric acid* and 50 mL of *water* and add 0.5 mL of *iron(III) chloride solution R1*.

Allow to stand for 2 hours before use.

Dimethylaminobenzaldehyde Solution, Alcoholic

Dissolve 1 g of *4-dimethylaminobenzaldehyde* in 30 mL of *ethanol (96%)* and add 180 mL of *butan-1-ol* and 30 mL of *hydrochloric acid*.

Prepare immediately before use and discard if a pink colour develops.

Dimethylaminobenzaldehyde Solution R1

Dissolve 0.2 g of *dimethylaminobenzaldehyde R* in 20 mL of *ethanol (96 per cent) R* and add 0.5 mL of *hydrochloric acid R*. Shake the solution with *activated charcoal R* and filter. The colour of the reagent is less intense than that of *iodine solution R3*. Prepare immediately before use.

Dimethylaminobenzaldehyde Solution R2

Dissolve 0.2 g of *dimethylaminobenzaldehyde R*, without heating, in a mixture of 4.5 mL of *water R* and 5.5 mL of *hydrochloric acid R*. Prepare immediately before use.

Dimethylaminobenzaldehyde Solution R6

Dissolve 0.125 g of *dimethylaminobenzaldehyde R* in a cooled mixture of 35 mL of *water R* and 65 mL of *sulfuric acid R*. Add 0.1 mL of a 50 g/L solution of *ferric chloride R*. Before use allow to stand for 24 h, protected from light.

Storage: when stored at room temperature, use within 1 week; when stored in a refrigerator use within several months.

Dimethylaminobenzaldehyde Solution R7

Dissolve 1.0 g of *dimethylaminobenzaldehyde R* in 50 mL of *hydrochloric acid R* and add 50 mL of *ethanol (96 per cent) R*.

Storage: protected from light; use within 4 weeks.

Dimethylaminobenzaldehyde Solution R8

Dissolve 0.25 g of *dimethylaminobenzaldehyde R* in a mixture of 5 g of *phosphoric acid R*, 45 g of *water R* and 50 g of *anhydrous acetic acid R*. Prepare immediately before use.

Dimethylaminobenzaldehyde Solution R9

Dissolve 1.0 g of *dimethylaminobenzaldehyde R* in 3.5 mL of perchloric acid (600 g/L $HClO_4$) and slowly add 6.5 mL of *2-propanol R*. Prepare immediately before use.

4-Dimethylaminocinnamaldehyde 3-(4-Dimethylaminophenyl)prop-2-enal; $C_{11}H_{13}NO$ = 175.2 (*6203-18-5*)

Orange or orange-brown crystals or powder. Sensitive to light.

mp: about 138 °C.

4-Dimethylaminocinnamaldehyde Solution

Dissolve 2 g of *4-dimethylaminocinnamaldehyde R* in a mixture of 100 mL of *hydrochloric acid R1* and 100 mL of *anhydrous ethanol R*. Dilute the solution to four times its volume with *anhydrous ethanol R* immediately before use.

Dimethylaminoethanol 2-(Dimethylamino)ethan-1-ol; $C_4H_{11}NO$ = 89.1 (*108-01-0*)

Colourless or slightly yellow liquid, miscible with water.

bp: about 135 °C.

2-(Dimethylamino)ethyl Methacrylate 2-(Dimethylamino)ethyl 2-methylpropenoate; $C_8H_{15}NO_2$ = 157.2 (*2867-47-2*)

d_4^{20}: about 0.930.

bp: about 187 °C.

Dimethylaminonaphthalenesulfonyl Chloride Dimethylaminonaphthalenesulphonyl chloride; 5-Dimethylaminonaphthalene-1-sulphonyl chloride; Dansyl chloride; $C_{12}H_{12}ClNO_2S$ = 269.8 (*605-65-2*)

Yellow, crystalline powder, slightly soluble in water, soluble in methanol.

mp: about 70 °C.

3-Dimethylaminophenol 3-(Dimethylamino)phenol; $C_8H_{11}NO$ = 137.2 (*99-07-0*)

Grey powder, slightly soluble in water.

mp: about 80 °C.

2-(Dimethylamino)thioacetamide Hydrochloride $C_4H_{11}ClN_2S$ = 154.7 (*27366-72-9*)

Dimethylaniline *N,N*-Dimethylaniline; (*121-69-7*)

See *N,N-Dimethylaniline R*.

***N,N*-Dimethylaniline** $C_8H_{11}N$ = 121.2 (*121-69-7*)

Clear, oily liquid, almost colourless when freshly distilled, darkening on storage to reddish-brown, practically insoluble in water, freely soluble in ethanol (96 per cent).

n_D^{20}: about 1.558.

Distillation range (*2.2.11*). Not less than 95 per cent distils between 192 °C and 194 °C.

2,3-Dimethylaniline 2,3-Xylidine; $C_8H_{11}N$ = 121.2 (*87-59-2*)

Yellowish liquid, sparingly soluble in water, soluble in ethanol (96 per cent).

d_{20}^{20}: 0.993 to 0.995.

n_D^{20}: about 1.569.

bp: about 224 °C.

2,4-Dimethylaniline 2,4-Xylidene; $C_8H_{11}N$ = 121.2 (*95-68-1*)

General reagent grade of commerce.

2,6-Dimethylaniline 2,6-Xylidine; $C_8H_{11}N$ = 121.2 (*87-62-7*)

Colourless liquid, sparingly soluble in water, soluble in ethanol (96 per cent).

d_{20}^{20}: about 0.98.

2,6-Dimethylaniline Hydrochloride 2,6-Dimethylbenzenamide hydrochloride; 2,6-Xylidine hydrochloride; $C_8H_{12}ClN$ = 157.6 (*21436-98-6*)

Content: minimum 98.0 per cent.

2,4-Dimethyl-6-*tert*-butylphenol 6-*tert*-Butyl-2,4-dimethylphenol; $C_{12}H_{18}O$ = 178.3 (*1879-09-0*)

Dimethyl Carbonate Carbonic acid dimethyl ester; $C_3H_6O_3$ = 90.1 (*616-38-6*)

Liquid, insoluble in water, miscible with ethanol (96 per cent).

d_4^{17}: 1.065.

n_D^{20}: 1.368.

bp: about 90 °C.

Dimethyl-β-cyclodextrin Heptakis(2,6-di-*O*-methyl) cyclomaltoheptaose; Cycloheptakis-(1→4)-(2,6-di-*O*-methyl-α-D-glucopyranosyl); $2^A,2^B,2^C,2^D,2^E,2^F,2^G,6^A,6^B,6^C,6^D,6^E,6^F,6^G$-Tetradeca-*O*-methyl-β-cyclodextrin; $C_{56}H_{98}O_{35}$ = 1331 (*51166-71-3*)

White or almost white powder.

Dimethyldecylamine *N,N*-dimethyldecylamine; $C_{12}H_{27}N$ = 185.4 (*1120-24-7*)

Content: minimum 98.0 per cent *m/m*.

bp: about 234 °C.

1,7-dimethyl-3,7-dihydro-1*H*-purine-2,6-dione paraxanthine; 1,7-dimethylxanthine; $C_7H_8N_4O_2$ = 180.16 (*611-59-6*)

mp: about 298°

General reagent grade of commerce.

***N,N*-Dimethyldodecylamine *N*-oxide** $C_{14}H_{31}NO$ = 229.4 (*1643-20-5*)

mp: about 132°.

General reagent grade of commerce.

1,1-Dimethylethylamine 2-Amino-2-methylpropane; *tert*-Butylamine; $C_4H_{11}N$ = 73.1 (*75-64-9*)

Liquid, miscible with ethanol (96 per cent).

d_{20}^{20}: about 0.694.

n_D^{20}: about 1.378.

bp: about 46 °C.

1,1-Dimethylethyl Methyl Ether *tert*-Butyl methyl ether; 2-methoxy-2-methylpropane; $C_5H_{12}O$ = 88.1 (*1634-04-4*)

Colourless, clear, flammable liquid.

n_D^{20}: about 1.376.

Absorbance (*2.2.25*): maximum 0.30 at 240 nm, 0.10 at 255 nm, 0.01 at 280 nm, determined using *water R* as compensation liquid.

1,1-Dimethylethyl Methyl Ether R1 2-Methoxy-2-methylpropane; *tert*-Butyl methyl ether; $C_5H_{12}O$ = 88.1 (*1634-04-4*)

Content: minimum 99.5 per cent.

d_{20}^{20}: about 0.741.

n_D^{20}: about 1.369.

bp: about 55 °C.

Dimethylformamide C_3H_7NO = 73.1 (*68-12-2*)

Clear, colourless neutral liquid, miscible with water and with ethanol (96 per cent).

d_{20}^{20}: 0.949 to 0.952.

bp: about 153 °C.

Water (*2.5.12*): maximum 0.1 per cent.

Dimethylformamide Diethylacetal *N,N*-Dimethylformamide diethylacetal; $C_7H_{17}NO_2$ = 147.2 (*1188-33-6*)

n_D^{20}: about 1.40.

bp: 128 °C to 130 °C.

***N,N*-Dimethylformamide Dimethylacetal** 1,1-Dimethoxytrimethylamine; $C_5H_{13}NO_2$ = 119.2 (*4637-24-5*)

Clear, colourless liquid.

d_{20}^{20}: about 0.896.

n_D^{20}: about 1.396.

bp: about 103 °C.

Dimethylglyoxime 2,3-Butanedione dioxime; $C_4H_8N_2O_2$ = 116.1 (*95-45-4*)

White or almost white, crystalline powder or colourless crystals, practically insoluble in cold water, very slightly soluble in boiling water, soluble in ethanol (96 per cent).

mp: about 240 °C, with decomposition.

Sulfated ash (*2.4.14*): maximum 0.05 per cent.

1,3-Dimethyl-2-imidazolidinone *N,N'*-Dimethylethylene urea; 1,3-Dimethyl-2-imidazolidone; $C_5H_{10}N_2O$ = 114.2 (*80-73-9*)

n_D^{20}: 1.4720.

bp: about 224 °C.

***N,N*-Dimethyl-*p*-nitrosoaniline** $C_8H_{10}N_2O$ = 150.2 (*138-89-6*)

mp: about 86°.

General reagent grade of commerce.

Green crystals or a green, crystalline powder.

***N,N*-Dimethyloctylamine** Octyldimethylamine; $C_{10}H_{23}N$ = 157.3 (*7378-99-6*)

Colourless liquid.

d_{20}^{20}: about 0.765.

n_D^{20}: about 1.424.

bp: about 195 °C.

2,5-Dimethylphenol *p*-Xylenol; $C_8H_{10}O$ = 122.2 (*95-87-4*)

White or almost white crystals.

2,6-Dimethylphenol $C_8H_{10}O$ = 122.2 (*576-26-1*)

Colourless needles, slightly soluble in water, very soluble in ethanol (96 per cent).

bp: about 203 °C.

mp: 46 °C to 48 °C.

3,4-Dimethylphenol $C_8H_{10}O$ = 122.2 (*95-65-8*)

White or almost white crystals, slightly soluble in water, freely soluble in ethanol (96 per cent).

bp: about 226 °C.

mp: 25 °C to 27 °C.

***N,N*-Dimethyl-L-phenylalanine** (2*S*)-2-(Dimethylamino)-3-phenylpropanoic acid; $C_{11}H_{15}NO_2$ = 193.2 (*17469-89-5*)

mp: about 226 °C.

***N,N*-Dimethyl-*p*-phenylenediamine Dihydrochloride** $C_8H_{12}N_2,2HCl$ = 209.1 (*536-46-9*)

General reagent grade of commerce.

Darkens readily on exposure to air.

Dimethyl Phthalate $C_{10}H_{10}O_4$ = 194.2 (*131-11-3*)

General reagent grade of commerce.

A colourless or faintly coloured liquid; weight per mL, about 1.19 g.

N,N'-Dimethylpiperazine 1,4-Dimethylpiperazine; Dimethylpiperazine; $C_6H_{14}N_2$ = 114.2 (*106-58-1*)

A colourless liquid, miscible with water and with ethanol (96 per cent).

d_{20}^{20}: about 0.85.

n_D^{20}: about 1.446.

bp: about 131 °C.

Dimethylstearamide Dimethylstearylamide; *N,N*-dimethyloctadecanamide; $C_{20}H_{41}NO$ = 311.6

White or almost white solid mass, soluble in many organic solvents, including acetone.

mp: about 51 °C.

Dimethylstearylamide See *dimethylstearamide R*.

Dimethyl Sulfone Dimethyl sulphone; $C_2H_6O_2S$ = 94.1 (*67-71-0*)

White or almost white, crystalline powder, freely soluble in water, soluble in acetone and ethanol (96 per cent).

mp: 108 °C to 110 °C.

Dimethyl Sulfoxide Dimethyl sulphoxide; (*67-68-5*)

See *Dimethyl sulfoxide (0763)*.

Dimethyl sulfoxide used in spectrophotometry complies with the following additional test.

Absorbance (*2.2.25*): maximum 1.00 at 262 nm, 0.46 at 270 nm, 0.16 at 290 nm, 0.01 at 340 nm and higher wavelengths, determined using *water R* as compensation liquid.

Dimethyl Sulfoxide R1 Dimethyl sulphoxide R1

Content: minimum 99.7 per cent, determined by gas chromatography.

Dimethyl Sulfoxide R2 Dimethyl sulphoxide R2

Content: minimum 99.9 per cent, determined by gas chromatography.

Residue on evaporation: maximum 0.0005 per cent.

Water (2.5.32): maximum 0.005 per cent.

N,N-Dimethyltetradecylamine $C_{16}H_{35}N$ = 241.5

d_{20}^{20}: about 0.80.

bp: about 260°.

General reagent grade of commerce containing 98.0 to 101.0% w/w of $C_{16}H_{35}N$.

A clear or almost clear, colourless or slightly yellow liquid.

Complies with the following tests.

Water Not more than 0.3%, Appendix IX C, Method I A.

Assay Dissolve 0.2 g in 10 mL of *ethanol (96%)* and titrate with *0.1M hydrochloric acid VS* using *methyl red solution* as indicator until a red colour is produced. Each mL of *0.1M hydrochloric acid VS* is equivalent to 24.15 mg of $C_{16}H_{35}N$.

Dimethyl Yellow CI 11020; 4-dimethylaminoazobenzene; $C_{14}H_{15}N_3$ = 225.3 (*60-11-7*)

Produces a red colour in moderately acidic alcoholic solutions and a yellow colour in weakly acidic and alkaline solutions (pH range, 2.8 to 4.6).

Complies with the following test.

Homogeneity Carry out the method for *thin-layer chromatography*, Appendix III A, using *silica gel G* as the coating substance and *dichloromethane* as the mobile phase. Apply to the plate 10 µL of a 0.01% w/v solution in *dichloromethane*. The chromatogram shows only one spot.

Dimethyl Yellow and Oracet Blue Solution

Dissolve 10 mg of *dimethyl yellow* and 10 mg of *oracet blue B* in 300 mL of *dichloromethane*.

Dimethyl Yellow and Oracet Blue 2R Solution

Dissolve 10 mg of *dimethyl yellow* and 10 mg of *oracet blue 2R* in 300 mL of *dichloromethane*.

Dimethyl Yellow Solution

A 0.2% w/v solution of *dimethyl yellow* in *ethanol (90%)*.

Complies with the following test.

Sensitivity A solution containing 2 g of *ammonium chloride* in 25 mL of *carbon dioxide-free water* to which is added 0.1 mL of the dimethyl yellow solution is yellow. Not more than 0.10 mL of *0.1M hydrochloric acid VS* is required to change the colour of the solution to red (pH range, 2.8 to 4.6).

Dimeticone (*9006-65-9*)

See *Dimeticone (0138)*.

Dimidium Bromide 3,8-Diamino-5-methyl-6-phenylphenanthridinium bromide; $C_{20}H_{18}BrN_3$ = 380.3 (*518-67-2*)

Dark-red crystals, slightly soluble in water at 20 °C, sparingly soluble in water at 60 °C and in ethanol (96 per cent).

Dimidium Bromide-Sulfan Blue Mixed Solution

Dimidium bromide-sulphan blue mixed solution

Dissolve separately 0.5 g of *dimidium bromide R* and 0.25 g of *sulfan blue R* in 30 mL of a hot mixture of 1 volume of *anhydrous ethanol R* and 9 volumes of *water R*, stir, mix the two solutions, and dilute to 250 mL with the same mixture of solvents. Mix 20 mL of this solution with 20 mL of a 14.0 per cent *V/V* solution of *sulfuric acid R* previously diluted with about 250 mL of *water R* and dilute to 500 mL with *water R*.

Storage: protected from light.

1,3-Dinitrobenzene Dinitrobenzene; $C_6H_4N_2O_4$ = 168.1 (*99-65-0*)

Yellowish crystalline powder or crystals, practically insoluble in water, slightly soluble in ethanol (96 per cent).

mp: about 90 °C.

Dinitrobenzene Solution

A 10 g/L solution of *dinitrobenzene R* in *ethanol (96 per cent) R*.

3,5-Dinitrobenzoic Acid Dinitrobenzoic acid; $C_7H_4N_2O_6$ = 212.1 (*99-34-3*)

Almost colourless crystals, slightly soluble in water, very soluble in ethanol (96 per cent).

mp: about 206 °C.

Dinitrobenzoic Acid Solution

A 20 g/L solution of *dinitrobenzoic acid R* in *ethanol (96 per cent) R*.

Dinitrobenzoyl Chloride 3,5-Dinitrobenzoyl chloride; $C_7H_3ClN_2O_5$ = 230.6 (*99-33-2*)

Translucent, yellow or greenish-yellow powder or yellowish crystals, soluble in acetone and in toluene.

mp: about 68 °C.

Suitability test. To 1 mL of *anhydrous ethanol R* and 0.1 g of *dinitrobenzoyl chloride R* add 0.05 mL of *dilute sulfuric acid R* and boil under a reflux condenser for 30 min. After evaporation on a water-bath add 5 mL of *heptane R* to the residue and heat to boiling. Filter the hot solution. Wash the crystals formed on cooling to room temperature with a small quantity of *heptane R* and dry in a desiccator. The crystals melt (*2.2.14*) between 92 °C and 95 °C.

2,4-Dinitrophenylhydrazine Dinitrophenylhydrazine; $C_6H_6N_4O_4$ = 198.1 (*119-26-6*)

Reddish-orange crystals, very slightly soluble in water, slightly soluble in ethanol (96 per cent).

mp: about 203 °C (instantaneous method).

Dinitrophenylhydrazine-aceto-hydrochloric Solution

Dissolve 0.2 g of *dinitrophenylhydrazine R* in 20 mL of *methanol R* and add 80 mL of a mixture of equal volumes of *acetic acid R* and *hydrochloric acid R1*. Prepare immediately before use.

Dinitrophenylhydrazine-hydrochloric Solution

Dissolve by heating 0.50 g of *dinitrophenylhydrazine R* in *dilute hydrochloric acid R* and dilute to 100 mL with the same solvent. Allow to cool and filter. Prepare immediately before use.

Dinitrophenylhydrazine-sulfuric Acid Solution

Dinitrophenylhydrazine-sulphuric acid solution

Dissolve 1.5 g of *dinitrophenylhydrazine R* in 50 mL of a 20 per cent *V/V* solution of *sulfuric acid R*. Prepare immediately before use.

Dinonyl Phthalate $C_{26}H_{42}O_4$ = 418.6 (*28553-12-0*)

Colourless to pale yellow, viscous liquid.

d_{20}^{20}: 0.97 to 0.98.

n_D^{20}: 1.482 to 1.489.

Acidity. Shake 5.0 g with 25 mL of *water R* for 1 min. Allow to stand, filter the separated aqueous layer and add 0.1 mL of *phenolphthalein solution R*. Not more than 0.3 mL of *0.1 M sodium hydroxide* is required to change the colour of the solution (0.05 per cent, calculated as phthalic acid).

Water (*2.5.12*): maximum 0.1 per cent.

Dioctadecyl Disulfide Dioctadecyl disulphide; $C_{36}H_{74}S_2$ = 571.1 (*2500-88-1*)

White or almost white powder, practically insoluble in water.

mp: 53 °C to 58 °C.

2,2′-Di(octadecyloxy)-5,5′-spirobi(1,3,2-dioxaphosphorinane) 2,2′-Di(octadecyloxy)-5,5′-spirobi(1,3,2-dioxaphosphorin-ane); $C_{41}H_{82}O_6P_2$ = 733

White or almost white, waxy solid, practically insoluble in water, soluble in hydrocarbons.

mp: 40 °C to 70 °C.

Dioctadecyl 3,3′-Thiodipropionate $C_{42}H_{82}O_4S$ = 683 (*693-36-7*)

White or almost white, crystalline powder, practically insoluble in water, freely soluble in methylene chloride, sparingly soluble in acetone, in ethanol (96 per cent) and in light petroleum.

mp: 58 °C to 67 °C.

Di-*n*-octyl Phthalate Dioctyl benzene-1,2-dicarboxylate; $C_{24}H_{38}O_4$ = 390.6 (*117-84-0*)

Colourless viscous liquid, insoluble in water.

Density: about 0.98 g/mL (20 °C).

Dioctyl Sodium Sulfosuccinate Docusate sodium; Sodium 1,4-bis[(2-ethylhexyl)oxy]-1,4-dioxobutane-2-sulfonate; 1,4-bis(2-ethylhexyl) sulfobutanedioate sodium salt; Sodium dioctyl sulfosuccinate; $C_{20}H_{37}NaO_7S$ = 444.6 (*577-11-7*)

White or almost white, waxy solid.

Diosgenin (25*R*)-Spirost-5-en-3β-ol; $C_{27}H_{42}O_3$ = 414.6 (*512-04-9*)

1,4-Dioxan Dioxan; $C_4H_8O_2$ = 88.1 (*123-91-1*)

Clear, colourless liquid, miscible with water and with most organic solvents.

d_{20}^{20}: about 1.03.

Freezing point (*2.2.18*): minimum 11.0 °C.

Water (*2.5.12*): maximum 0.5 per cent.

Do not distil if the dioxan does not comply with the test for peroxides.

Peroxides. Place 8 mL of *potassium iodide and starch solution R* in a 12 mL ground-glass-stoppered cylinder about 1.5 cm in diameter. Fill completely with the substance to be examined, shake vigorously and allow to stand in the dark for 30 min. No colour is produced.

Dioxan used for liquid scintillation is of a suitable analytical grade.

Dioxan Solution

Dissolve 1.00 g of *dioxan R* in *water R* and dilute to 100.0 mL with the same solvent. Dilute 5.0 mL of this solution to 100.0 mL with *water R* (0.5 mg/mL of dioxan).

Dioxan Solution R1

Dilute 10.0 mL of *dioxan solution R* to 50.0 mL with *water R*. (0.1 mg/mL of dioxan).

Dioxan Solution R2

Dilute 2.0 mL of *dioxan solution R* to 50.0 mL with *water R* (0.02 mg/mL of dioxan).

Diphenylamine $C_{12}H_{11}N$ = 169.2 (*122-39-4*)

White or almost white crystals, slightly soluble in water, soluble in ethanol (96 per cent).

mp: about 55 °C.

Storage: protected from light.

Diphenylamine Solution

A 1 g/L solution of *diphenylamine R* in *sulfuric acid R*.

Storage: protected from light.

Diphenylamine Solution R1

A 10 g/L solution of *diphenylamine R* in *sulfuric acid R*. The solution is colourless.

Diphenylamine Solution R2

Dissolve 1 g of *diphenylamine R* in 100 mL of *glacial acetic acid R* and add 2.75 mL of *sulfuric acid R*. Use immediately.

9,10-Diphenylanthracene Diphenylanthracene; $C_{26}H_{18}$ = 330.4 (*1499-10-1*)

Yellowish or yellow, crystalline powder, practically insoluble in water.

mp: about 248 °C.

***N,N′*-Diphenylbenzidine** *N,N′*-Diphenylbiphenyl-4,4′-diamine; Diphenylbenzidine; $C_{24}H_{20}N_2$ = 336.4 (*531-91-9*)

White or faintly grey, crystalline powder, practically insoluble in water, slightly soluble in acetone and in ethanol (96 per cent).

mp: about 248 °C.

Nitrates. Dissolve 8 mg in a cooled mixture of 5 mL of *water R* and 45 mL of *nitrogen-free sulfuric acid R*. The solution is colourless or very pale blue.

Sulfated ash (*2.4.14*): maximum 0.1 per cent.

Storage: protected from light.

Diphenylboric Acid Aminoethyl Ester $C_{14}H_{16}BNO$ = 225.1 (*524-95-8*)

White or slightly yellow, crystalline powder, practically insoluble in water, soluble in ethanol (96 per cent).

mp: about 193 °C.

1,5-Diphenylcarbazide 1,5-diphenylcarbonodihydrazide; Diphenylcarbazide; $C_{13}H_{14}N_4O$ = 242.3 (*140-22-7*)

White or almost white, crystalline powder which gradually becomes pink on exposure to air, very slightly soluble in water, soluble in acetone, in ethanol (96 per cent) and in glacial acetic acid.

mp: about 170 °C.

Sulfated ash (*2.4.14*): maximum 0.1 per cent.

Storage: protected from light.

Diphenylcarbazide Solution

Dissolve 0.2 g of *diphenylcarbazide R* in 10 mL of *glacial acetic acid R* and dilute to 100 mL with *anhydrous ethanol R*. Prepare immediately before use.

1,5-Diphenylcarbazone Diphenylcarbazone; $C_{13}H_{12}N_4O$ = 240.3 (*538-62-5*)

Orange-yellow, crystalline powder, practically insoluble in water, freely soluble in ethanol (96 per cent).

mp: about 157 °C, with decomposition.

Diphenylcarbazone Mercuric Reagent

Solution A. Dissolve 0.1 g of *diphenylcarbazone R* in *anhydrous ethanol R* and dilute to 50 mL with the same solvent.

Solution B. Dissolve 1 g of *mercuric chloride R* in *anhydrous ethanol R* and dilute to 50 mL with the same solvent.

Mix equal volumes of the two solutions.

2,2-Diphenylglycine Amino(diphenyl)acetic acid; $C_{14}H_{13}NO_2$ = 227.26 (*3060-50-2*)

1,2-Diphenylhydrazine Hydrazobenzene; 1,2-Diphenyldiazane; $C_{12}H_{12}N_2$ = 184.3 (*122-66-7*)

Orange powder.

mp: about 125 °C.

Diphenylmethanol Benzhydrol; $C_{13}H_{12}O$ = 184.2 (*91-01-0*)

White or almost white, crystalline powder.

mp: about 66 °C.

Diphenyloxazole 2,5-Diphenyloxazole; $C_{15}H_{11}NO$ = 221.3 (*92-71-7*)

White or almost white powder, practically insoluble in water, soluble in methanol, sparingly soluble in dioxan and in glacial acetic acid.

mp: about 70 °C.

$A_{1\,cm}^{1\%}$: about 1260 determined at 305 nm in *methanol R*.

Diphenyloxazole used for liquid scintillation is of a suitable analytical grade.

Diphenylphenylene Oxide Polymer 2,6-Diphenyl-*p*-phenylene oxide polymer

White or almost white, porous beads. The size range of the beads is specified after the name of the reagent in the tests where it is used.

Dipotassium Edetate Dipotassium dihydrogen ethylenediaminetetra-acetate; $C_{10}H_{14}N_2K_2O_8,2H_2O$ = 404.5 (*25102-12-9*)

General reagent grade of commerce.

Dipotassium Hydrogen Orthophosphate Dipotassium hydrogen phosphate; K_2HPO_4 = 174.2 (*7758-11-4*)

White or almost white, crystalline powder, hygroscopic, very soluble in water, slightly soluble in ethanol (96 per cent).

Storage: in an airtight container.

Dipotassium Hydrogen Phosphate Trihydrate $K_2HPO_4,3H_2O$ = 228.2 (*16788-57-1*)

Colourless or white or almost white powder or crystals, freely soluble in water.

Dipotassium Sulfate Potassium sulfate; Dipotassium sulphate; Potassium sulphate; K_2SO_4 = 174.3 (*7778-80-5*)

Colourless crystals, soluble in water.

Dipotassium (+)-Tartrate Potassium tartrate; $C_4H_4K_2O_6,{}^1/_2H_2O$ = 235.3 (*921-53-9*)

White or almost white, granular powder or crystals, very soluble in water, very slightly soluble in ethanol (96 per cent).

2,2′-Dipyridyl 2,2′-Bipyridine; $C_{10}H_8N_2$ = 156.2 (*366-18-7*)

mp: about 72°.

General reagent grade of commerce.

2,2′-Dipyridylamine *N*-(Pyridin-2-yl)pyridin-2-amine; $C_{10}H_9N_3$ = 171.2 (*1202-34-2*)

mp: about 95 °C.

Disodium Arsenate Sodium arsenate heptahydrate; $Na_2HAsO_4,7H_2O$ = 312.0 (*10048-95-0*)

Crystals, efflorescent in warm air, freely soluble in water, soluble in glycerol, slightly soluble in ethanol (96 per cent). The aqueous solution is alcaline to litmus.

d_{20}^{20}: about 1.87.

mp: about 57 °C when rapidly heated.

Disodium Bicinchoninate 2,2′-Biquinoline-4-4′-dicarboxylate disodium salt; $C_{20}H_{10}N_2Na_2O_4$ = 388.3 (*979-88-4*)

Disodium Catechol-3,5-disulfonate 4,5-Dihydroxy-1,3-benzene disulfonic acid disodium salt monohydrate, 4,5-dihydroxy-1,3-benzene disulphonic acid disodium salt monohydrate, disodium catechol-3,5- disulphonate; $C_6H_4O_8Na,H_2O$ = 332 (*149-45-1*)

General reagent grade of commerce.

Disodium Edetate Disodium dihydrogen ethylenediaminetetra-acetate, dihydrate; Sodium edetate; (*6381-92-6*)

See *Disodium edetate (0232)*.

Disodium Ethanedisulfonate Disodium ethanedisulphonate; 1,2-ethanedisulfonic acid disodium salt; 1,2-ethanedisulphonic acid disodium salt; $C_2H_4Na_2O_6S_2$ = 234.2 (*5325-43-9*)

General reagent grade of commerce.

Disodium Hydrogen Citrate Sodium acid citrate; Disodium hydrogen 2-hydroxypropane-1,2,3-tricarboxylate sesquihydrate; $C_6H_6Na_2O_7,1{}^1/_2H_2O$ = 263.1 (*144-33-2*)

White or almost white powder, soluble in less than 2 parts of water, practically insoluble in ethanol (96 per cent).

Disodium Hydrogen Orthophosphate Disodium Hydrogen Phosphate; Disodium hydrogen phosphate dodecahydrate; (*10039-32-4*)

See *Disodium phosphate dodecahydrate (0118)*.

Disodium Hydrogen Orthophosphate, Anhydrous Disodium hydrogen phosphate, anhydrous; Na_2HPO_4 = 142.0 (*7558-79-4*)

Disodium Hydrogen Orthophosphate Dihydrate Disodium hydrogen phosphate dihydrate; (*10028-24-7*)

See *Disodium phosphate dihydrate (0602)*.

Disodium Hydrogen Orthophosphate Heptahydrate Disodium hydrogen phosphate heptahydrate; $Na_2HPO_4,7H_2O$ = 268.1 (*7782-85-6*)

Disodium Hydrogen Phosphate Heptahydrate $Na_2HPO_4,7H_2O$ = 268.1 (*7782-85-6*)

Disodium Hydrogen Phosphate Solution

A 90 g/L solution of *disodium hydrogen phosphate dodecahydrate R*.

Ditalimphos *O,O*-Diethyl (1,3-dihydro-1,3-dioxo-2*H*-isoindol-2-yl)phosphonothioate; $C_{12}H_{14}NO_4PS$ = 299.3 (*5131-24-8*)

Very slightly soluble in water, in ethyl acetate and in anhydrous ethanol.

A suitable certified reference solution may be used.

5,5′-Dithiobis(2-nitrobenzoic) Acid 3-Carboxy-4-nitrophenyldisulphide; $C_{14}H_8N_2O_8S_2$ = 396.4 (*69-78-3*)

Yellow powder sparingly soluble in ethanol (96 per cent).

mp: about 242 °C.

Dithioerythritol $C_4H_{10}O_2S_2$ = 154.3 (*6892-68-8*)

(2*R*,3*S*)-1,4-Disulfanylbutane-2,3-diol. DTE.

mp: about 83 °C.

Dithiol Toluene-3,4-dithiol; 4-Methylbenzene-1,2-dithiol; $C_7H_8S_2$ = 156.3 (*496-74-2*)

White or almost white crystals, hygroscopic, soluble in methanol and in solutions of alkali hydroxides.

mp: about 30 °C.

Storage: in an airtight container.

Dithiol Reagent Toluenedithiol reagent

To 1 g of *dithiol R* add 2 mL of *thioglycollic acid R* and dilute to 250 mL with a 20 g/L solution of *sodium hydroxide R*. Prepare immediately before use.

Dithiothreitol *threo*-1,4-Dimercaptobutane-2,3-diol; $C_4H_{10}O_2S_2$ = 154.2 (*27565-41-9*)

Slightly hygroscopic needles, freely soluble in water, in acetone and in anhydrous ethanol.

Storage: in an airtight container.

Dithizone 1,5-Diphenylthiocarbazone; $C_{13}H_{12}N_4S$ = 256.3 (*60-10-6*)

A bluish-black, brownish-black or black powder, practically insoluble in water, soluble in ethanol (96 per cent).

Storage: protected from light.

Dithizone R1 1,5-Diphenylthiocarbazone; $C_{13}H_{12}N_4S$ = 256.3 (*60-10-6*)

Content: minimum 98.0 per cent.

Bluish-black, brownish-black or black powder, practically insoluble in water, soluble in ethanol (96 per cent).

Storage: protected from light.

Dithizone Solution

A 0.5 g/L solution of *dithizone R* in *chloroform R*. Prepare immediately before use.

Dithizone Solution R2

Dissolve 40.0 mg of *dithizone R* in *chloroform R* and dilute to 1000.0 mL with the same solvent. Dilute 30.0 mL of the solution to 100.0 mL with *chloroform R*.

Assay. Dissolve a quantity of *mercuric chloride R* equivalent to 0.1354 g of $HgCl_2$ in a mixture of equal volumes of *dilute sulfuric acid R* and *water R* and dilute to 100.0 mL with the same mixture of solvents. Dilute 2.0 mL of this solution to 100.0 mL with a mixture of equal volumes of *dilute sulfuric acid R* and *water R*. (This solution contains 20 ppm of Hg). Transfer 1.0 mL of the solution to a separating funnel and add 50 mL of *dilute sulfuric acid R*, 140 mL of *water R* and 10 mL of a 200 g/L solution of *hydroxylamine hydrochloride R*. Titrate with the dithizone solution; after each addition, shake the mixture twenty times and towards the end of the titration allow to separate and discard the chloroform layer. Titrate until a bluish-green colour is obtained. Calculate the equivalent in micrograms of mercury per millilitre of the dithizone solution from the expression $20/V$, where V is the volume in millilitres of the dithizone solution used in the titration.

Divanadium Pentoxide Vanadium(V) oxide; V_2O_5 = 181.9 (*1314-62-1*)

Content: minimum 98.5 per cent.

Yellow-brown or rust-brown powder, slightly soluble in water, soluble in strong mineral acids and in solutions of alkali hydroxides with formation of salts.

Appearance of solution. Heat 1 g for 30 min with 10 mL of *sulfuric acid R*. Allow to cool and dilute to 10 mL with the same acid. The solution is clear (*2.2.1*).

Sensitivity to hydrogen peroxide. Dilute 1.0 mL of the solution prepared for the test for appearance of solution cautiously to 50.0 mL with *water R*. To 0.5 mL of the solution add 0.1 mL of a solution of hydrogen peroxide (0.1 g/L of H_2O_2) prepared from *dilute hydrogen peroxide solution R*. The solution has a distinct orange colour compared with a blank prepared from 0.5 mL of the solution to be examined and 0.1 mL of *water R*. After the addition of 0.4 mL of a solution of hydrogen peroxide (0.1 g/L of H_2O_2) prepared from *dilute hydrogen peroxide solution R*, the orange solution becomes orange-yellow.

Loss on ignition: maximum 1.0 per cent, determined on 1.00 g at 700 ± 50 °C.

Assay. Dissolve 0.200 g with heating in 20 mL of a 70 per cent *m/m* solution of *sulfuric acid R*. Add 100 mL of *water R* and *0.02 M potassium permanganate* until a reddish colour is obtained. Decolorise the excess of potassium permanganate by the addition of a 30 g/L solution of *sodium nitrite R*. Add 5 g of *urea R* and 80 mL of a 70 per cent *m/m* solution of *sulfuric acid R*. Cool. Using 0.1 mL of *ferroin R* as indicator, titrate the solution immediately with *0.1 M ferrous sulfate* until a greenish-red colour is obtained.

1 mL of *0.1 M ferrous sulfate* is equivalent to 9.095 mg of V_2O_5.

Divanadium Pentoxide Solution in Sulfuric Acid
Divanadium Pentoxide Solution in Sulphuric Acid

Dissolve 0.2 g of *divanadium pentoxide R* in 4 mL of *sulfuric acid R* and dilute to 100 mL with *water R*.

Divinylbenzene and Vinylpyrrolidone Copolymer for Chromatography

Chromatographic reagent grade of commerce.

Spherical particles (30 µm) of an *m*-divinylbenzene and *N*-vinylpyrrolidone copolymer.

Docosahexaenoic Acid Methyl Ester DHA methyl ester; Cervonic acid methyl ester; (all-*Z*)-Docosa-4,7,10,13,16,19-hexaenoic acid methyl ester; $C_{23}H_{34}O_2$ = 342.5 (*301-01-9*)

Content: minimum 90.0 per cent, determined by gas chromatography.

Docusate Sodium (*577-11-7*)

See *Docusate sodium (1418)*.

Dodecan-1-ol Lauryl alcohol; $C_{12}H_{26}O$ = 186.3 (*112-53-8*)

d_{20}^{20}: about 0.820.

mp: 24 °C to 27 °C.

Content: minimum 98.0 per cent, determined by gas chromatography.

4-Dodecylresorcinol $CH_3(CH_2)_{11}C_6H_3$-1,3-$(OH)_2$ = 278.43 (*24305-56-4*)

General reagent grade of commerce.

Dodecyltrimethylammonium Bromide *N,N,N*-Trimethyldodecan-1-aminium bromide; $C_{15}H_{34}BrN$ = 308.4 (*1119-94-4*)

White or almost white crystals.

mp: about 246 °C.

Domiphen Bromide Dodecyldimethyl-2-phenoxyethylammonium bromide; $C_{22}H_{40}BrNO$ = 414.5 (*538-71-6*)

General reagent grade of commerce.

D-Dopa (2*R*)-2-Amino-3-(3,4-dihydroxyphenyl)propanoic acid; 3-Hydroxy-D-tyrosine; 3,4-Dihydroxy-D-phenylalanine; $C_9H_{11}NO_4$ = 197.2 (*5796-17-8*)

$[\alpha]_D^{20}$: + 9.5 to + 11.5, determined on a 10 g/L solution in *1 M hydrochloric acid*.

mp: about 277 °C.

Dotriacontane *n*-Dotriacontane; $C_{32}H_{66}$ = 450.9 (*544-85-4*)

White or almost white plates, practically insoluble in water, sparingly soluble in hexane.

mp: about 69 °C.

Impurities. Not more than 0.1 per cent of impurities with the same t_R value as α-tocopherol acetate, determined by the gas chromatographic method prescribed in the monograph *α-Tocopherol acetate (0439)*.

Doxycycline

See *Doxycycline monohydrate (0820)*.

β-Ecdysterone (2β,3β,5β,22*R*)-2,3,14,20,22,25-Hexahydroxycholest-7-en-6-one; $C_{27}H_{44}O_7$ = 480.6 (*5289-74-7*)

Echinacoside β-(3′,4′-Dihydroxyphenyl)-ethyl-*O*-α-L-rhamnopyranosyl (1→3)-*O*-β-D-[β-D-glucopyranosyl(1→6)]-(4-*O*-caffeoyl)-glucopyranoside; $C_{35}H_{46}O_{20}$ = 787 (*82854-37-3*)

Pale yellow powder, odourless.

Edotreotide *N*-[[4,7,10-Tris(carboxymethyl)-1,4,7,10-tetraazacyclododecan-1-yl]acetyl]-D-phenylalanyl-L-cysteinyl-L-tyrosyl-D-tryptophyl-L-lysyl-L-threonyl-*N*-[(1*R*,2*R*)-2-hydroxy-1-(hydroxymethyl)propyl]-L-cysteinamide cyclic (2→7)-disulfide; DOTATOC; DOTA-[Tyr^3]-octreotide; $C_{65}H_{92}N_{14}O_{18}S_2$ = 1422 (*204318-14-9*)

White or almost white powder.

Content: minimum 95.0 per cent.

***n*-Eicosane** $C_{20}H_{42}$ = 282.6 (*112-95-8*)

mp: about 37°.

General reagent grade of commerce.

Electrolyte Reagent for the Determination of Water
Electrolyte reagent for the micro determination of water

Commercially available anhydrous reagent or a combination of anhydrous reagents for the coulometric titration of water, containing suitable organic bases, sulfur dioxide and iodide dissolved in a suitable solvent.

Embonic Acid $C_{23}H_{16}O_6$ = 388.4 (*130-85-8*)

General reagent grade of commerce.

Emodin 1,3,8-Trihydroxy-6-methylanthraquinone; $C_{15}H_{10}O_5$ = 270.2 (*518-82-1*)

Orange-red needles, practically insoluble in water, soluble in ethanol (96 per cent) and in solutions of alkali hydroxides.

Chromatography. Thin-layer chromatography (*2.2.27*) as prescribed in the monograph *Rhubarb (0291)*; the chromatogram shows only one principal spot.

Endoprotease LysC

Microbial extracellular proteolytic enzyme secreted by *Achromobacter lyticus*. A lyophilised powder, free of salts.

α-Endosulfan α-Endosulphan; $C_9H_6Cl_6O_3S$ = 406.9 (*959-98-8*)

bp: about 200 °C.

mp: about 108 °C.

A suitable certified reference solution (10 ng/µL in cyclohexane) may be used.

β-Endosulfan β-Endosulphan; $C_9H_6Cl_6O_3S$ = 406.9 (*33213-65-9*)

bp: about 390 °C.

mp: about 207 °C.

A suitable certified reference solution (10 ng/µL in cyclohexane) may be used.

Endrin $C_{12}H_8Cl_6O$ = 380.9 (*72-20-8*)

A suitable certified reference solution (10 ng/µL in cyclohexane) may be used.

Eosin CI 45380; acid red 87; $C_{20}H_6Br_4Na_2O_5$ = 691.9 (*17372-87-1*)

General reagent grade of commerce.

A red powder.

(−)-Epicatechin (2*R*,3*R*)-2-(3,4-Dihydroxyphenyl)-3,4-dihydro-2*H*-1-benzopyran-3,5,7-triol; $C_{15}H_{14}O_6$ = 290.3 (*490-46-0*)

4-epichlortetracycline hydrochloride $C_{22}H_{23}ClN_2O_8 \cdot HCl$ = 515.34 (*101342-45-4*)

General reagent grade of commerce.

(−)-Epigallocatechin-3-*O*-gallate (2*R*,3*R*)-5,7-Dihydroxy-2-(3,4,5-trihydroxyphenyl)-3,4-dihydro-2*H*-1-benzopyran-3-yl 3,4,5-trihydroxybenzoate; $C_{22}H_{18}O_{11}$ = 458.4 (*989-51-5*)

Epilactose $C_{12}H_{22}O_{11}$ = 342.3 (*20869-27-6*)

4-*O*-β-D-Galactopyranosyl-D-mannopyranose.

Content: minimum 98 per cent.

4-Epioxytetracycline $C_{22}H_{24}N_2O_9$ = 460.4 (*14206-58-7*)

Analytical reagent grade of commerce.

Erucamide (*Z*)-Docos-13-enoamide; $C_{22}H_{43}NO$ = 337.6 (*112-84-5*)

Yellowish or white powder or granules, practically insoluble in water, very soluble in methylene chloride, soluble in anhydrous ethanol.

mp: about 70 °C.

Erythritol (*149-32-6*)

See *Erythritol (1803)*.

Esculetin 6,7-Dihydroxy-2*H*-1-benzopyran-2-one; Aesculetin; $C_9H_6O_4$ = 178.1 (*305-01-1*)

Esculin 6-(β-D-Glucopyranosyloxy)-7-hydroxy-2*H*-chromen-2-one; $C_{15}H_{16}O_9, 1^1/_2H_2O$ = 367.3 (*531-75-9*)

White or almost white powder or colourless crystals, sparingly soluble in water and in ethanol (96 per cent), freely soluble in hot water and in hot ethanol (96 per cent).

Chromatography (*2.2.27*). Thin-layer chromatography (*2.2.27*) as prescribed in the monograph *Eleutherococcus (1419)*; the chromatogram shows only one principal spot.

Estradiol Estra-1,3,5(10)-triene-3,17β-diol; β-Estradiol; $C_{18}H_{24}O_2$ = 272.4 (*50-28-2*)

Prisms stable in air, practically insoluble in water, freely soluble in ethanol (96 per cent), soluble in acetone and in dioxan, sparingly soluble in vegetable oils.

mp: 173 °C to 179 °C.

17α-Estradiol $C_{18}H_{24}O_2$ = 272.4 (*57-91-0*)

White or almost white, crystalline powder or colourless crystals.

mp: 220 °C to 223 °C.

Estragole 4-Allylanisole; $C_{10}H_{12}O$ = 148.2 (*140-67-0*)

Liquid, miscible with ethanol (96 per cent).

n_D^{20}: about 1.52.

bp: about 216 °C.

Estragole used in gas chromatography complies with the following test.

Assay. Gas chromatography (*2.2.28*) as prescribed in the monograph *Anise oil (0804)*.

Test solution. The substance to be examined.

Content: minimum 98.0 per cent, calculated by the normalisation procedure.

Ethane C_2H_6 = 30.07 (*74-84-0*)

Content: minimum 99.0 per cent *V/V*.

Ethane-1,2-diol Ethylene glycol; $C_2H_6O_2$ = 62.1 (*107-21-1*)

Content: minimum 99.0 per cent.

Colourless, slightly viscous liquid, hygroscopic, miscible with water and with ethanol (96 per cent).

d_{20}^{20}: 1.113 to 1.115.

n_D^{20}: about 1.432.

bp: about 198 °C.

mp: about −12 °C.

Acidity. To 10 mL add 20 mL of *water R* and 1 mL of *phenolphthalein solution R*. Not more than 0.15 mL of *0.02 M sodium hydroxide* is required to change the colour of the indicator to pink.

Water (*2.5.12*): maximum 0.2 per cent

Ethanol Absolute ethanol; (*64-17-5*)

See *Ethanol, anhydrous R*.

Ethanol, Absolute Ethanol; C_2H_6O = 46.07 (*64-17-5*)

d_{20}^{20}: 0.791 to 0.794.

bp: 78° to 79°.

Analytical reagent grade of commerce containing not less than 99.5% v/v of C_2H_6O.

A colourless, hygroscopic liquid.

Store protected from light at a temperature not exceeding 30°.

Ethanol, Anhydrous (*64-17-5*)

See *Ethanol, anhydrous (1318)*.

Ethanol R1, Absolute Ethanol R1

Complies with the requirements prescribed for the monograph *Ethanol, anhydrous (1318)* with the following additional requirement.

Methanol. Gas chromatography (*2.2.28*).

Test solution. The substance to be examined.

Reference solution. Dilute 0.50 mL of *anhydrous methanol R* to 100.0 mL with the substance to be examined. Dilute 1.0 mL of this solution to 100.0 mL with the substance to be examined.

Column:
— *material*: glass;
— *size*: *l* = 2 m, Ø = 2 mm;
— *stationary phase*: *ethylvinylbenzene-divinylbenzene copolymer R* (75-100 µm).

Carrier gas: *nitrogen for chromatography R*.

Flow rate: 30 mL/min.

Temperature:
— *column*: 130 °C;
— *injection port*: 150 °C;
— *detector*: 200 °C.

Detection: flame-ionisation.

Injection: 1 µL of the test solution and 1 µL of the reference solution, alternately, three times.

After each chromatography, heat the column to 230 °C for 8 min. Integrate the methanol peak. Calculate the percentage methanol content from the following expression:

$$\frac{a \times b}{c - b}$$

a = percentage *V/V* content of methanol in the reference solution,
b = area of the methanol peak in the chromatogram obtained with the test solution,
c = area of the methanol peak in the chromatogram obtained with the reference solution.

Limit:
— *methanol*: maximum 0.005 per cent *V/V*.

Ethanol (96 per cent) (*64-17-5*)

See *Ethanol (96 per cent) (1317)*.

Ethanol (96%)

Alcohol

Analytical reagent grade ethanol of commerce containing not less than 95.1% v/v and not more than 96.9% v/v of C_2H_6O.

A colourless liquid; weight per mL, about 0.81 g.

Diluted ethanols may be prepared by diluting the volumes of *ethanol (96%)* indicated in the following table to 1000 mL with *water*.

Strength % v/v	Volume of *ethanol (96%)* (approx) ml	Weight per ml g
90	934	0.83
85	885	0.85
80	831	0.86
70	727	0.89
65	676	0.90
60	623	0.91
50	519	0.93
45	468	0.94
25	259	0.97
20	207	0.975
10	104	0.986

Ethanol (96%), Aldehyde-free

Aldehyde-free alcohol

Mix 1200 mL of *ethanol (96%)* with 5 mL of a 40% w/v solution of *silver nitrate* and 10 mL of a cooled 50% w/v solution of *potassium hydroxide*. Shake, allow to stand for a few days and filter. Distil the filtrate immediately before use.

Ethanol (*x*% v/v)

Mix appropriate volumes of *water R* and *ethanol (96 per cent) R*, allowing for the effects of warming and volume contraction inherent to the preparation of such a mixture, to obtain a solution whose final content of ethanol corresponds to the value of *x*.

Ethanolamine 2-Aminoethanol; C_2H_7NO = 61.1 (*141-43-5*)

Clear, colourless, viscous, hygroscopic liquid, miscible with water and with methanol.

d_{20}^{20}: about 1.014.

n_D^{20}: about 1.454.

mp: about 11 °C.

Storage: in an airtight container.

Ether Diethyl ether; $C_4H_{10}O$ = 74.1 (*60-29-7*)

Clear, colourless, volatile and very mobile liquid, very flammable, hygroscopic, soluble in water, miscible with ethanol (96 per cent).

d_{20}^{20}: 0.713 to 0.715.

bp: 34 °C to 35 °C.

Do not distil if the ether does not comply with the test for peroxides.

Peroxides. Place 8 mL of *potassium iodide and starch solution R* in a 12 mL ground-glass-stoppered cylinder about 1.5 cm in diameter. Fill completely with the substance to be examined, shake vigorously and allow to stand in the dark for 30 min. No colour is produced.

The name and concentration of any added stabilisers are stated on the label.

Storage: in an airtight container, protected from light, at a temperature not exceeding 15 °C.

Ether, Peroxide-free See *Anaesthetic ether (0367)*.

Shake 1000 mL of *ether* with 20 mL of a solution of 30 g of *iron(II) sulfate* in 55 mL of *water* and 3 mL of *sulfuric acid*. Continue shaking until a small sample no longer produces a

blue colour when shaken with an equal volume of a 2% w/v solution of *potassium iodide* and 0.1 mL of *starch mucilage*.

Ethion $C_9H_{22}O_4P_2S_4$ = 384.5 (*563-12-2*)

mp: −24 °C to −25 °C.

A suitable certified reference solution (10 ng/µL in cyclohexane) may be used.

Ethoxychrysoidine Hydrochloride 4-*p*-Ethoxyphenylazo-*m*-phenylenediamine hydrochloride; $C_{14}H_{17}ClN_4O$ = 292.8 (*2313-87-3*)

Reddish powder, soluble in ethanol (96 per cent).

Ethoxychrysoidine Solution

A 1 g/L solution of *ethoxychrysoidine hydrochloride R* in *ethanol (96 per cent) R*.

Test for sensitivity. To a mixture of 5 mL of *dilute hydrochloric acid R* and 0.05 mL of the ethoxy-chrysoidine solution add 0.05 mL of *0.0167 M bromide-bromate*. The colour changes from red to light yellow within 2 min.

2-Ethoxyethanol Ethylene glycol monoethyl ether; $C_4H_{10}O_2$ = 90.1 (*110-80-5*)

Content: minimum 99.0 per cent.

Clear, colourless liquid, miscible with water, with acetone and with ethanol (96 per cent).

d_{20}^{20}: about 0.93.

n_D^{25}: about 1.406.

bp: about 135 °C.

(2-ethoxyphenoxy) acetic acid $C_{10}H_{12}O_4$ = 196.2 g/mol (*3251-30-7*)

General reagent grade of commerce

Ethyl Acetate $C_4H_8O_2$ = 88.1 (*141-78-6*)

Clear, colourless liquid, soluble in water, miscible with ethanol (96 per cent).

d_{20}^{20}: 0.901 to 0.904.

bp: 76 °C to 78 °C.

Ethyl Acetate, Treated

Disperse 200 g of *sulfamic acid R* in *ethyl acetate R* and make up to 1000 mL with the same solvent. Stir the suspension obtained for three days and filter through a filter paper.

Storage: use within 1 month.

Ethyl Acetate R1 $C_4H_8O_2$ = 88.1 (*141-78-6*)

Suitable for gas chromatography ECD and FID.

Liquid, miscible in water (i.e. 85.3 g/L).

d_{20}^{20}: 0.90.

Content: minimum 99.8 per cent.

Evaporation residue: maximum 3.0 mg/L.

Water: maximum 0.02 per cent.

Ethyl Acrylate Ethyl prop-2-enoate; $C_5H_8O_2$ = 100.1 (*140-88-5*)

Colourless liquid.

d_{20}^{20}: about 0.924.

n_D^{20}: about 1.406.

bp: about 99 °C.

mp: about −71 °C.

Ethyl Benzoate $C_9H_{10}O_2$ = 150.2 (*93-89-0*)

A clear, colourless, refractive liquid, practically insoluble in water, miscible with ethanol (96 per cent) and with light petroleum.

d_4^{25}: about 1.050.

n_D^{20}: about 1.506.

bp: 211 °C to 213 °C.

Ethyl 5-Bromovalerate Ethyl 5-bromopentanoate; $C_7H_{13}BrO_2$ = 209.1 (*14660-52-7*)

Clear, colourless liquid.

d_{20}^{20}: about 1.321.

bp: 104 °C to 109 °C.

Ethyl Cinnamate $C_{11}H_{12}O_2$ = 176.2 (*103-36-6*)

General reagent grade of commerce.

A colourless or very pale yellow liquid; weight per mL, about 1.05 g.

Ethyl Clorazepate Ethyl (3*RS*)-7-chloro-2-oxo-5-phenyl-2,3-dihydro-1*H*-1,4-benzodiazepine-3-carboxylate; $C_{18}H_{15}ClN_2O_3$ = 342.8 (*5606-55-3*)

Ethyl Cyanoacetate $C_5H_7NO_2$ = 113.1 (*105-56-6*)

Colourless or pale yellow liquid, slightly soluble in water, miscible with ethanol (96 per cent).

bp: 205 °C to 209 °C, with decomposition.

Ethyl Formate Ethyl methanoate; $C_3H_6O_2$ = 74.1 (*109-94-4*)

Clear, colourless, flammable liquid, freely soluble in water, miscible with ethanol (96 per cent).

d_{20}^{20}: about 0.919.

n_D^{20}: about 1.36.

bp: about 54 °C.

Ethyl 4-Hydroxybenzoate (*120-47-8*)

See *Ethyl parahydroxybenzoate R*.

Ethyl Methanesulfonate $C_3H_8O_3S$ = 124.2 (*62-50-0*)

Clear, colourless liquid.

Content: minimum 99.0 per cent.

Density: about 1.206 g/cm^3 (20 °C).

n_D^{20}: about 1.418.

bp: about 213 °C.

Ethyl Parahydroxybenzoate Ethyl 4-hydroxybenzoate; (*120-47-8*)

See *Ethyl parahydroxybenzoate (0900)*.

4-[(Ethylamino)methyl]pyridine $C_8H_{12}N_2$ = 136.2 (*33403-97-3*)

Pale yellow liquid.

d_{20}^{20}: about 0.98.

n_D^{20}: about 1.516.

bp: about 98 °C.

Ethylbenzene C_8H_{10} = 106.2 (*100-41-4*)

Content: minimum 99.5 per cent *m/m*, determined by gas chromatography.

Clear, colourless liquid, practically insoluble in water, soluble in acetone, and in ethanol (96 per cent).

d_{20}^{20}: about 0.87.

n_D^{20}: about 1.496.

bp: about 135 °C.

Ethyl Benzenesulfonate $C_8H_{10}O_3S$ = 186.2 (*515-46-8*)

Content: minimum 97.0 per cent.

Colourless or slightly yellow liquid, slightly soluble in water, miscible with ethanol (96 per cent).

Density: about 1.22 g/mL (25 °C).

4-Ethylcatechol 3,4-Dihydroxyethylbenzene; $C_8H_{10}O_2$ = 138.2

General reagent grade of commerce.

Ethylene Bis[3,3-di(3-(1,1-dimethyl)ethyl-4-hydroxyphenyl)butyrate] Ethylene bis[3,3-di(3-*tert*-butyl-4-hydroxyphenyl)butyrate]; $C_{50}H_{66}O_8$ = 795 (*32509-66-3*)

Crystalline powder, practically insoluble in water and in light petroleum, very soluble in acetone and in methanol.

mp: about 165 °C.

Ethylene Bis[3,3-di(3-*tert*-butyl-4-hydroxyphenyl) butyrate] (*32509-66-3*)

See *ethylene bis[3,3-di(3-(1,1-dimethylethyl)-4-hydroxyphenyl) butyrate] R*.

Ethylene Glycol Monododecyl Ether 2-(Dodecyloxy) ethan-1-ol; $C_{14}H_{30}O_2$ = 230.4 (*4536-30-5*)

Colourless or faintly green liquid.

Ethylene Oxide Oxirane; C_2H_4O = 44.05 (*75-21-8*)

Colourless, flammable gas, very soluble in water and in anhydrous ethanol.

Liquefaction point: about 12 °C.

Ethylene Oxide Solution

Weigh a quantity of cool *ethylene oxide stock solution R* equivalent to 2.5 mg of ethylene oxide into a cool flask and dilute to 50.0 g with *macrogol 200 R1*. Mix well and dilute 2.5 g of this solution to 25.0 mL with *macrogol 200 R1* (5 µg of ethylene oxide per gram of solution). *Prepare immediately before use.*

The solution can be prepared using commercially available reagents instead of *ethylene oxide stock solution R*, making appropriate dilutions.

Ethylene Oxide Solution R1

Dilute 1.0 mL of cooled *ethylene oxide stock solution R* (check the exact volume by weighing) to 50.0 mL with *macrogol 200 R1*. Mix well and dilute 2.5 g of this solution to 25.0 mL with *macrogol 200 R1*. Calculate the exact amount of ethylene oxide in parts per million from the volume determined by weighing and taking the relative density of *macrogol 200 R1* as 1.127. *Prepare immediately before use.*

The solution can be prepared using commercially available reagents instead of *ethylene oxide stock solution R*, making appropriate dilutions.

Ethylene Oxide Solution R2

Weigh 1.00 g of cold *ethylene oxide stock solution R* (equivalent to 2.5 mg of ethylene oxide) into a cold flask containing 40.0 g of cold *macrogol 200 R1*. Mix and determine the exact mass and dilute to a calculated mass to obtain a solution containing 50 µg of ethylene oxide per gram of solution. Weigh 10.00 g into a flask containing about 30 mL of *water R*, mix and dilute to 50.0 mL with *water R* (10 µg/mL of ethylene oxide). *Prepare immediately before use.*

The solution can be prepared using commercially available reagents instead of *ethylene oxide stock solution R*, making appropriate dilutions.

Ethylene Oxide Solution R3

Dilute 10.0 mL of *ethylene oxide solution R2* to 50.0 mL with *water R* (2 µg/mL of ethylene oxide). *Prepare immediately before use.*

Ethylene Oxide Solution R4

Dilute 1.0 mL of *ethylene oxide stock solution R1* to 100.0 mL with *water R*. Dilute 1.0 mL of this solution to 25.0 mL with *water R*.

Ethylene Oxide Solution R5

A 50 g/L solution of *ethylene oxide R* in *methylene chloride R*.

Either use a commercially available reagent or prepare the solution corresponding to the above-mentioned composition.

Ethylene Oxide Stock Solution

All operations carried out in the preparation of these solutions must be conducted in a fume cupboard. The operator must protect both hands and face by wearing polyethylene protective gloves and an appropriate face mask.

Store all solutions in an airtight container in a refrigerator at 4 °C to 8 °C. Carry out all determinations three times.

Into a dry, clean test-tube, cooled in a mixture of 1 part of *sodium chloride R* and 3 parts of crushed ice, introduce a slow current of *ethylene oxide R* gas, allowing condensation onto the inner wall of the test-tube. Using a glass syringe, previously cooled to −10 °C, inject about 300 µL (corresponding to about 0.25 g) of liquid *ethylene oxide R* into 50 mL of *macrogol 200 R1*. Determine the absorbed quantity of ethylene oxide by weighing before and after absorption (M_{eo}). Dilute to 100.0 mL with *macrogol 200 R1*. Mix well before use.

Assay. To 10 mL of a 500 g/L suspension of *magnesium chloride R* in *anhydrous ethanol R* add 20.0 mL of *0.1 M alcoholic hydrochloric acid R* in a flask. Stopper and shake to obtain a saturated solution and allow to stand overnight to equilibrate. Weigh 5.00 g of ethylene oxide stock solution (2.5 g/L) into the flask and allow to stand for 30 min. Titrate with 0.1 M alcoholic potassium hydroxide R determining the end-point potentiometrically (*2.2.20*).

Carry out a blank titration, replacing the substance to be examined with the same quantity of *macrogol 200 R1*.

Ethylene oxide content in milligrams per gram is given by:

$$\frac{(V_0 - V_1) \times f \times 4.404}{m}$$

V_0, V_1 = volumes of *0.1 M alcoholic potassium hydroxide* used respectively for the blank titration and the assay,
f = factor of the alcoholic potassium hydroxide solution,
m = mass of the sample taken, in grams.

Ethylene Oxide Stock Solution R1

A 50 g/L solution of *ethylene oxide R* in *methanol R*.

Either use a commercially available reagent or prepare the solution corresponding to the aforementioned composition.

Ethylene Oxide Stock Solution R2

A 50 g/L solution of *ethylene oxide R* in *methylene chloride R*.

Either use a commercially available reagent or prepare the solution corresponding to the aforementioned composition.

Ethylenediamine 1,2-Diaminoethane; $C_2H_8N_2$ = 60.1 (*107-15-3*)

Clear, colourless, fuming liquid, strongly alkaline, miscible with water and with ethanol (96 per cent).

bp: about 116 °C.

Ethylenediaminetetra-acetic Acid (Ethylenedinitrilo) tetra-acetic acid; $C_{10}H_{16}N_2O_8$ = 292.2 (*60-00-4*)

White or almost white crystalline powder, very slightly soluble in water.

mp: about 250 °C, with decomposition.

2-Ethylhexane-1,3-diol $C_8H_{18}O_2$ = 146.2 (*94-96-2*)

Slightly oily liquid, soluble in anhydrous ethanol, 2-propanol, propylene glycol and castor oil.

d_{20}^{20}: about 0.942.

n_D^{20}: about 1.451.

bp: about 244 °C.

2-Ethylhexanoic Acid 2-Ethylhexoic acid; $C_8H_{16}O_2$ = 144.2 (*149-57-5*)

Colourless liquid.

d_{20}^{20}: about 0.91.

n_D^{20}: about 1.425.

Related substances. Gas chromatography (*2.2.28*).

Injection: 1 µL of the test solution.

Test solution: suspend 0.2 g of the 2-ethylhexanoic acid in 5 mL of *water R*, add 3 mL of *dilute hydrochloric acid R* and 5 mL of *hexane R*, shake for 1 min, allow the layers to separate and use the upper layer. Carry out the chromatographic procedure as prescribed in the test for 2-ethylhexanoic acid in the monograph on *Amoxicillin sodium (0577)*.

Limit: the sum of the area of any peaks, apart from the principal peak and the peak due to the solvent, is not greater than 2.5 per cent of the area of the principal peak.

1,1′-Ethylidenebis(tryptophan) $C_{24}H_{26}N_4O_4$ = 434.5 (*132685-02-0*)

mp: about 223°, with decomposition.

General reagent grade of commerce containing not less than 98.0% of $C_{24}H_{26}N_4O_4$.

Assay Proceed as described in the test for 1,1′-Ethylidenebis(tryptophan) and other related substances in the monograph for Tryptophan. The area of the principal peak in the chromatogram obtained with reference solution (a) is not less than 98.0% of the area of all the peaks.

***N*-Ethylmaleimide** 1-Ethyl-1*H*-pyrrole-2,5-dione; $C_6H_7NO_2$ = 125.1 (*128-53-0*)

Colourless crystals, sparingly soluble in water, freely soluble in ethanol (96 per cent).

mp: 41 °C to 45 °C.

Storage: at a temperature of 2 °C to 8 °C.

2-Ethyl-2-methylsuccinic Acid 2-Ethyl-2-methylbutanedioic acid; $C_7H_{12}O_4$ = 160.2 (*631-31-2*)

mp: 104 °C to 107 °C.

2-Ethylpyridine C_7H_9N = 107.2 (*100-71-0*)

Colourless or brownish liquid.

d_{20}^{20}: about 0.939.

n_D^{20}: about 1.496.

bp: about 149 °C.

1-Ethylquinaldinium Iodide 1-Ethyl-2-methylquinolinium iodide; $C_{12}H_{14}IN$ = 299.2 (*606-55-3*)

General reagent grade of commerce.

Ethyl Toluenesulfonate Ethyl 4-methylbenzenesulfonate; Ethyl tosilate; $C_9H_{12}O_3S$ = 200.3 (*80-40-0*)

Content: minimum 97.0 per cent.

Density: about 1.17 g/mL (25 °C).

bp: about 160 °C.

mp: about 33 °C.

Ethylvinylbenzene-Divinylbenzene Copolymer

Porous, rigid, cross-linked polymer beads. Several grades are available with different sizes of bead. The size range of the beads is specified after the name of the reagent in the tests where it is used.

Eugenol 4-Allyl-2-methoxyphenol; $C_{10}H_{12}O_2$ = 164.2 (*97-53-0*)

Colourless or pale yellow, oily liquid, darkening on exposure to air and light and becoming more viscous, practically insoluble in water, miscible with ethanol (96 per cent) and with fatty and essential oils.

d_{20}^{20}: about 1.07.

bp: about 250 °C.

Eugenol used in gas chromatography complies with the following additional test.

Assay. Gas chromatography (*2.2.28*) as prescribed in the monograph *Clove oil (1091)*.

Test solution. The substance to be examined.

Content: minimum 98.0 per cent, calculated by the normalisation procedure.

Storage: protected from light.

Euglobulins, Bovine

Use fresh bovine blood collected into an anticoagulant solution (for example, sodium citrate solution). Discard any haemolysed blood. Centrifuge at 1500-1800 *g* at 15-20 °C to obtain a supernatant plasma poor in platelets.

To 1 L of bovine plasma add 75 g of *barium sulfate R* and shake for 30 min. Centrifuge at not less than 1500-1800 *g* at 15-20 °C and draw off the clear supernatant. Add 10 mL of a 0.2 mg/mL solution of *aprotinin R* and shake to ensure mixing. In a container with a minimum capacity of 30 L in a chamber at 4 °C introduce 25 L of *distilled water R* at 4 °C and add about 500 g of solid carbon dioxide. Immediately add, while stirring, the supernatant obtained from the plasma. A white precipitate is formed. Allow to settle at 4 °C for 10-15 h. Remove the clear supernatant solution by siphoning. Collect the precipitate by centrifuging at 4 °C. Suspend the precipitate by dispersing mechanically in 500 mL of *distilled water R* at 4 °C, shake for 5 min and collect the precipitate by centrifuging at 4 °C. Disperse the precipitate mechanically in 60 mL of a solution containing 9 g/L of *sodium chloride R* and 0.9 g/L *sodium citrate R* and adjust to pH 7.2-7.4 by adding a 10 g/L solution of *sodium hydroxide R*. Filter through a sintered glass filter (*2.1.2*); to facilitate the dissolution of the precipitate crush the particles of the precipitate with a suitable instrument. Wash the filter and the instrument with 40 mL of the chloride-citrate solution described above and dilute to 100 mL with the same solution. Freeze-dry the solution. The yields are generally 6 g to 8 g of euglobulins per litre of bovine plasma.

Test for suitability. For this test, prepare the solutions using *phosphate buffer solution pH 7.4 R* containing 30 g/L of *bovine albumin R*.

Into a test-tube 8 mm in diameter placed in a water-bath at 37 °C introduce 0.2 mL of a solution of a reference preparation of urokinase containing 100 IU/mL and 0.1 mL of a solution of *human thrombin R* containing 20 IU/mL. Add rapidly 0.5 mL of a solution containing 10 mg of bovine euglobulins per millilitre. A firm clot forms in less than 10 s. Note the time that elapses between the addition of the solution of bovine euglobulins and the lysis of the clot. The lysis time does not exceed 15 min.

Storage: protected from moisture at 4 °C; use within 1 year.

Euglobulins, Human

For the preparation, use fresh human blood collected into an anticoagulant solution (for example sodium citrate solution) or human blood for transfusion that has been collected in plastic blood bags and which has just reached its expiry date. Discard any haemolysed blood. Centrifuge at 1500-1800 *g* at 15 °C to obtain a supernatant plasma poor in platelets. Iso-group plasmas may be mixed.

To 1 L of the plasma add 75 g of *barium sulfate R* and shake for 30 min. Centrifuge at not less than 15 000 *g* at 15 °C and draw off the clear supernatant. Add 10 mL of a solution of *aprotinin R* containing 0.2 mg/mL and shake to ensure mixing. In a container with a minimum capacity of 30 L in a chamber at 4 °C introduce 25 L of *distilled water R* at 4 °C and add about 500 g of solid carbon dioxide. Immediately add while stirring the supernatant obtained from the plasma. A white precipitate is formed. Allow to settle at 4 °C for 10-15 h. Remove the clear supernatant solution by siphoning. Collect the precipitate by centrifuging at 4 °C. Suspend the precipitate by dispersing mechanically in 500 mL of *distilled water R* at 4 °C, shake for 5 min and collect the precipitate by centrifuging at 4 °C. Disperse the precipitate mechanically in 60 mL of a solution containing 9 g/L of *sodium chloride R* and 0.9 g/L of *sodium citrate R*, and adjust the pH to 7.2-7.4 by adding a 10 g/L solution of *sodium hydroxide R*. Filter through a sintered-glass filter (*2.1.2*); to facilitate the dissolution of the precipitate crush the particles of the precipitate with a suitable instrument. Wash the filter and the instrument with 40 mL of the chloride-citrate solution described above and dilute to 100 mL with the same solution. Freeze-dry the solution. The yields are generally 6 g to 8 g of euglobulins per litre of human plasma.

Test for suitability. For this test, prepare the solutions using *phosphate buffer solution pH 7.2 R* containing 30 g/L of *bovine albumin R*. Into a test-tube 8 mm in diameter placed in a water-bath at 37 °C introduce 0.1 mL of a solution of a reference preparation of streptokinase containing 10 IU of streptokinase activity per millilitre and 0.1 mL of a solution of *human thrombin R* containing 20 IU/mL. Add rapidly 1 mL of a solution containing 10 mg of human euglobulins per millilitre. A firm clot forms in less than 10 s. Note the time that elapses between the addition of the solution of human euglobulins and the lysis of the clot. The lysis time does not exceed 15 min.

Storage: in an airtight container at 4 °C; use within 1 year.

Evodiamine (13b*S*)-14-Methyl-8,13,13b,14-tetrahydroindolo[2′,3′:3,4]pyrido[2,1-*b*]quinazolin-5(7*H*)-one; $C_{19}H_{17}N_3O$ = 303.4 (*518-17-2*)

Extraction Resin

Solid phase extraction resin containing 2,2′-oxybis(*N*,*N*-dioctylacetamide) (*N*,*N*,*N*′,*N*′-tetra-*n*-octyldiglycolamide).

Fargesin $C_{21}H_{22}O_6$ = 370.4 (*31008-19-2*)

5-[(3*SR*,3a*RS*,6*RS*,6a*RS*)-6-(3,4-Dimethoxyphenyl)-1,3,3a,4,6,6a-hexahydrofuro[3,4-*c*]furan-3-yl]-1,3-benzodioxole.

(*E*,*E*)-Farnesol *trans*,*trans*-Farnesol; (2*E*,6*E*)-3,7,11-Trimethyldodeca-2,6,10-trien-1-ol; $C_{15}H_{26}O$ = 222.4 (*106-28-5*)

Fast Blue B Salt 3,3′-Dimethoxy(biphenyl)-4,4′-bisdiazonium dichloride; $C_{14}H_{12}Cl_2N_4O_2$ = 339.2 (*84633-94-3*)

Schultz No. 490

Colour Index No. 37235

Dark green powder, soluble in water. It is stabilised by addition of zinc chloride.

Storage: in an airtight container, at a temperature between 2 °C and 8 °C.

Fast Blue B Salt Solution

Dissolve 140 mg of *fast blue B salt R* in 10 mL of *water R* and mix with 50 mL of *methylene chloride R* and 140 mL of *methanol R*.

Storage: protected from light at a temperature of 4 °C; use within 1 week.

Fast Red B Salt 2-Methoxy-4-nitrobenzenediazonium hydrogen naphthalene-1,5-disulfonate; $C_{17}H_{13}N_3O_9S_2$ = 467.4 (*49735-71-9*)

Schultz No. 155

Colour Index No. 37125

Orange-yellow powder, soluble in water, slightly soluble in ethanol (96 per cent).

Storage: in an airtight container, protected from light, at 2 °C to 8 °C.

Fenbufen $C_{16}H_{14}O_3$ = 254.3 (*36330-85-5*)

General reagent grade of commerce.

Fenchlorphos $C_8H_8Cl_3O_3PS$ = 321.5 (*299-84-3*)

mp: about 35 °C.

A suitable certified reference solution (10 ng/µL in cyclohexane) may be used.

Fenchone (1*R*)-1,3,3-Trimethylbicyclo[2.2.1]heptan-2-one; $C_{10}H_{16}O$ = 152.2 (*7787-20-4*)

Oily liquid, miscible with ethanol (96 per cent), practically insoluble in water.

n_D^{20}: about 1.46.

bp_{15mm}: 192 °C to 194 °C.

Fenchone used in gas chromatography complies with the following test.

Assay. Gas chromatography (*2.2.28*) as prescribed in the monograph *Bitter fennel (0824)*.

Test solution. The substance to be examined.

Content: minimum 98.0 per cent, calculated by the normalisation procedure.

Fenvalerate $C_{25}H_{22}ClNO_3$ = 419.9 (*51630-58-1*)

bp: about 300 °C.

A suitable certified reference solution (10 ng/µL in cyclohexane) may be used.

Ferric Chloride-ferricyanide-arsenite Reagent

Immediately before use mix 10 mL of a 27 g/L solution of *ferric chloride R* in *dilute hydrochloric acid R*, 7 mL of *potassium ferricyanide solution R*, 3 mL of *water R* and 10 mL of *sodium arsenite solution R*.

Ferric Chloride Solution R3

Dissolve 2.0 g of *ferric chloride R* in *anhydrous ethanol R* and dilute to 100.0 mL with the same solvent.

Ferric Chloride Solution, Radiolabelled [^{59}Fe], Concentrated

A commercially available solution of [^{59}Fe]ferric chloride (approximate specific activity: 100-1000 MBq/mg of Fe).

Ferric Chloride Solution, Radiolabelled [^{59}Fe]

Dilute the *concentrated radiolabelled [^{59}Fe]ferric chloride solution* in *sodium citrate buffer solution pH 7.8* to obtain a solution with an activity of 3.7×10^4 Bq/mL

Ferrocyphen Dicyanobis(1,10-phenanthroline)iron(II); Ferrocyphene; $C_{26}H_{16}FeN_6$ = 468.3 (*14768-11-7*)

Violet-bronze, crystalline powder, practically insoluble in water and in ethanol (96 per cent).

Storage: protected from light and moisture.

Ferrocyphen Solution

Dissolve, without warming, 0.5 g of *ferrocyphen* in 50 mL of *sulfuric acid*.

Ferroin Solution Ferroin; (*14634-91-4*)

Dissolve 0.7 g of *ferrous sulfate R* and 1.76 g of *phenanthroline hydrochloride R* in 70 mL of *water R* and dilute to 100 mL with the same solvent.

Test for sensitivity. To 50 mL of *dilute sulfuric acid R* add 0.1 mL of *ferroin R*. After the addition of 0.1 mL of *0.1 M ammonium and cerium nitrate* the colour changes from red to light blue.

Ferulic Acid 4-Hydroxy-3-methoxycinnamic acid; 3-(4-Hydroxy-3-methoxyphenyl)propenoic acid; $C_{10}H_{10}O_4$ = 194.2 (*1135-24-6*)

Faint yellow powder, freely soluble in methanol.

mp: 172.9 °C to 173.9 °C.

Ferulic acid used in the assay of eleutherosides in Eleutherococcus (1419) complies with the following additional test.

Assay. Liquid chromatography (*2.2.29*) as prescribed in the monograph *Eleutherococcus (1419)*.

Content: minimum 99 per cent, calculated by the normalisation procedure.

Fibrin Blue

Mix 1.5 g of fibrin with 30 mL of a 5 g/L solution of *indigo carmine R* in 1 per cent *V/V dilute hydrochloric acid R*. Heat the mixture to 80 °C and maintain at this temperature whilst stirring for about 30 min. Allow to cool. Filter. Wash extensively by resuspension in 1 per cent *V/V dilute hydrochloric acid R* and mixing for about 30 min; filter. Repeat the washing operation three times. Dry at 50 °C. Grind.

Fibrinogen (*9001-32-5*)

See *Human fibrinogen, freeze-dried (0024)*.

Fixing Solution

To 250 mL of *methanol R*, add 0.27 mL of *formaldehyde R* and dilute to 500.0 mL with *water R*.

Fixing Solution for Isoelectric Focusing in Polyacrylamide Gel

A solution containing 35 g of *sulfosalicylic acid R* and 100 g of *trichloroacetic acid R* per litre of *water R*.

Flufenamic Acid 2-[[3-(Trifluoromethyl)phenyl]amino] benzoic acid; $C_{14}H_{10}F_3NO_2$ = 281.2 (*530-78-9*)

Pale yellow, crystalline powder or needles, practically insoluble in water, freely soluble in ethanol (96 per cent).

mp: 132 °C to 135 °C.

Flumazenil (*78755-81-4*)

See *Flumazenil (1326)*.

Flunitrazepam (*1622-62-4*)

See *Flunitrazepam (0717)*.

Fluoranthene $C_{16}H_{10}$ = 202.3 (*206-44-0*)

bp: about 384°.

mp: 105° to 110°.

General reagent grade of commerce.

Fluorene Diphenylenemethane; $C_{13}H_{10}$ = 166.2 (*86-73-7*)

White or almost white crystals, freely soluble in anhydrous acetic acid, soluble in hot ethanol (96 per cent).

mp: 113 °C to 115 °C.

9-Fluorenone $C_{13}H_{18}O$ = 180.2 (*486-25-9*)

mp: about 83°.

General reagent grade of commerce.

Fluorenone Solution

Dissolve 50 mg of *9-fluorenone* in 10 mL of warm *methanol*, transfer to a 500-mL graduated flask with the aid of 190 mL of *methanol* and add sufficient *water* to produce 500 mL. The solution should be clear. Immediately before use dilute 10 mL of this solution to 100 mL with *methanol*.

(9-Fluorenyl)methyl Chloroformate Fluoren-9-ylmethyl chloromethanoate; $C_{15}H_{11}ClO_2$ = 258.7 (*28920-43-6*)

mp: about 63 °C.

Fluorescamine 4-Phenylspiro[furan-2(3*H*),1'(3'*H*)-isobenzofuran]-3,3'-dione; $C_{17}H_{10}O_4$ = 278.3 (*38183-12-9*)

mp: 154 °C to 155 °C.

Fluorescein 3',6'-Dihydroxyspiro[isobenzofurane-1(3*H*),9'-[9*H*]xanthen]-3-one; $C_{20}H_{12}O_5$ = 332.3 (*2321-07-5*)

Orange-red powder, practically insoluble in water, soluble in warm ethanol (96 per cent), soluble in alkaline solutions. In solution, fluorescein displays a green fluorescence.

mp: about 315 °C.

Fluorescein Sodium Disodium 2-(3-oxo-6-oxido-3H-xanthen-9-yl)benzoate; Sodium fluoresceinate; $C_{20}H_{10}Na_2O_5$ = 376.3 (*518-47-8*)

Schultz No. 880

Colour Index No. 45350

Orange-red powder, freely soluble in water. Aqueous solutions display an intense yellowish-green fluorescence.

Fluorocholine Chloride *N*-(Fluoromethyl)-2-hydroxy-*N*,*N*-dimethylethan-1-aminium chloride; $C_5H_{13}ClFNO$ = 157.6 (*459424-38-5*)

Colourless, hygroscopic crystals.

mp: about 184 °C.

2-Fluoro-2-deoxy-D-glucose $C_6H_{11}FO_5$ = 182.2 (*86783-82-6*)

White or almost white crystalline powder.

mp: 174 °C to 176 °C.

2-Fluoro-2-deoxy-D-mannose $C_6H_{11}FO_5$ = 182.1 (*38440-79-8*)

Colourless semi-solid.

1-Fluoro-2,4-dinitrobenzene 2,4-Dinitrofluorobenzene; Fluorodinitrobenzene; $C_6H_3FN_2O_4$ = 186.1 (*70-34-8*)

Pale yellow liquid or crystals, soluble in propylene glycol.

mp: about 29 °C.

Content: minimum 99.0 per cent, determined by gas chromatography.

1-Fluoro-2,4-dinitrophenyl-5-L-alaninamide N^{α}-(5-Fluoro-2,4-dinitrophenyl)-L-alaninamide; Marfey's reagent; FDAA; $C_9H_9FN_4O_5$ = 272.2 (*95713-52-3*)

Yellow or orange powder.

mp: about 228 °C.

Enantiomeric purity: minimum 99.5 per cent.

DL-6-Fluorodopa Hydrochloride (2*RS*)-2-Amino-3-(2-fluoro-4,5-dihydroxyphenyl)propanoic acid hydrochloride; 2-Fluoro-5-hydroxy-DL-tyrosine hydrochloride; $C_9H_{11}ClFNO_4$ = 251.6

White or almost white powder.

Fluoroethyl(2-hydroxyethyl)dimethylammonium Chloride *N*-(2-Fluoroethyl)-2-hydroxy-*N*,*N*-dimethylethan-1-aminium chloride; $C_6H_{15}ClFNO$ = 171.6 (*479407-08-4*)

Slightly yellow powder.

Fluoroethyl-D-tyrosine Hydrochloride (2*R*)-2-Amino-3-[4-(2-fluoroethoxy)phenyl]propanoic acid hydrochloride; $C_{11}H_{15}FNO_3Cl$ = 263.7

Content: minimum 95 per cent.

Colourless or almost colourless crystals.

Fluoroethyl-L-tyrosine Hydrochloride (2*S*)-2-Amino-3-[4-(2-fluoroethoxy)phenyl]propanoic acid hydrochloride; $C_{11}H_{15}FNO_3Cl$ = 263.7

Content: minimum 95 per cent.

Colourless or almost colourless crystals.

6-Fluorolevodopa Hydrochloride (2*S*)-2-Amino-3-(2-fluoro-4,5-dihydroxyphenyl)propanoic acid hydrochloride; 2-Fluoro-5-hydroxy-L-tyrosine hydrochloride; $C_9H_{11}ClFNO_4$ = 251.6 (*144334-59-8*)

Colourless or almost colourless solid, soluble in water.

Fluoromisonidazole (2*RS*)-1-Fluoro-3-(2-nitro-1*H*-imidazol-1-yl)propan-2-ol; FMISO; $C_6H_8FN_3O_3$ = 189.1 (*13551-89-8*)

Content: minimum 95 per cent.

Yellow crystals.

1-Fluoro-2-nitro-4-trifluoromethylbenzene 4-Fluoro-3-nitrobenzotrifluoride; 1-Fluoro-2-nitro-4-(trifluoromethyl) benzene; $C_7H_3F_4NO_2$ = 209.1 (*367-86-2*)

mp: about 197 °C.

Folic Acid (*75708-92-8*)

See *Folic acid hydrate (0067)*.

Formaldehyde (*50-00-0*)

See *Formaldehyde solution R*.

Formaldehyde Solution R1

Complies with the requirements prescribed in the monograph *Formaldehyde solution (35 per cent) (0826)* with the following modification.

Content: 36.5 per cent *m/m* to 38.0 per cent *m/m* of formaldehyde (CH_2O; M_r 30.03).

Formaldehyde Solution See *Formaldehyde solution (35 per cent) (0826)*.

Formamide CH_3NO = 45.0 (*75-12-7*)

Clear, colourless, oily liquid, hygroscopic, miscible with water and with ethanol (96 per cent). It is hydrolysed by water.

d_{20}^{20}: about 1.134.

bp: about 210 °C.

Content: minimum 99.5 per cent.

Storage: in an airtight container.

Formamide R1

Complies with the requirements prescribed for *formamide R* with the following additional requirement.

Water (*2.5.12*): maximum 0.1 per cent determined with an equal volume of *anhydrous methanol R*.

Formamide, Treated

Disperse 1.0 g of *sulfamic acid R* in 20.0 mL of *formamide R* containing 5 per cent *V/V* of *water R*.

Formic Acid Formic acid, anhydrous; CH_2O_2 = 46.03 (*64-18-6*)

Content: minimum 98.0 per cent *m/m*.

Colourless liquid, corrosive, miscible with water and with ethanol (96 per cent).

d_{20}^{20}: about 1.22.

Assay. Weigh accurately a conical flask containing 10 mL of *water R*, quickly add about 1 mL of the acid and weigh again. Add 50 mL of *water R* and titrate with *1 M sodium hydroxide*, using 0.5 mL of *phenolphthalein solution R* as indicator.

1 mL of *1 M sodium hydroxide* is equivalent to 46.03 mg of CH_2O_2.

Formononetin 7-Hydroxy-3-(4-methoxyphenyl)-4-benzopyrone; 7-Hydroxy-3-(4-methoxyphenyl)chromone; 7-Hydroxy-4′-methoxyisoflavone; $C_{16}H_{12}O_4$ = 268.26 (*485-72-3*)

General reagent grade of commerce.

Forsythoside A (2*R*,3*S*,4*R*,5*R*,6*R*)-6-[2-(3,4-Dihydroxyphenyl)ethoxy]-4,5-dihydroxy-2-[[[(2*R*,3*R*,4*R*,5*R*,6*S*)-3,4,5-trihydroxy-6-methyloxan-2-yl]oxy]methyl]oxan-3-yl (2*E*)-3-(3,4-dihydroxyphenyl)prop-2-enoate; 2-(3,4-Dihydroxyphenyl)ethyl 6-*O*-(6-deoxy-α-L-mannopyranosyl)-4-*O*-[(2*E*)-3-(3,4-dihydroxyphenyl)prop-2-enoyl]-β-D-glucopyranoside; $C_{29}H_{36}O_{15}$ = 625 (*79916-77-1*)

D-Fructose Laevulose; Fructose; (*57-48-7*)

See *Fructose (0188)*.

Fuchsin, Basic (*632-99-5*)

A mixture of rosaniline hydrochloride ($C_{20}H_{20}ClN_3$; M_r 337.9; Colour Index No. 42510; Schultz No. 780) and *para*-rosaniline hydrochloride ($C_{19}H_{18}ClN_3$; M_r 323.8; Colour Index No. 42500; Schultz No. 779).

If necessary, purify in the following manner. Dissolve 1 g in 250 mL of *dilute hydrochloric acid R*. Allow to stand for 2 h at room temperature, filter and neutralise with *dilute sodium hydroxide solution R* and add 1 mL to 2 mL in excess. Filter the precipitate through a sintered-glass filter (40) (*2.1.2*) and wash with *water R*. Dissolve the precipitate in 70 mL of *methanol R*, previously heated to boiling, and add 300 mL of *water R* at 80 °C. Allow to cool to room temperature, filter and dry the crystals *in vacuo*.

Crystals with a greenish-bronze sheen, soluble in water and in ethanol (96 per cent).

Storage: protected from light.

Fuchsin Solution, Basic

Dissolve 0.1 g of *basic fuchsin* in 3 mL of *methanol*, dilute to 100 mL with *water*, mix and filter.

Fuchsin Solution, Decolorised

Dissolve 0.1 g of *basic fuchsin R* in 60 mL of *water R*. Add a solution containing 1 g of *anhydrous sodium sulfite R* or 2 g of *sodium sulfite heptahydrate R* in 10 mL of *water R*. Slowly and with continuous shaking add 2 mL of *hydrochloric acid R*. Dilute to 100 mL with *water R*. Allow to stand protected from light for at least 12 h, decolorise with *activated charcoal R* and filter. If the solution becomes cloudy, filter before use. If on standing the solution becomes violet, decolorise again by adding *activated charcoal R*.

Test for sensitivity. To 1.0 mL add 1.0 mL of *water R* and 0.1 mL of *aldehyde-free alcohol R*. Add 0.2 mL of a solution containing 0.1 g/L of formaldehyde (CH_2O, M_r 30.03). A pale-pink colour develops within 5 min.

Storage: protected from light.

Fuchsin Solution R1, Decolorised

To 1 g of *basic fuchsin R* add 100 mL of *water R*. Heat to 50 °C and allow to cool with occasional shaking. Allow to stand for 48 h, shake and filter. To 4 mL of the filtrate add 6 mL of *hydrochloric acid R*, mix and dilute to 100 mL with *water R*. Allow to stand for at least 1 h before use.

L-Fucose Fucose; $C_6H_{12}O_5$ = 164.2 (*6696-41-9*)

White or almost white powder, soluble in water and in ethanol (96 per cent).

$[\alpha]_D^{20}$: about −76, determined on a 90 g/L solution 24 h after dissolution.

mp: about 140 °C.

Fumaric Acid (*E*)-Butenedioic acid; $C_4H_4O_4$ = 116.1 (*110-17-8*)

White or almost white crystals, slightly soluble in water, soluble in ethanol (96 per cent), slightly soluble in acetone.

mp: about 300 °C.

Furfuraldehyde Furan-2-aldehyde; Furfural; $C_5H_4O_2$ = 96.1 (*98-01-1*)

Clear, colourless to brownish-yellow, oily liquid, miscible in 11 parts of water, miscible with ethanol (96 per cent).

d_{20}^{20}: 1.155 to 1.161.

Distillation range (*2.2.11*). Not less than 95 per cent distils between 159 °C and 163 °C.

Storage: in a dark place.

Gadolinium Chloride Hexahydrate Gadolinium trichloride hexahydrate; $GdCl_3,6H_2O$ = 371.7 (*13450-84-5*)

Content: minimum 99.9 per cent.

Gadolinium Sulfate Octahydrate $Gd_2(SO_4)_3,8H_2O$ = 747 (*13450-87-8*)

Colourless, crystalline powder.

D-Galactose Galactose; $C_6H_{12}O_6$ = 180.2 (*59-23-4*)

White or almost white, crystalline powder, freely soluble in water.

$[\alpha]_D^{20}$: + 79 to + 81, determined on a 100 g/L solution in *water R* containing about 0.05 per cent of NH_3.

1,6-Galactosylgalactose 6-*O*-β-D-Galactopyranosyl-D-galactopyranose; $C_{12}H_{22}O_{11}$ = 342.3 (*5077-31-6*)

White or almost white powder.

Galacturonic Acid D-(+)-galacturonic acid; (2*S*,3*R*,4*S*,5*R*)-2,3,4,5-Tetrahydroxy-6-oxo-hexanoic acid; $C_6H_{10}O_7$ = 194.1 (*685-73-4*)

$[\alpha]_D^{20}$: about + 53°, determined on a 100 g/L solution.

Gallic Acid 3,4,5-Trihydroxybenzoic acid monohydrate; $C_7H_6O_5,H_2O$ = 188.1 (*5995-86-8*)

Crystalline powder or long needles, colourless or slightly yellow, soluble in water, freely soluble in hot water, in ethanol (96 per cent) and in glycerol.

It loses its water of crystallisation at 120 °C.

mp: about 260 °C, with decomposition.

Chromatography. Thin-layer chromatography (*2.2.27*) as prescribed in the monograph *Bearberry leaf (1054)*; the chromatogram shows only one principal spot.

Gallium (^{68}Ga) Chloride Solution $^{68}GaCl_3$ = 174.3

Solution containing gallium-68 in the form of gallium chloride in *dilute hydrochloric acid R*.

Content: 90 per cent to 110 per cent of the declared gallium-68 radioactivity at the date and time stated on the label.

Gallium PSMA-11 $C_{44}H_{59}GaN_6O_{17}$ = 1014

Complex of gallium with (3*S*,7*S*)-22-[3-[[[2-[[[5-(2-carboxyethyl)-2-hydroxyphenyl]methyl](carboxymethyl)amino]ethyl](carboxymethyl)amino]methyl]-4-hydroxyphenyl]-5,13,20-trioxo-4,6,12,19-tetraazadocosane-1,3,7-tricarboxylic acid (PSMA-11).

Colourless or almost white powder.

Content: minimum 95.0 per cent (anhydrous and trifluoroacetic acid-free substance).

Ganoderic Acid A (25*R*)-7β,15α-Dihydroxy-3,11,23-trioxolanost-8-en-26-oic acid; $C_{30}H_{44}O_7$ = 516.7 (*81907-62-2*)

Gastric Juice, Artificial

Dissolve 2.0 g of *sodium chloride R* and 3.2 g of *pepsin powder R* in *water R*. Add 80 mL of *1 M hydrochloric acid* and dilute to 1000 mL with *water R*.

Gastrodin 4-(Hydroxymethyl)phenyl α-D-glucopyranoside; (2*R*,3*S*,4*S*,5*R*,6*S*)-2-(Hydroxymethyl)-6-[4-(hydroxymethyl)phenoxy]oxane-3,4,5-triol; $C_{13}H_{18}O_7$ = 286.3 (*62499-27-8*)

GC Concentrical Column

A commercially available system consisting of 2 concentrically arranged tubes. The outer tube is packed with molecular sieves and the inner tube is packed with a porous polymer mixture. The main application is the separation of gases.

Gelatin (*9000-70-8*)

See *Gelatin (0330)*.

Gelatin, Hydrolysed

Dissolve 50 g of *gelatin R* in 1000 mL of *water R*. Autoclave in saturated steam at 121 °C for 90 min and freeze dry.

Geniposide Methyl (1*S*,4a*S*,7a*S*)-1-(β-D-glucopyranosyloxy)-7-(hydroxymethyl)-1,4a,5,7a-tetrahydrocyclopenta[*c*]pyran-4-carboxylate; $C_{17}H_{24}O_{10}$ = 388.4 (*24512-63-8*)

Geraniol (*E*)-3,7-Dimethylocta-2,6-dien-1-ol; $C_{10}H_{18}O$ = 154.2 (*106-24-1*)

Oily liquid, slight odour of rose, practically insoluble in water, miscible with ethanol (96 per cent).

Geraniol used in gas chromatography complies with the following additional test.

Assay. Gas chromatography (*2.2.28*) as prescribed in the monograph *Citronella oil (1609)*.

Content: minimum 98.5 per cent, calculated by the normalisation procedure.

Storage: in an airtight container, protected from light

Geranyl Acetate (*E*)-3,7-Dimethylocta-2,6-dien-1-yl acetate; $C_{12}H_{20}O_2$ = 196.3 (*105-87-3*)

Colourless or slightly yellow liquid, slight odour of rose and lavender.

Geranyl acetate used in gas chromatography complies with the following additional test.

Assay. Gas chromatography (*2.2.28*) as prescribed in the monograph *Bitter-orange-flower oil (1175)*.

Test solution. The substance to be examined.

Content: minimum 98.0 per cent, calculated by the normalisation procedure.

Ginsenoside Rb1 (20*S*)-3β-Di-D-glucopyranosyl-20-di-D-glucopyranosylprotopanaxadiol; (20*S*)-3β-[(2-*O*-β-D-Glucopyranosyl-β-D-glucopyranosyl)oxy]-20-[(6-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]-5α-dammar-24-en-12β-ol; (20*S*)-3β-[(2-*O*-β-D-Glucopyranosyl-β-D-glucopyranosyl)oxy]-20-[(6-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy]-4,4,8,14-tetramethyl-18-nor-5α-cholest-24-en-12β-ol; $C_{54}H_{92}O_{23},3H_2O$ = 1163 (*41753-43-9*)

A colourless solid, soluble in water, in anhydrous ethanol and in methanol.

$[\alpha]_D^{20}$: + 11.3 determined on a 10 g/L solution in *methanol R*.

mp: about 199 °C.

Water (2.5.12): maximum 6.8 per cent.

Assay. Liquid chromatography (*2.2.29*) as prescribed in the monograph *Ginseng (1523)*.

Test solution. Dissolve 3.0 mg, accurately weighed, of *ginsenoside Rb1* in 10 mL of *methanol R*.

Content: minimum 95.0 per cent, calculated by the normalisation procedure.

Ginsenoside Re (3β,6α,12β)-20-(β-D-Glucopyranosyloxy)-3,12-dihydroxydammar-24-en-6-yl 2-*O*-(6-deoxy-α-L-mannopyranosyl)-β-D-glucopyranoside; $C_{48}H_{82}O_{18}$ = 947.2 (*52286-59-6*)

Colourless solid, soluble in water, in ethanol (96 per cent) and in methanol.

Ginsenoside Rf (20*S*)-6-*O*-[β-D-Glucopyranosyl-(1→2)-β-D-glycopyranoside]-dammar-24-ene-3β,6α,12β,20-tetrol; $C_{42}H_{72}O_{14},2H_2O$ = 837 (*52286-58-5*)

A colourless solid, soluble in water, in anhydrous ethanol and in methanol.

$[\alpha]_D^{20}$: + 12.8 determined on a 10 g/L solution in *methanol R*.

mp: about 198 °C.

Ginsenoside Rg1 (20*S*)-6β-D-Glucopyranosyl-D-glucopyranosylprotopanaxatriol; (20*S*)-6α,20-Bis(β-D-glucopyranosyloxy)-5α-dammar-24-ene-3β,12β-diol; (20*S*)-6α,20-Bis(β-D-glucopyranosyloxy)-4,4,8,14-tetramethyl-18-nor-5α-cholest-24-ene-3β,12β-diol; $C_{42}H_{72}O_{14},2H_2O$ = 837 (*22427-39-0*)

A colourless solid, soluble in water, in anhydrous ethanol and in methanol.

$[\alpha]_D^{20}$: + 31.2 determined on a 10 g/L solution in *methanol R*.

mp: 188 °C to 191 °C.

Water (2.5.12): maximum 4.8 per cent.

Assay. Liquid chromatography (*2.2.29*) as prescribed in the monograph *Ginseng (1523)*.

Test solution. Dissolve 3.0 mg, accurately weighed, of *ginsenoside Rg1* in 10 mL of *methanol R*.

Content: minimum 95.0 per cent, calculated by the normalisation procedure.

Ginsenoside Rg2 3β,12β,20-Trihydroxydammar-24-en-6α-yl 2-*O*-(6-deoxy-α-L-mannopyranosyl)-β-D-glucopyranoside; $C_{42}H_{72}O_{13}$ = 785 (*52286-74-5*)

Ginsenoside Ro (3β)-28-(β-D-Glucopyranosyloxy)-28-oxoolean-12-en-3-yl 2-*O*-β-D-glucopyranosyl-β-D-glucopyranosiduronic acid; $C_{48}H_{76}O_{19}$ = 957 (*34367-04-9*)

Gitoxin Glycoside of *Digitalis purpurea* L; 3β-(*O*-2,6-Dideoxy-β-d-*ribo*-hexopyranosyl-(1→4)-*O*-2,6-dideoxy-β-d-*ribo*-hexopyranosyl-(1→4)-2,6-dideoxy-β-d-*ribo*-hexopyranosyloxy)-14,16β-dihydroxy-5β,14β-card-20(22)-enolide; $C_{41}H_{64}O_{14}$ = 781 (*4562-36-1*)

A white or almost white, crystalline powder, practically insoluble in water and in most common organic solvents, soluble in pyridine.

$[\alpha]_D^{20}$: + 20 to + 24, determined on a 5 g/L solution in a mixture of equal volumes of *chloroform R* and *methanol R*.

Chromatography. Thin-layer chromatography (*2.2.27*) as prescribed in the monograph *Digitalis leaf (0117)*; the chromatogram shows only one principal spot.

Glucosamine Hydrochloride D-Glucosamine hydrochloride; $C_6H_{14}ClNO_5$ = 215.6 (*66-84-2*)

Crystals, soluble in water.

$[\alpha]_D^{20}$: + 100, decreasing to + 47.5 after 30 min, determined on a 100 g/L solution.

Glucose (*50-99-7*)

See *Glucose (0177)*.

D-Glucose Dextrose; (*50-99-7*)

See *glucose R*.

D-Glucose Monohydrate $C_6H_{12}O_6,H_2O$ = 198.2 (*5996-10-1*)

$[\alpha]_D^{20}$: about +52.5 (anhydrous) (10% w/v in water containing about 0.2% of NH_3).

General reagent grade of commerce.

Colourless crystals or a white to cream, crystalline powder.

D-Glucuronic Acid $C_6H_{10}O_7$ = 194.1 (*6556-12-3*)

Content: minimum 96.0 per cent, calculated with reference to the substance dried *in vacuo* (*2.2.32*).

Soluble in water and in ethanol (96 per cent).

Shows mutarotation: $[\alpha]_D^{24}$: + 11.7 → + 36.3.

Assay. Dissolve 0.150 g in 50 mL of *anhydrous methanol R* while stirring under nitrogen. Titrate with *0.1 M tetrabutylammonium hydroxide*, protecting the solution from atmospheric carbon dioxide throughout solubilisation and titration. Determine the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M tetrabutylammonium hydroxide* is equivalent to 19.41 mg of $C_6H_{10}O_7$.

Glutamic Acid (*56-86-0*)

See *Glutamic acid (0750)*.

L-Glutamine (*S*)-2,5-Diamino-5-oxopentanoic acid; $C_5H_{10}N_2O_3$ = 146.2 (*56-85-9*)

White crystalline powder.

mp: about 185 °C, with decomposition.

Glutamyl Endopeptidase for Peptide Mapping Endoproteinase Glu-C of high purity from *Staphylococcus aureus* strain V8 (EC 3.4.21.19); (*137010-42-5*)

L-γ-Glutamyl-L-cysteine $C_8H_{14}N_2O_5S$ = 250.3 (*636-58-8*)

Glutaraldehyde $C_5H_8O_2$ = 100.1 (*111-30-8*)

Oily liquid, soluble in water.

n_D^{25}: about 1.434.

bp: about 188 °C.

Glutaric Acid Pentanedioic acid; $C_5H_8O_4$ = 132.1 (*110-94-1*)

White or almost white, crystalline powder.

L-Glutathione, Oxidised Bis(L-γ-glutamyl-L-cysteinylglycine) disulfide; $C_{20}H_{32}N_6O_{12}S_2$ = 612.6 (*27025-41-8*)

Glycerol Propane-1,2,3-triol; (*56-81-5*)

See *Glycerol (0496)*.

Glycerol R1

Complies with the requirements prescribed for the monograph *Glycerol (0496)* and free from diethylene glycol when examined as prescribed in the test for impurity A and related substances in that monograph.

Glycerol (85%) See *Glycerol (85 per cent) (0497)*.

Glycerol (85 per cent) R1

Complies with the requirements prescribed for the monograph *Glycerol 85 per cent (0497)* and free from diethylene glycol when examined as prescribed in the test for impurity A and related substances in that monograph.

Glycerol 1-decanoate (2*RS*)-2,3-Dihydroxypropyl decanoate; α-Monocaprin; 1-Monodecanoyl-*rac*-glycerol; $C_{13}H_{26}O_4$ = 246.3 (*2277-23-8*)

Content: about 99 per cent.

Glycerol 1-octanoate (2*RS*)-2,3-Dihydroxypropyl octanoate; α-Monocaprylin; 1-Monooctanoyl-*rac*-glycerol; $C_{11}H_{22}O_4$ = 218.3 (*502-54-5*)

Content: about 99 per cent.

Glycidol $C_3H_6O_2$ = 74.1 (*556-52-5*)

Slightly viscous liquid, miscible with water.

d_4^{20}: about 1.115.

n_D^{20}: about 1.432.

Glycine Aminoacetic acid; (*56-40-6*)

See *Glycine (0614)*.

Glycine Anhydride Piperazine-2,5-dione (2,5-DKP); $C_4H_6N_2O_2$ = 114.1 (*106-57-0*)

Glycolic Acid 2-Hydroxyacetic acid; $C_2H_4O_3$ = 76.0 (*79-14-1*)

Crystals, soluble in water, in acetone, in ethanol (96 per cent) and in methanol.

mp: about 80 °C.

18α-Glycyrrhetinic Acid (20β)-3β-Hydroxy-11-oxo-18α-olean-12-en-29-oic acid; $C_{30}H_{46}O_4$ = 470.7 (*1449-05-4*)

White or almost white powder, practically insoluble in water, soluble in anhydrous ethanol, sparingly soluble in methylene chloride.

β-Glycyrrhetinic Acid Glycyrrhetinic Acid; Glycyrrhetic acid; $C_{30}H_{46}O_4$ = 470.7 (*471-53-4*)

A mixture of α- and β-glycyrrhetic acids in which the β-isomer is predominant.

White or yellowish-brown powder, practically insoluble in water, soluble in anhydrous ethanol and in glacial acetic acid.

$[\alpha]_D^{20}$: + 145 to + 155, determined on a 10.0 g/L solution in *anhydrous ethanol R*.

Chromatography. Thin-layer chromatography (*2.2.27*) using *silica gel* GF_{254} *R* as the coating substance; prepare the slurry using a 0.25 per cent *V/V* solution of *phosphoric acid R*. Apply to the plate 5 µL of a 5 g/L solution of the glycyrrhetic acid in a mixture of equal volumes of *chloroform R* and *methanol R*. Develop over a path of 10 cm using a mixture of 5 volumes of *methanol R* and 95 volumes of *chloroform R*. Examine the chromatogram in ultraviolet light at 254 nm. The chromatogram shows a dark spot (R_F about 0.3) corresponding to β-glycyrrhetic acid and a smaller spot (R_F about 0.5) corresponding to α-glycyrrhetic acid. Spray with *anisaldehyde solution R* and heat at 100-105 °C for 10 min. Both spots are coloured bluish-violet. Between them a smaller bluish-violet spot may be present.

Glycyrrhizic Acid $C_{42}H_{62}O_{16}$ = 822.93 (*1405-86-3*)

Analytical reagent grade of commerce.

Glyoxal Bis(2-hydroxyanil) Bis(2-hydroxyphenylimino) ethane; Glyoxalhydroxyanil; $C_{14}H_{12}N_2O_2$ = 240.3 (*1149-16-2*)

White or almost white crystals, soluble in hot ethanol (96 per cent).

mp: about 200 °C.

Glyoxal Sodium Bisulfite Glyoxal sodium bisulphite; $C_2H_4Na_2O_8S_2,H_2O$ = 284.2

General reagent grade of commerce.

A white or cream powder.

Glyoxal Solution (*107-22-2*)

Contains about 40 per cent (*m/m*) glyoxal.

Assay. In a ground-glass stoppered flask place 1.000 g of glyoxal solution, 20 mL of a 70 g/L solution of *hydroxylamine hydrochloride R* and 50 mL of *water R*. Allow to stand for 30 min and add 1 mL of *methyl red mixed solution R* and titrate with *1 M sodium hydroxide* until the colour changes from red to green. Carry out a blank titration.

1 mL of *1 M sodium hydroxide* is equivalent to 29.02 mg of glyoxal ($C_2H_2O_2$).

Gonadotrophin, Chorionic (*9002-61-3*)

See *Chorionic gonadotrophin (0498)*.

Gonadotrophin, Serum See *Equine serum gonadotrophin for veterinary use (0719)*.

Gramine 1-(1*H*-Indol-3-yl)-*N*,*N*-dimethylmethanamine; $C_{11}H_{14}N_2$ = 174.2 (*87-52-5*)

Flakes, practically insoluble in water, soluble in ethanol (96 per cent), slightly soluble in acetone.

mp: 132 °C to 134 °C.

Guaiacol 2-Methoxyphenol; 1-Hydroxy-2-methoxybenzene; $C_7H_8O_2$ = 124.1 (*90-05-1*)

Crystalline mass or colourless or yellowish liquid, hygroscopic, slightly soluble in water, very soluble in methylene chloride, freely soluble in ethanol (96 per cent).

bp: about 205 °C.

mp: about 28 °C.

Guaiacum Resin Gum guaiac

Resin obtained from the heartwood of *Guaiacum officinale* L. and *Guaiacum sanctum* L.

Reddish-brown or greenish-brown, hard, glassy fragments; fracture shiny.

Guaiazulene 1,4-Dimethyl-7-isopropylazulene; $C_{15}H_{18}$ = 198.3 (*489-84-9*)

Dark-blue crystals or blue liquid, very slightly soluble in water, miscible with fatty and essential oils and with liquid paraffin, sparingly soluble in ethanol (96 per cent), soluble in 500 g/L sulfuric acid and 80 per cent *m/m* phosphoric acid, giving a colourless solution.

mp: about 30 °C.

Storage: protected from light and air.

Guaiphenesin Guaifenesin; guaiacol glyceryl ether; $C_{10}H_{14}O_4$ = 198.2 (*93-14-1*)

mp: about 79°.

General reagent grade of commerce.

Guanidine Hydrochloride CH_5N_3HCl = 95.5 (*50-01-1*)

Crystalline powder, freely soluble in water and in ethanol (96 per cent).

Guanine 2-Aminopurin-6-one; $C_5H_5N_5O$ = 151.1 (*73-40-5*)

Amorphous white or almost white powder, practically insoluble in water, slightly soluble in ethanol (96 per cent). It dissolves in ammonia and in dilute solutions of alkali hydroxides.

Haemoglobin (*9008-02-0*)

Nitrogen: 15 per cent to 16 per cent.

Iron: 0.2 per cent to 0.3 per cent.

Loss on drying (*2.2.32*): maximum 2 per cent.

Sulfated ash (*2.4.14*): maximum 1.5 per cent.

Haemoglobin Solution

Transfer 2 g of *haemoglobin R* to a 250 mL beaker and add 75 mL of *dilute hydrochloric acid R2*. Stir until solution is complete. Adjust the pH to 1.6 ± 0.1 using *1 M hydrochloric acid*. Transfer to a 100 mL flask with the aid of *dilute hydrochloric acid R2*. Add 25 mg of *thiomersal R*. Prepare daily, store at 5 ± 3 °C and readjust to pH 1.6 before use.

Storage: at 2 °C to 8 °C.

Hamamelitannin (2*R*,3*R*,4*R*)-2-Formyl-2,3,4-trihydroxypentane-1,5-diyl bis(3,4,5-trihydroxybenzoate); 2-*C*-[(Galloyloxy)methyl]-D-ribose 5-gallate; $C_{20}H_{20}O_{14}$ = 484.4 (*469-32-9*)

Harpagoside $C_{24}H_{30}O_{11}$ = 494.5

White or almost white, crystalline powder, very hygroscopic, soluble in water and in ethanol (96 per cent).

Storage: in an airtight container.

Hederacoside C *O*-6-Deoxy-α-L-mannopyranosyl-(1→4)-*O*-β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl (4*R*)-3β-[[2-*O*(-6-deoxy-α-L-mannopyranosyl)-α-L-arabinopyranosyl] oxy]-23-hydroxyolean-12-en-28-oate; $C_{59}H_{96}O_{26}$ = 1221 (*14216-03-6*)

Colourless crystals or white or almost white powder.

mp: about 220 °C.

Hederacoside C used in liquid chromatography complies with the following additional test.

Assay. Liquid chromatography (*2.2.29*) as prescribed in the monograph *Ivy leaf (2148)*.

Test solution. Dissolve 5.0 mg of hederacoside C in 5.0 mL of *methanol R*.

Content: minimum 95 per cent, calculated by the normalisation procedure.

Hederagenin Astrantiagenin E; Caulosapogenin; 3β,23-Dihydroxy-4α-olean-12-en-28-oic acid; $C_{30}H_{48}O_4$ = 472.7 (*465-99-6*)

α-Hederin (+)-(4*R*)-3β-[[2-*O*-(6-Deoxy-α-L-mannopyranosyl)-α-L-arabinopyranosyl]oxy]-23-hydroxyolean-12-en-28-oic acid; $C_{41}H_{66}O_{12}$ = 751.0 (*27013-91-8*)

White or almost white powder.

mp: about 256 °C.

Helium Helium for chromatography; He = 4.003 (*7440-59-7*)

Content: minimum 99.995 per cent *V/V* of He.

Heparin (*9041-08-1*)

See *Heparin sodium (0333)*.

Heparinase I Heparin lyase (EC 4.2.2.7); (*9025-39-2*)

Enzyme from *Flavobacterium heparinum* that performs eliminative cleavage of polysaccharides containing (1→4)-linked D-glucuronate or L-iduronate residues and (1→4)-α-linked 2-sulfoamino-2-deoxy-6-sulfo-D-glucose residues to give oligosaccharides with terminal 4-deoxy-α-D-gluc-4-enuronosyl groups at their non-reducing ends.

Heparinase II (*149371-12-0*)

Enzyme from *Flavobacterium heparinum* that depolymerises sulfated polysaccharide chains containing 1→4 linkages between hexosamines and uronic acid residues (both iduronic and glucuronic acid residues). The reaction yields oligosaccharide products (mainly disaccharides) containing unsaturated uronic acids.

Heparinase III Heparin-sulfate lyase (EC 4.2.2.8); (*37290-86-1*)

Enzyme from *Flavobacterium heparinum* that depolymerises selectively sulfated polysaccharide chains containing 1→4 linkages between hexosamines and glucuronic acid residues to give oligosaccharide products (mainly disaccharides) containing unsaturated uronic acids.

Heptachlor $C_{10}H_5Cl_7$ = 373.3 (*76-44-8*)

bp: about 135 °C.

mp: about 95 °C.

A suitable certified reference solution (10 ng/µL in cyclohexane) may be used.

Heptachlor Epoxide $C_{10}H_5Cl_7O$ = 389.3 (*1024-57-3*)

bp: about 200 °C.

mp: about 160 °C.

A suitable certified reference solution (10 ng/µL in cyclohexane) may be used.

Heptafluorobutyric Acid HFBA; $C_4HF_7O_2$ = 214.0 (*375-22-4*)

Clear, colourless liquid. Corrosive.

d_{20}^{20}: about 1.645.

n_D^{20}: about 1.300.

bp: about 120 °C.

Content: minimum 99.5 per cent.

Heptafluorobutyric Anhydride $C_8F_{14}O_3$ = 410.1 (*336-59-4*)

n_D^{20}: about 1.287.

bp: about 108°.

Use a grade of commerce suitable for derivatisation.

Heptafluoro-*N*-methyl-*N*-(trimethylsilyl)butanamide 2,2,3,3,4,4,4-Heptafluoro-*N*-methyl-*N*-(trimethylsilyl)butyramide; $C_8H_{12}F_7NOSi$ = 299.3 (*53296-64-3*)

Clear, colourless liquid, flammable.

n_D^{20}: about 1.351.

bp: about 148 °C.

***N*-Heptane** Heptane; C_7H_{16} = 100.2 (*142-82-5*)

Colourless, flammable liquid, practically insoluble in water, miscible with anhydrous ethanol.

d_{20}^{20}: 0.683 to 0.686.

n_D^{20}: 1.387 to 1.388.

Distillation range (*2.2.11*). Not less than 95 per cent distils between 97 °C and 98 °C.

***n*-Heptylamine** $C_7H_{17}N$ = 115.22 (*111-68-2*)

General reagent grade of commerce.

Boiling point, about 156 °C.

2-Heptylamine 2-Aminoheptane; $C_7H_{17}N$ = 115.2

bp: about 143°.

General reagent grade of commerce.

Hesperidin (*S*)-7-[[6-*O*-(6-Deoxy-α-L-mannopyranosyl)-β-D-glucopyranosyl]oxy]-5-hydroxy-2-(3-hydroxy-4-methoxyphenyl)-2,3-dihydro-4*H*-1-benzopyran-4-one; $C_{28}H_{34}O_{15}$ = 611 (*520-26-3*)

Hygroscopic powder, slightly soluble in water and in methanol.

mp: 258 °C to 262 °C.

Hexachlorobenzene C_6Cl_6 = 284.8 (*118-74-1*)

bp: about 332 °C.

mp: about 230 °C.

A suitable certified reference solution (10 ng/µL in cyclohexane) may be used.

α-Hexachlorocyclohexane $C_6H_6Cl_6$ = 290.8 (*319-84-6*)

bp: about 288 °C.

mp: about 158 °C.

A suitable certified reference solution (10 ng/µL in cyclohexane) may be used.

β-Hexachlorocyclohexane $C_6H_6Cl_6$ = 290.8 (*319-85-7*)

A suitable certified reference solution (10 ng/µL in cyclohexane) may be used.

δ-Hexachlorocyclohexane $C_6H_6Cl_6$ = 290.8 (*319-86-8*)

A suitable certified reference solution (10 ng/µL in cyclohexane) may be used.

Hexacosane $C_{26}H_{54}$ = 366.7 (*630-01-3*)

Colourless or white or almost white flakes.

mp: about 57 °C.

Hexadimethrine Bromide 1,5-Dimethyl-1,5-diazaundecamethylene polymethobromide; Poly(1,1,5,5-tetramethyl-1,5-azonia-undecamethylene dibromide); $(C_{13}H_{30}Br_2N_2)_n$ (*28728-55-4*)

White or almost white, amorphous powder, hygroscopic, soluble in water.

Storage: in an airtight container.

2,2′,2″,6,6′,6″-Hexa-(1,1-dimethylethyl)-4,4′,4″-[2,4,6-trimethyl-1,3,5-benzenetriyltrismethylene]triphenol 2,2′,2″,6,6′,6″-Hexa-*tert*-butyl-4,4′,4″-[(2,4,6-trimethyl-1,3,5-benzenetriyl)trismethylene]triphenol; $C_{54}H_{78}O_3$ = 775

Crystalline powder, practically insoluble in water, soluble in acetone, slightly soluble in ethanol (96 per cent).

mp: about 244 °C.

1,1,1,3,3,3-Hexafluoropropan-2-ol $C_3H_2F_6O$ = 168.0 (*920-66-1*)

Content: minimum 99.0 per cent, determined by gas chromatography.

Clear, colourless liquid, miscible with water and with anhydrous ethanol.

d_{20}^{20}: about 1.596.

bp: about 59 °C.

Hexamethyldisilazane $C_6H_{19}NSi_2$ = 161.4 (*999-97-3*)

Clear, colourless liquid.

d_{20}^{20}: about 0.78.

n_D^{20}: about 1.408.

bp: about 125 °C.

Storage: in an airtight container.

Hexamine 1,3,5,7-tetra-azatricyclo [3.3.1.13,7]decane; Hexamethylenetetramine; $C_6H_{12}N_4$ = 140.2 (*100-97-0*)

Colourless, crystalline powder, very soluble in water.

***n*-Hexane** Hexane; C_6H_{14} = 86.2 (*110-54-3*)

Colourless, flammable liquid, practically insoluble in water, miscible with anhydrous ethanol.

d_{20}^{20}: 0.659 to 0.663.

n_D^{20}: 1.375 to 1.376.

Distillation range (*2.2.11*). Not less than 95 per cent distils between 67 °C and 69 °C.

Hexane used in spectrophotometry complies with the following additional test.

Absorbance (*2.2.25*): maximum 0.01 from 260 nm to 420 nm, determined using *water R* as compensation liquid.

Hexane, Purified

A grade of *hexane* containing not more than 0.002% w/v of non-volatile matter.

Hexylamine Hexan-1-amine; $C_6H_{15}N$ = 101.2 (*111-26-2*)

Colourless liquid, slightly soluble in water, soluble in ethanol (96 per cent).

d_{20}^{20}: about 0.766.

n_D^{20}: about 1.418.

bp: 127 °C to 131 °C.

Hibifolin 2-(3,4-Dihydroxyphenyl)-3,5,7-trihydroxy-4-oxo-4*H*-1-benzopyran-8-yl β-D-glucopyranosiduronic acid; Gossypetin 8-*O*-glucuronide; Gossypetin 8-*O*-β-D-glucuropyranoside; $C_{21}H_{18}O_{14}$ = 494.4 (*55366-56-8*)

Storage: protected from light, at a temperature not exceeding 8 °C, in a dry place.

Histamine Dihydrochloride (*56-92-8*)

See *Histamine dihydrochloride (0143)*.

Histamine Phosphate Histamine acid phosphate; $C_5H_9N_3,2H_3PO_4$ = 307.1 (*23297-93-0*)

Of the British Pharmacopoeia.

Histamine Solution

A 9 g/L solution of *sodium chloride R* containing 0.1 µg per millilitre of histamine base (as the phosphate or dihydrochloride).

Histidine (2*S*)-2-Amino-3-(1*H*-imidazol-4-yl)propanoic acid; (*71-00-1*)

Histidine Monohydrochloride (*RS*)-2-Amino-3-(imidazol-4-yl)propionic acid hydrochloride monohydrate; $C_6H_{10}ClN_3O_2,H_2O$ = 209.6 (*123333-71-1*)

Crystalline powder or colourless crystals, soluble in water.

mp: about 250 °C, with decomposition.

Chromatography. Thin-layer chromatography (*2.2.27*) as prescribed in the monograph *Histamine dihydrochloride (0143)*; the chromatogram shows only one principal spot.

Holmium Oxide Diholmium trioxide; Ho_2O_3 = 377.9 (*12055-62-8*)

Yellowish powder, practically insoluble in water.

Holmium Perchlorate Solution

A 40 g/L solution of *holmium oxide R* in a solution of *perchloric acid R* containing 141 g/L of $HClO_4$.

DL-Homocysteine (2*RS*)-2-Amino-4-sulfanylbutanoic acid; $C_4H_9NO_2S$ = 135.2 (*454-29-5*)

White or almost white, crystalline powder.

mp: about 232 °C.

L-Homocysteine Thiolactone Hydrochloride (3*S*)-3-Aminodihydrothiophen-2(3*H*)-one hydrochloride; C_4H_8ClNOS = 153.6 (*31828-68-9*)

White or almost white, crystalline powder.

mp: about 202 °C.

Homoorientin 2-(3,4-Dihydroxyphenyl)-6-β-D-glucopyranosyl-5,7-dihydroxy-4*H*-1-benzopyran-4-one; Isoorientin; Luteolin-6-*C*-glucoside; $C_{21}H_{20}O_{11}$ = 448.4 (*4261-42-1*)

Honokiol 3′,5-Di(prop-2-enyl)biphenyl-2,4′-diol; 3′,5-Diallyl-2,4′-dihydroxybiphenyl; 3′,5-Di-2-propenyl-[1,1′-biphenyl]-2,4′-diol; $C_{18}H_{18}O_2$ = 266.3 (*35354-74-6*)

Human Tissue Factor Solution

Solution containing human tissue factor, which may be produced by recombinant DNA technology, combined with

phospholipids and calcium buffers. Suitable stabilisers may be added.

Hyaluronate Solution

Dilute *potassium hyaluronate stock solution* with an equal volume of *phosphate-buffered saline pH 6.4*.

Use on the day of preparation.

Hyaluronidase Diluent

Mix 100 mL of *phosphate buffer solution pH 6.4 R* with 100 mL of *water R*. Dissolve 0.140 g of *hydrolysed gelatin R* in the solution at 37 °C.

Storage: use within 2 h.

Hydrastine Hydrochloride (3*S*)-6,7-Dimethoxy-3-[(5*R*)-6-methyl-5,6,7,8-tetrahydro-1,3-dioxolo[4,5-*g*]isoquinolin-5-yl]isobenzofuran-1(3*H*)-one hydrochloride; $C_{21}H_{22}ClNO_6$ = 419.9 (*5936-28-7*)

White or almost white powder, hygroscopic, very soluble in water and in ethanol (96 per cent).

$[\alpha]_D^{17}$: about + 127.

mp: about 116 °C.

Hydrastine hydrochloride used in liquid chromatography complies with the following additional test.

Assay. Liquid chromatography (*2.2.29*) as prescribed in the monograph *Goldenseal rhizome (1831)*.

Content: minimum 98 per cent, calculated by the normalisation procedure.

Hydrazine Diazane; H_4N_2 = 32.05 (*302-01-2*)

Slightly oily liquid, colourless, with a strong odour of ammonia, miscible with water. Dilute solutions in water are commercially available.

n_D^{20}: about 1.470.

bp: about 113 °C.

mp: about 1.5 °C.

Caution: toxic and corrosive.

Hydrazine Hydrate N_2H_4,H_2O = 50.06 (*10217-52-4*)

Analytical reagent grade of commerce.

A colourless liquid; weight per mL, about 1.03 g.

Hydrazine Sulfate Hydrazine sulphate; $H_6N_2O_4S$ = 130.1 (*10034-93-2*)

Colourless crystals, sparingly soluble in cold water, soluble in hot water (50 °C) and freely soluble in boiling water, practically insoluble in ethanol (96 per cent).

Content: minimum 99 per cent.

Hydrindantin 2,2′-Dihydroxy-2,2′-bi-indan-1,1′,3,3′-tetraone dihydrate; $C_{18}H_{10}O_6,2H_2O$ = 358.3 (*5950-69-6*)

mp: about 258°.

General reagent grade of commerce.

Hydriodic Acid HI = 127.9 (*10034-85-2*)

Prepare by distilling hydriodic acid over red phosphorus, passing *carbon dioxide R* or *nitrogen R* through the apparatus during the distillation. Use the colourless or almost colourless, constant-boiling mixture (55 per cent to 58 per cent of HI) distilling between 126 °C and 127 °C.

Place the acid in small, amber, glass-stoppered bottles previously flushed with *carbon dioxide R* or *nitrogen R*, seal with paraffin.

Storage: in a dark place.

Hydrobromic Acid, 30 per cent (*10035-10-6*)

A 30 per cent solution of hydrobromic acid in *glacial acetic acid R*.

Degas with caution the contents before opening.

Hydrobromic Acid, 47 per cent

A 47 per cent *m/m* solution of hydrobromic acid.

Hydrobromic Acid, Dilute

Place 5.0 mL of *30 per cent hydrobromic acid R* in amber vials equipped with polyethylene stoppers. Seal under *argon R* and store in the dark. Add 5.0 mL of *glacial acetic acid R* immediately before use. Shake.

Storage: in the dark.

Hydrobromic Acid RI, Dilute

Contains 7,9 g/L of HBr.

Dissolve 16.81 g of *47 per cent hydrobromic acid R* in *water R* and dilute to 1000 mL with the same solvent.

Hydrocarbons (Type L), Low-vapour-pressure

Unctuous mass, soluble in benzene and in toluene.

Hydrochloric Acid (*7647-01-0*)

See *Concentrated hydrochloric acid (0002)*.

Solutions of molarity *x*M should be prepared by diluting 85*x* mL of hydrochloric acid to 1000 mL with *water*.

0.1M Hydrochloric Acid, Alcoholic

Dilute 9.0 mL of *hydrochloric acid R* to 1000.0 mL with *aldehyde-free alcohol R*.

2M Hydrochloric Acid

Dilute 206.0 g of *hydrochloric acid R* to 1000.0 mL with *water R*.

3M Hydrochloric Acid

Dilute 309.0 g of *hydrochloric acid R* to 1000.0 mL with *water R*.

6M Hydrochloric Acid

Dilute 618.0 g of *hydrochloric acid R* to 1000.0 mL with *water R*.

Hydrochloric Acid, Brominated

To 1 mL of *bromine solution R* add 100 mL of *hydrochloric acid R*.

Hydrochloric Acid, Dilute

Contains 73 g/L of HCl.

Dilute 20 g of *hydrochloric acid R* to 100 mL with *water R*.

Hydrochloric Acid, Dilute, Heavy Metal-free

Complies with the requirements prescribed for *dilute hydrochloric acid R* with the following maximum contents of heavy metals.

As: 0.005 ppm.

Cd: 0.003 ppm.

Cu: 0.003 ppm.

Fe: 0.05 ppm.

Hg: 0.005 ppm.

Ni: 0.004 ppm.

Pb: 0.001 ppm.

Zn: 0.005 ppm.

Hydrochloric Acid, Ethanolic

Dilute 5.0 mL of *1 M hydrochloric acid* to 500.0 mL with *ethanol (96 per cent) R*.

Solutions of the requisite molarity may be obtained by diluting *hydrochloric acid* with *ethanol (96%)* in place of water as directed under *hydrochloric acid*.

Hydrochloric Acid, Heavy Metal-free

Complies with the requirements prescribed for *hydrochloric acid R* with the following maximum contents of heavy metals.

As: 0.005 ppm.

Cd: 0.003 ppm.

Cu: 0.003 ppm.

Fe: 0.05 ppm.

Hg: 0.005 ppm.

Ni: 0.004 ppm.

Pb: 0.001 ppm.

Zn: 0.005 ppm.

Hydrochloric Acid, Lead-free

Complies with the requirements prescribed for *hydrochloric acid R* with the following additional requirement.

Lead: maximum 20 ppb.

Atomic emission spectrometry (*2.2.22, Method I*).

Test solution. In a quartz crucible evaporate 200 g of the acid to be examined almost to dryness. Take up the residue in 5 mL of nitric acid prepared by sub-boiling distillation of *nitric acid R* and evaporate to dryness. Take up the residue in 5 mL of nitric acid prepared by sub-boiling distillation of *nitric acid R*.

Reference solutions. Prepare the reference solutions using *lead standard solution (0.1 ppm Pb) R* diluted with nitric acid prepared by sub-boiling distillation of *nitric acid R*.

Wavelength: 220.35 nm.

Hydrochloric Acid, Methanolic

Dilute 4.0 mL of *hydrochloric acid R* to 1000.0 mL with *methanol R2*.

Hydrochloric Acid R1

Contains 250 g/L of HCl.

Dilute 70 g of *hydrochloric acid R* to 100 mL with *water R*.

Hydrochloric Acid R1, Dilute

Contains 0.37 g/L of HCl.

Dilute 1.0 mL of *dilute hydrochloric acid R* to 200.0 mL with *water R*.

Hydrochloric Acid R2, Dilute

Dilute 30 mL of *1 M hydrochloric acid* to 1000 mL with *water R*; adjust to pH 1.6 ± 0.1.

Hydrochloric Acid, Dilute R3

Contains 3.7 g/L of HCl.

Dilute 10.0 mL of *dilute hydrochloric acid R* to 200.0 mL with *water R*.

Hydrochloric Acid, Stannated

Use stannated hydrochloric acid low in arsenic, grade of commerce, or prepare by adding 1 mL of *tin(II) chloride solution* to 100 mL of *hydrochloric acid*.

Hydrocortisone $C_{21}H_{30}O_5$ = 362.5 (*50-23-7*)

mp: about 214°, with decomposition.

General reagent grade of commerce.

Hydrocortisone Acetate (*50-03-3*)

See *Hydrocortisone acetate (0334)*.

Hydrofluoric Acid HF = 20.01 (*7664-39-3*)

Content: minimum 40.0 per cent *m/m*.

Clear, colourless liquid.

Loss on ignition: not more than 0.05 per cent *m/m*; evaporate the hydrofluoric acid in a platinum crucible and gently ignite the residue to constant mass.

Assay. Weigh accurately a glass-stoppered flask containing 50.0 mL of *1 M sodium hydroxide*. Introduce 2 g of the hydrofluoric acid and weigh again. Titrate the solution with *0.5 M sulfuric acid*, using 0.5 mL of *phenolphthalein solution R* as indicator.

1 mL of *1 M sodium hydroxide* is equivalent to 20.01 mg of HF.

Storage: in a polyethylene container.

Hydrogen Hydrogen for chromatography; H_2 = 2.016 (*1333-74-0*)

Content: minimum 99.95 per cent *V/V*.

Hydrogen Peroxide Solution (200 vol) H_2O_2 = 34.02 (*7724-84-1*)

General reagent grade of commerce containing about 60% w/v of H_2O_2.

A colourless liquid; weight per mL, about 1.18 g.

Hydrogen Peroxide Solution (100 vol) See *Strong hydrogen peroxide solution*

Hydrogen Peroxide Solution (20 vol)

Analytical reagent grade of commerce containing about 6% w/v of H_2O_2 or *hydrogen peroxide solution (100 vol)* diluted with 4 volumes of *water*.

A colourless liquid; weight per mL, about 1.02 g.

Hydrogen Peroxide Solution (10 vol) See *Dilute hydrogen peroxide solution*

Hydrogen Peroxide Solution, Dilute (*7722-84-1*)

See *Hydrogen peroxide solution (3 per cent) (0395)*.

Hydrogen Peroxide Solution, Strong (*7722-84-1*)

See *Hydrogen peroxide solution (30 per cent) (0396)*.

Hydrogen Sulfide Hydrogen sulphide; H_2S = 34.08 (*7783-06-4*)

Gas, slightly soluble in water.

Hydrogen Sulfide R1 Hydrogen sulphide R1; H_2S = 34.08 (*7783-06-4*)

Content: minimum 99.7 per cent *V/V*.

Hydrogen Sulfide Solution Hydrogen sulphide solution

A recently prepared solution of *hydrogen sulfide R* in *water R*. The saturated solution contains about 0.4 per cent to 0.5 per cent of H_2S at 20 °C.

Hydroquinone Quinol; $C_6H_6O_2$ = 110.1 (*123-31-9*)

Fine, colourless or white or almost white needles, darkening on exposure to air and light, soluble in water and in ethanol (96 per cent).

mp: about 173 °C.

Storage: protected from light and air.

Hydroquinone Solution

Dissolve 0.5 g of *hydroquinone R* in *water R*, add 20 µL of *sulfuric acid R* and dilute to 50 mL with *water R*.

4′-Hydroxyacetophenone 1-(4-Hydroxyphenyl)ethan-1-one; $C_8H_8O_2$ = 136.2 (*99-93-4*)

4-Hydroxybenzaldehyde $C_7H_6O_2$ = 122.2 (*123-08-0*)

mp: about 118°.

General reagent grade of commerce.

Colourless needles.

2-Hydroxybenzimidazole 1*H*-benzimidazol-2-ol; $C_7H_6N_2O$ = 134.1 (*615-16-7*)

4-Hydroxybenzohydrazide *p*-Hydroxybenzohydrazide; $C_7H_8N_2O_2$ = 152.2 (*5351-23-5*)

4-Hydroxybenzoic Acid Parahydroxybenzoic acid; $C_7H_6O_3$ = 138.1 (*99-96-7*)

Crystals, slightly soluble in water, very soluble in ethanol (96 per cent), soluble in acetone.

mp: 214 °C to 215 °C.

4-Hydroxybiphenyl See *Biphenyl-4-ol*

4-Hydroxycoumarin 4-Hydroxy-2*H*-1-benzopyran-2-one; $C_9H_6O_3$ = 162.2 (*1076-38-6*)

White or almost white powder, freely soluble in methanol.

Content: minimum 98.0 per cent.

6-Hydroxydopa (2*RS*)-2-Amino-3-(2,4,5-trihydroxyphenyl)propanoic acid; 2,5-Dihydroxy-DL-tyrosine; $C_9H_{11}NO_5$ = 213.2 (*21373-30-8*)

mp: about 257 °C.

2-[4-(2-Hydroxyethyl)piperazin-1-yl]ethane-1-sulfonic Acid 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulphonic acid; HEPES; $C_8H_{18}N_2O_4S$ = 238.3 (*7365-45-9*)

White or almost white powder.

mp: about 236 °C, with decomposition

4-Hydroxyisophthalic Acid 4-Hydroxybenzene-1,3-dicarboxylic acid; $C_8H_6O_5$ = 182.1 (*636-46-4*)

Needles or platelets, very slightly soluble in water, freely soluble in ethanol (96 per cent).

mp: about 314 °C, with decomposition.

Hydroxylamine Hydrochloride Hydroxylammonium chloride; NH_4ClO = 69.5 (*5470-11-1*)

White or almost white, crystalline powder, very soluble in water, soluble in ethanol (96 per cent).

Hydroxylamine Hydrochloride Solution R2

Dissolve 2.5 g of *hydroxylamine hydrochloride R* in 4.5 mL of hot *water R* and add 40 mL of *ethanol (96 per cent) R* and 0.4 mL of *bromophenol blue solution R2*. Add *0.5 M alcoholic potassium hydroxide* until a greenish-yellow colour is obtained. Dilute to 50.0 mL with *ethanol (96 per cent) R*.

Hydroxylamine Solution, Alcoholic

Dissolve 3.5 g of *hydroxylamine hydrochloride R* in 95 mL of *ethanol (60 per cent V/V) R*, add 0.5 mL of a 2 g/L solution of *methyl orange R* in *ethanol (60 per cent V/V) R* and sufficient *0.5 M potassium hydroxide in alcohol (60 per cent V/V)* to give a pure yellow colour. Dilute to 100 mL with *ethanol (60 per cent V/V) R*.

Hydroxylamine Solution, Alkaline

Immediately before use, mix equal volumes of a 139 g/L solution of *hydroxylamine hydrochloride R* and a 150 g/L solution of *sodium hydroxide R*.

Hydroxylamine Solution R1, Alkaline

Solution A. Dissolve 12.5 g of *hydroxylamine hydrochloride R* in *methanol R* and dilute to 100 mL with the same solvent.

Solution B. Dissolve 12.5 g of *sodium hydroxide R* in *methanol R* and dilute to 100 mL with the same solvent.

Mix equal volumes of solution A and solution B immediately before use.

Hydroxymethylfurfural 5-Hydroxymethylfurfural; $C_6H_6O_3$ = 126.1 (*67-47-0*)

Acicular crystals, freely soluble in water, in acetone and in ethanol (96 per cent).

mp: about 32 °C.

Hydroxynaphthol Blue, Sodium Salt Trisodium 2,2′-dihydroxy-1,1′-azonaphthalene-3′,4,6′-trisulfonate; $C_{20}H_{11}N_2Na_3O_{11}S_3$ = 620 (*63451-35-4*)

4-(4-Hydroxyphenyl)butan-2-one $C_{10}H_{12}O_2$ = 164.2 (*5471-51-2*)

mp: about 82°.

General reagent grade of commerce.

2-Hydroxypropylbetadex for Chromatography Betacyclodextrin modified by the bonding of (*R*) or (*RS*) propylene oxide groups on the hydroxyl groups; **Hydroxypropyl-β-cyclodextrin** (*94035-02-6*)

See *Hydroxypropylbetadex (1804)*.

pH (*2.2.3*): 5.0 to 7.5 for a 20 g/L solution.

8-Hydroxyquinoline Quinolin-8-ol; Hydroxyquinoline; C_9H_7NO = 145.2 (*148-24-3*)

White or slightly yellowish, crystalline powder, slightly soluble in water, freely soluble in acetone, in ethanol (96 per cent) and in dilute mineral acids.

mp: about 75 °C.

Sulfated ash (*2.4.14*): maximum 0.05 per cent.

12-Hydroxystearic Acid 12-Hydroxyoctadecanoic acid; $C_{18}H_{36}O_3$ = 300.5 (*106-14-9*)

White or almost white powder.

mp: 71 °C to 74 °C.

5-Hydroxyuracil 2,4,5-trihydroxypyrimidine; $C_4H_4N_2O_3$ = 128.1 (*496-76-4*)

White or almost white, crystalline powder.

mp: about 310 °C, with decomposition.

Chromatography. Thin-layer chromatography (*2.2.27*) as prescribed in the monograph *Fluorouracil (0611)*; the chromatogram shows a principal spot with an R_F of about 0.3.

Storage: in an airtight container.

Hyoscine Hydrobromide Scopolamine hydrobromide; (*6533-68-2*)

See *Hyoscine hydrobromide (0106)*.

Hyoscyamine Sulfate Hyoscyamine sulphate; (*620-61-1*)

See *Hyoscyamine sulfate (0501)*.

Hypericin 1,3,4,6,8,13-Hexahydroxy-10,11-dimethylphenanthro[1,10,9,8-*opqra*]perylene-7,14-dione; $C_{30}H_{16}O_8$ = 504.4 (*548-04-9*)

Content: minimum 85 per cent.

Hyperoside 2-(3,4-Dihydroxyphenyl)-3-β-D-galactopyranosyloxy-5,7-dihydroxychromen-4-one; $C_{21}H_{20}O_{12}$ = 464.4

Faint yellow needles, soluble in methanol.

Absorbance (*2.2.25*). A solution in *methanol R* shows 2 absorption maxima at about 257 nm and at about 359 nm.

Hypophosphorous Reagent

Dissolve with the aid of gentle heat, 10 g of *sodium hypophosphite R* in 20 mL of *water R* and dilute to 100 mL with *hydrochloric acid R*. Allow to settle and decant or filter through glass wool.

Hypoxanthine 6-Hydroxypurine; $C_5H_4N_4O$ = 136.1 (*68-94-0*)

White or almost white, crystalline powder, very slightly soluble in water, sparingly soluble in boiling water, soluble in dilute acids and in dilute alkali hydroxide solutions, decomposes without melting at about 150 °C.

Chromatography. Thin-layer chromatography (*2.2.27*) as prescribed in the monograph *Mercaptopurine (0096)*; the chromatogram shows only one principal spot.

Ibuprofen (*15687-27-1*)

See *Ibuprofen (0721)*.

Imidazole Glyoxaline; $C_3H_4N_2$ = 68.1 (*288-32-4*)

White or almost white, crystalline powder, soluble in water and in ethanol (96 per cent).

mp: about 90 °C.

Imidazole, Recrystallised

Twice recrystallise 25 g of *imidazole* from 100 mL of *toluene*, cool in ice with stirring, wash with *ether* and dry at room temperature at a pressure of 2 kPa over *anhydrous silica gel*, or use a purified grade of commerce.

Complies with the following test.

Light absorption *Absorbance* of an 8% w/v solution at 325 nm, not more than 0.10, Appendix II B.

Imidazole Solution

Dissolve 8.25 g of *recrystallised imidazole* in 60 mL of *water*, adjust the pH to 6.75 to 6.85 with 5M *hydrochloric acid* and add sufficient *water* to produce 100 mL.

The hydrochloric acid used in preparing this reagent must be free from stabilising mercury compounds.

Imidazole–Mercury Reagent

Dissolve 8.25 g of *recrystallised imidazole* in 60 mL of *water* and add 10 mL of 5M *hydrochloric acid*. Stir the solution magnetically and add, dropwise, 10 mL of a 0.27% w/v solution of *mercury(II) chloride*. If a cloudy solution results, discard and prepare a further solution adding the mercury chloride solution more slowly. Adjust the pH to 6.75 to 6.85 with 5M *hydrochloric acid* (about 4 mL is required) and add sufficient *water* to produce 100 mL.

Iminodiacetic Acid 2,2′-Iminodiacetic acid; $C_4H_7NO_4$ = 133.1 (*142-73-4*)

Iminodibenzyl 10,11-Dihydrodibenz[*b*,*f*]azepine; $C_{14}H_{13}N$ = 195.3 (*494-19-9*)

Pale yellow, crystalline powder, practically insoluble in water, freely soluble in acetone.

mp: about 106 °C.

Imipramine Hydrochloride (*113-52-0*)

See *Imipramine hydrochloride (0029)*.

Imperatorin 9-[(3-Methylbut-2-enyl)oxy]-7*H*-furo[3,2-*g*][1]benzopyran-7-one; $C_{16}H_{14}O_4$ = 270.3 (*482-44-0*)

2-Indanamine Hydrochloride 2-Aminoindane hydrochloride; $C_9H_{12}ClN$ = 169.7 (*2338-18-3*)

2,3-Dihydro-1*H*-inden-2-amine hydrochloride.

Indian Sandalwood Oil Sandalwood Oil; East Indian Sandalwood Oil; Santalum Album; $C_{30}H_{48}O_2$ = 440.70 (*8006-87-9*)

General reagent grade of commerce.

Indigo Indigotin; 1,1′,3,3′-Tetrahydro-2-2′-bi(indolylidene)-3,3′-dione; $C_{16}H_{10}N_2O_2$ = 262.3 (*482-89-3*)

Indigo Carmine Acid blue 74; $C_{16}H_8N_2Na_2O_8S_2$ = 466.3 (*860-22-0*)

Schultz No. 1309

Colour Index No. 73015

It usually contains sodium chloride.

Blue or violet-blue powder or blue granules with a coppery lustre, sparingly soluble in water, practically insoluble in ethanol (96 per cent). It is precipitated from an aqueous solution by sodium chloride.

Indigo Carmine Solution

To a mixture of 10 mL of *hydrochloric acid R* and 990 mL of 200 g/L *nitrogen-free sulfuric acid R* add 0.2 g of *indigo carmine R*.

The solution complies with the following test: add 10 mL to a solution of 1.0 mg of *potassium nitrate R* in 10 mL of *water R*, rapidly add 20 mL of *nitrogen-free sulfuric acid R* and heat to boiling. The blue colour is discharged within 1 min.

Indigo Carmine Solution R1

Dissolve 4 g of *indigo carmine R* in about 900 mL of *water R* added in several portions. Add 2 mL of *sulfuric acid R* and dilute to 1000 mL with *water R*.

Assay. Place in a 100 mL conical flask with a wide neck 10.0 mL of *nitrate standard solution (100 ppm NO_3) R*, 10 mL of *water R*, 0.05 mL of the *indigo carmine solution R1*, and then in a single addition, but with caution, 30 mL of *sulfuric acid R*. Titrate the solution immediately, using the *indigo carmine solution R1*, until a stable blue colour is obtained.

The number of millilitres used, *n*, is equivalent to 1 mg of NO_3.

Indirubin $C_{16}H_{10}N_2O_2$ = 262.3 (*479-41-4*)

1,1′,2′,3-Tetrahydro-2,3′-bi(indolylidene)-2′,3-dione.

Indometacin (*53-86-1*)

See *Indometacin (0092)*.

Industrial Methylated Spirit (95%)

Of the British Pharmacopoeia.

Inosine 9-β-D-Ribofuranosylhypoxanthine; 9-β-D-Ribofuranosyl-1,9-dihydro-6*H*-purin-6-one; $C_{10}H_{12}N_4O_5$ = 268.2 (*58-63-9*)

mp: 222 °C to 226 °C.

***myo*-Inositol**

See *myo-Inositol (1805)*.

Iodic Acid HIO_3 = 175.9 (*7782-68-5*)

Analytical reagent grade of commerce.

Iodine (*7553-56-2*)

See *Iodine (0031)*.

To prepare 0.05M iodine dissolve 20 g of *potassium iodide* in the minimum amount of *water*, add 13 g of *iodine*, allow to dissolve and add sufficient *water* to produce 1000 mL. Weaker solutions should be prepared using proportionately lesser amounts of reagents or by appropriate dilution.

Iodine-123 and Ruthenium-106 Spiking Solution

Prepare immediately before use. Mix 3.5 mL of an 18.5 kBq/mL solution of ruthenium-106 in the form of ruthenium trichloride in a mixture of equal volumes of *glacial acetic acid R* and *water R* with 200 µL of a 75 kBq/mL solution of iodine-123 in the form of sodium iodide in *water R*.

Iodine Bromide IBr = 206.8 (*7789-33-5*)

Bluish-black or brownish-black crystals, freely soluble in water, in ethanol (96 per cent) and in glacial acetic acid.

bp: about 116 °C.

mp: about 40 °C.

Storage: protected from light.

Iodine Bromide Solution

Dissolve 20 g of *iodine bromide R* in *glacial acetic acid R* and dilute to 1000 mL with the same solvent.

Storage: protected from light.

Iodine Chloride ICl = 162.4 (*7790-99-0*)

Black crystals, soluble in water, in acetic acid and in ethanol (96 per cent).

bp: about 97.4 °C.

Iodine Chloride Solution

Dissolve 1.4 g of *iodine chloride R* in *glacial acetic acid R* and dilute to 100 mL with the same acid.

Storage: protected from light.

Iodine Monochloride Reagent, Strong

Dissolve 10 g of *potassium iodide* and 6.4 g of *potassium iodate* in 75 mL of *water*, add 75 mL of *hydrochloric acid* and 5 mL of *chloroform*, shake and, if necessary, add dropwise with vigorous shaking 0.1M *potassium iodide* until a faint iodine colour appears in the chloroform layer. Add in the same manner 0.001M *potassium iodate* until the chloroform is just colourless.

Before use, readjust with either 0.1M *potassium iodide* or 0.001M *potassium iodate* as required.

Store in a cool place protected from light.

Iodine Monochloride Solution

Dissolve 8 g of *iodine trichloride* in about 200 mL of *glacial acetic acid* and separately dissolve 9 g of *iodine* in 300 mL of *dichloromethane*. Mix the two solutions and dilute to 1000 mL with *glacial acetic acid*.

Store in a stoppered bottle, protected from light and at a temperature not exceeding 15°.

Iodine Pentoxide, Recrystallised Iodine pentoxide; I_2O_5 = 333.8 (*12029-98-0*)

Content: minimum 99.5 per cent.

White or almost white, crystalline powder, or white or greyish-white granules, hygroscopic, very soluble in water forming HIO_3.

Stability on heating. Dissolve 2 g, previously heated for 1 h at 200 °C, in 50 mL of *water R*. A colourless solution is obtained.

Assay. Dissolve 0.100 g in 50 mL of *water R*, add 3 g of *potassium iodide R* and 10 mL of *dilute hydrochloric acid R*. Titrate the liberated iodine with *0.1 M sodium thiosulfate*, using 1 mL of *starch solution R* as indicator.

1 mL of *0.1 M sodium thiosulfate* is equivalent to 2.782 mg of I_2O_5.

Storage: in an airtight container, protected from light.

Iodine Solution, Alcoholic

A 10 g/L solution of *iodine R* in *ethanol (96 per cent) R*.

Storage: protected from light.

Iodine Solution, Chloroformic

A 5 g/L solution of *iodine R* in *chloroform R*.

Storage: protected from light.

Iodine Solution R1

To 10.0 mL of *0.05 M iodine* add 0.6 g of *potassium iodide R* and dilute to 100.0 mL with *water R*. Prepare immediately before use.

Iodine Solution R2

To 10.0 mL of *0.05 M iodine* add 0.6 g of *potassium iodide R* and dilute to 1000.0 mL with *water R*. Prepare immediately before use.

Iodine Solution R3

Dilute 2.0 mL of *iodine solution R1* to 100.0 mL with *water R*. Prepare immediately before use.

Iodine Solution R4

Dissolve 14 g of *iodine R* in 100 mL of a 400 g/L solution of *potassium iodide R*, add 1 mL of *dilute hydrochloric acid R* and dilute to 1000 mL with *water R*.

Storage: protected from light.

Iodine Solution R5

Dissolve 12.7 g of *iodine R* and 20 g of *potassium iodide R* in *water R* and dilute to 1000.0 mL with the same solvent (0.05 M solution).

Iodine Trichloride ICl_3 = 233.3 (*865-44-1*)

Analytical reagent grade of commerce.

Reddish orange crystals.

Iodoacetamide 2-Iodoacetamide; C_2H_4INO = 185.0 (*144-48-9*)

Slightly yellow, crystalline powder, soluble in water.

mp: about 92 °C.

Iodoacetic Acid $C_2H_3IO_2$ = 185.9 (*64-69-7*)

Colourless or white or almost white crystals, soluble in water and in ethanol (96 per cent).

mp: 82 °C to 83 °C.

2-Iodobenzoic Acid $C_7H_5IO_2$ = 248.0 (*88-67-5*)

White or slightly yellow, crystalline powder, slightly soluble in water, soluble in ethanol (96 per cent).

mp: about 160 °C.

Chromatography. Thin-layer chromatography (*2.2.27*), using cellulose for chromatography f_{254} R as the coating substance: apply to the plate 20 µL of a solution of the 2-iodobenzoic acid, prepared by dissolving 40 mg in 4 mL of *0.1 M sodium hydroxide* and diluting to 10 mL with *water R*. Develop over a path of about 12 cm using as the mobile phase the upper layer obtained by shaking together 20 volumes of *water R*, 40 volumes of *glacial acetic acid R* and 40 volumes of *toluene R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. The chromatogram shows only one principal spot.

3-Iodobenzylammonium Chloride 1-(3-Iodophenyl) methanamine hydrochloride; 1-(3-Iodophenyl) methanaminium chloride; *m*-Iodobenzylamine hydrochloride; C_7H_9ClIN = 269.5 (*3718-88-5*)

White or almost white crystals.

mp: 188 °C to 190 °C.

Iodoethane C_2H_5I = 156.0 (*75-03-6*)

Content: minimum 99 per cent.

Colourless or slightly yellowish liquid, darkening on exposure to air and light, miscible with ethanol (96 per cent) and most organic solvents.

d_{20}^{20}: about 1.95.

n_D^{20}: about 1.513.

bp: about 72 °C.

Storage: in an airtight container, protected from light.

2-Iodohippuric Acid 2-(2-Iodobenzamido)acetic acid; $C_9H_8INO_3,2H_2O$ = 341.1 (*147-58-0*)

White or almost white, crystalline powder, sparingly soluble in water.

mp: about 170 °C.

Water (*2.5.12*): 9 per cent to 13 per cent, determined on 1.000 g.

Chromatography. Thin-layer chromatography (*2.2.27*), using *cellulose for chromatography F_{254} R* as the coating substance: apply to the plate 20 µL of a solution of the 2-iodohippuric acid, prepared by dissolving 40 mg in 4 mL of *0.1 M sodium hydroxide* and diluting to 10 mL with *water R*. Develop over a path of about 12 cm using as the mobile phase the upper layer obtained by shaking together 20 volumes of *water R*, 40 volumes of *glacial acetic acid R* and 40 volumes of *toluene R*. Allow the plate to dry in air and examine in ultraviolet light at 254 nm. The chromatogram shows only one principal spot.

Iodomethane Methyl iodide; CH_3I = 141.9 (*74-88-4*)

Content: minimum 99.0 per cent.

Iodoplatinate Reagent

To 3 mL of a 100 g/L solution of *chloroplatinic acid R* add 97 mL of *water R* and 100 mL of a 60 g/L solution of *potassium iodide R*.

Storage: protected from light.

Iodoplatinate Reagent R1

Mix 2.5 mL of a 50 g/L solution of *chloroplatinic acid R*, 22.5 mL of a 100 g/L solution of *potassium iodide R* and 50 mL of *water R*.

Storage: protected from light, at a temperature of 2-8 °C.

2-Iodopropane Isopropyl iodide; C_3H_7I = 170.0 (*75-30-9*)

Content: minimum 99 per cent.

5-Iodouracil 5-Iodo-1*H*,3*H*-pyrimidine-2,4-dione; $C_4H_3IN_2O_2$ = 238.0 (*696-07-1*)

mp: about 276 °C, with decomposition.

Chromatography. Thin-layer chromatography (*2.2.27*) as prescribed in the monograph *Idoxuridine (0669)*: apply 5 µL of a 0.25 g/L solution; the chromatogram obtained shows only one principal spot.

Ion-exchange Resin, Strongly Acidic

Resin in protonated form with sulfonic acid groups attached to a lattice consisting of polystyrene cross-linked with 8 per cent of divinylbenzene. It is available as spherical beads; unless otherwise prescribed, the particle size is 0.3 mm to 1.2 mm.

Capacity. 4.5 mmol to 5 mmol per gram, with a water content of 50 per cent to 60 per cent.

Preparation of a column. Unless otherwise prescribed, use a tube with a fused-in sintered glass disc having a length of 400 mm, an internal diameter of 20 mm and a filling height of about 200 mm. Introduce the resin, mixing it with *water R* and pouring the slurry into the tube, ensuring that no air bubbles are trapped between the particles. When in use, the liquid must not be allowed to fall below the surface of the resin. If the resin is in its protonated form, wash with *water R* until 50 mL requires not more than 0.05 mL of *0.1 M sodium hydroxide* for neutralisation, using 0.1 mL of *methyl orange solution R* as indicator.

If the resin is in its sodium form or if it requires regeneration, pass about 100 mL of a mixture of equal volumes of *hydrochloric acid R1* and *water R* slowly through the column and then wash with *water R* as described above.

Ion-exclusion Resin for Chromatography

Resin with a sulfonate funtionalised latex cross-linked with divinylbenzene.

Irisflorentin 9-Methoxy-7-(3,4,5-trimethoxyphenyl)-8*H*-1,3-dioxolo[4,5-*g*][1]benzopyran-8-one; $C_{20}H_{18}O_8$ = 386.4 (*41743-73-1*)

Iron Fe = 55.85 (*7439-89-6*)

Grey powder or wire, soluble in dilute mineral acids.

Iron(III) Chloride, Anhydrous Iron(III) chloride; anhydrous ferric chloride; $FeCl_3$ = 162.2 (*7705-08-0*)

General reagent grade of commerce.

Greenish black crystals or crystalline powder turning orange on exposure to moist air.

Iron(III) Chloride Hexahydrate Iron trichloride hexahydrate; Ferric chloride; $FeCl_3,6H_2O$ = 270.3 (*10025-77-1*)

Yellowish-orange or brownish crystalline masses, deliquescent, very soluble in water, soluble in ethanol (96 per cent). On exposure to light, ferric chloride and its solutions are partly reduced.

Storage: in an airtight container.

Iron(III) Chloride Solution

Analytical reagent grade of commerce, diluted to contain about 15% w/v of $FeCl_3$.

Iron(III) Chloride Solution, Ethanolic

Carefully add 25 mL of *sulfuric acid* dropwise to 75 mL of well-cooled *absolute ethanol*, stirring constantly. Add 2 g of *anhydrous iron(III) chloride*, stir and filter.

Iron(III) Chloride Solution R1 Ferric chloride solution R1

A 105 g/L solution of *ferric chloride R*.

Iron(III) Chloride Solution R2 Ferric chloride solution R2

A 13 g/L solution of *ferric chloride R*.

Iron(III) Chloride-Sulfamic Acid Reagent Ferric chloride-sulfamic acid reagent

A solution containing 10 g/L of *ferric chloride R* and 16 g/L of *sulfamic acid R*.

Iron(III) Nitrate Ferric nitrate; $Fe(NO_3)_3,9H_2O$ = 404 (*7782-61-8*)

Content: minimum 99.0 per cent *m/m* of $Fe(NO_3)_3,9H_2O$.

Light-purple crystals or crystalline mass, very soluble in water.

Free acid: not more than 0.3 per cent (as HNO_3).

Iron(III) Nitrate Solution

A 0.1% w/v solution of *iron(III) nitrate* in 0.1% v/v *nitric acid*.

Iron Salicylate Solution

Dissolve 0.1 g of *ferric ammonium sulfate R* in a mixture of 2 mL of *dilute sulfuric acid R* and 48 mL of *water R* and dilute to 100 mL with *water R*. Add 50 mL of a 11.5 g/L solution of *sodium salicylate R*, 10 mL of *dilute acetic acid R*, 80 mL of a 136 g/L solution of *sodium acetate R* and dilute to 500 mL with *water R*. The solution should be recently prepared.

Storage: in an airtight container, protected from light.

Iron(II) Sulfate Ferrous sulphate; Iron(III) sulphate; Ferrous sulfate; (*7782-63-0*)

See *Ferrous sulfate heptahydrate (0083)*.

Iron(III) Sulfate Ferric sulphate; Iron(III) sulphate; Iron(III)trisulfate hydrated; Iron(III)trisulphate hydrated; Ferric sulfate; $Fe_2(SO_4)_3,xH_2O$ (*15244-10-7*)

Yellowish-white powder, very hygroscopic, decomposes in air, slightly soluble in water and in ethanol (96 per cent).

Storage: in an airtight container, protected from light.

Iron(III) Sulfate Pentahydrate Ferric sulphate pentahydrate; Iron(III) sulphate pentahydrate; Ferric sulfate pentahydrate; $Fe_2(SO_4)_3,5H_2O$ = 489.9 (*142906-29-4*)

White or yellowish powder.

Iron(III) Sulfate Solution Ferric sulfate solution

Dissolve 50 g of *ferric sulfate R* in an excess of *water R*, add 200 mL of *sulfuric acid R* and dilute to 1000 mL with *water R*.

Iron(II) Sulfate Solution R2 Ferrous sulfate solution R2

Dissolve 0.45 g of *ferrous sulfate R* in 50 mL of *0.1 M hydrochloric acid* and dilute to 100 mL with *carbon dioxide-free water R*. Prepare immediately before use.

Iron(II) Sulfate-Citrate Solution

Iron(II) sulphate-citrate solution

Dissolve 1 g of *sodium metabisulfite* in 200 mL of *water* and add 0.5 mL of 2M *hydrochloric acid*, 1.5 g of *iron(II) sulfate* and 10 g of *sodium citrate*.

Prepare immediately before use.

Isatin Indoline-2,3-dione; $C_8H_5NO_2$ = 147.1 (*91-56-5*)

Small, yellowish-red crystals, slightly soluble in water, soluble in hot water and in ethanol (96 per cent), soluble in solutions of alkali hydroxides giving a violet colour becoming yellow on standing.

mp: about 200 °C, with partial sublimation.

Sulfated ash (*2.4.14*): maximum 0.2 per cent.

Isatin Reagent

Dissolve 6 mg of *ferric sulfate R* in 8 mL of *water R* and add cautiously 50 mL of *sulfuric acid R*. Add 6 mg of *isatin R* and stir until dissolved.

The reagent should be pale yellow, but not orange or red.

Isoandrosterone Epiandrosterone; 3β-Hydroxy-5α-androstan-17-one; $C_{19}H_{30}O_2$ = 290.4 (*481-29-8*)

White or almost white powder, practically insoluble in water, soluble in organic solvents.

$[\alpha]_D^{20}$: + 88, determined on 20 g/L solution in *methanol R*.

mp: 172 °C to 174 °C.

ΔA (*2.2.41*): 14.24 × 10^3, determined at 304 nm on a 1.25 g/L solution.

Isobutyl Acetate $C_6H_{12}O_2$ = 116.2 (*110-19-0*)

bp: about 118°.

General reagent grade of commerce.

A colourless liquid; weight per mL, about 0.87 g.

***N*-Isobutyldodecatetraenamide** (*2E,4E,8Z,10EZ*)-*N*-2-(Methylpropyl)dodeca-2,4,8,10-tetraenamide; $C_{16}H_{25}NO$ = 247.4 (*866602-52-0*)

White or almost white or non-coloured crystals.

mp: about 70 °C.

***N*-Isobutyldodecatetraenamide Solution**

A solution of *N-isobutyldodecatetraenamide R*, exactly weighed, in *methanol R* at a concentration of about 10 mg/mL.

Isodrin 1,2,3,4,10,10-Hexachloro-1,4,4a,5,8,8a-hexahydro-*endo,endo*-1,4:5,8-dimethanonaphthalene; $C_{12}H_8Cl_6$ = 364.9 (*465-73-6*)

Practically insoluble in water, soluble in common organic solvents such as acetone.

A suitable certified reference solution may be used.

Isoeugenol 2-Methoxy-4-[(1*E*)-prop-1-en-1-yl]phenol; $C_{10}H_{12}O_2$ = 164.2 (*97-54-1*)

Isoeugenyl Acetate $C_{12}H_{14}O_3$ = 206.24 (*93-29-8*)

Analytical reagent grade of commerce.

Isoleucine (*73-32-5*)

See *Isoleucine (0770)*.

Isomalt $C_{12}H_{24}O_{11}$ = 344.3 (*64519-82-0*)

Mixture of 6-*O*-α-D-glucopyranosyl-D-glucitol and of 1-*O*-α-D-glucopyranosyl-D-mannitol.

White or almost white powder or granules, freely soluble in water.

Isomaltitol 6-*O*-α-D-Glucopyranosyl-D-glucitol; $C_{12}H_{24}O_{11}$ = 344.3 (*534-73-6*)

White or almost white powder, freely soluble in water.

Isomenthol (+)-*Isomenthol:* (1*S*,2*R*,5*R*)-2-isopropyl-5-methylcyclohexanol; (±)-*Isomenthol:* a mixture of equal parts of (1*S*,2*R*,5*R*)- and (1*R*,2*S*,5*S*)-2-isopropyl-5-methylcyclohexanol; $C_{10}H_{20}O$ = 156.3 (*23283-97-8*)

Colourless crystals, practically insoluble in water, very soluble in ethanol (96 per cent).

$[\alpha]_D^{20}$: (+)-*Isomenthol*: about + 24, determined on a 100 g/L solution in *ethanol (96 per cent) R*.

bp: (+)-*Isomenthol*: about 218 °C. (±)-*Isomenthol*: about 218 °C.

mp: (+)-*Isomenthol*: about 80 °C. (±)-*Isomenthol*: about 53 °C.

(+)-Isomenthone (1*R*)-*cis*-*p*-Menthan-3-one; (1*R*)-*cis*-2-Isopropyl-5-methylcyclohexanone; $C_{10}H_{18}O$ = 154.2

Contains variable amounts of menthone. A colourless liquid, very slightly soluble in water, soluble in ethanol (96 per cent).

d_{20}^{20}: about 0.904.

n_D^{20}: about 1.453.

$[\alpha]_D^{20}$: about + 93.2.

Isomenthone used in gas chromatography complies with the following additional test.

Assay. Gas chromatography (*2.2.28*) as prescribed in the monograph *Peppermint oil (0405)*.

Test solution. The substance to be examined.

Content: minimum 80.0 per cent, calculated by the normalisation procedure.

Isomethyleugenol 1,2-Dimethoxy-4-prop-1-enylbenzene; $C_{11}H_{14}O_2$ = 178.2 (*93-16-3*)

Isomethyleugenol used in gas chromatography complies with the following additional test.

Assay. Gas chromatography (*2.2.28*) as prescribed in the monograph *Niaouli oil, cineole type (2468)*.

Content: minimum 97.0 per cent, calculated by the normalisation procedure.

Isoniazid Isonicotinohydrazide; $C_6H_7N_3O$ = 137.1 (*54-85-3*)

mp: about 171°.

General reagent grade of commerce.

Colourless crystals or a white, crystalline powder.

Isoniazid Solution

Dissolve 0.1 g of *isoniazid* in 150 mL of *methanol*, add 0.12 mL of *hydrochloric acid* and dilute to 200 mL with *methanol*.

Isonicotinamide 4-Pyridinecarboxamide; Pyridine-4-carboxamide; $C_6H_6N_2O$ = 122.1 (*1453-82-3*)

White or almost white, crystalline powder, soluble in water.

Isonicotinic Acid Pyridine-4-carboxylic acid; $C_6H_5NO_2$ = 123.1 (*55-22-1*)

Creamish-white powder, sparingly soluble in water.

mp: about 311 °C.

Isopentyl Benzoate 3-Methylbutyl benzoate; Isoamyl benzoate; $C_{12}H_{16}O_2$ = 192.3 (*94-46-2*)

n_D^{20}: about 1.494.

bp: about 261 °C.

Colourless or pale yellow liquid.

Isopropylamine 2-Propylamine; C_3H_9N = 59.1 (*75-31-0*)

Colourless, highly volatile, flammable liquid.

n_D^{20}: about 1.374.

bp: 32 °C to 34 °C.

Isopropyl Methanesulfonate 1-methylethyl methanesulfonate; $C_4H_{10}O_3S$ = 138.2 (*926-06-7*)

Clear, colourless liquid.

Content: minimum 99.0 per cent.

Density: about 1.129 g/cm^3 (20 °C).

n_D^{20}: 1.418-1.421.

bp: about 82 °C at 6 mm Hg.

Isopropyl Myristate Isopropyl tetradecanoate; (*110-27-0*)

See *Isopropyl myristate (0725)*.

4-Isopropylphenol $C_9H_{12}O$ = 136.2 (*99-89-8*)

Content: minimum 98 per cent.

bp: about 212 °C.

mp: 59 °C to 61 °C.

Isopropyl Toluenesulfonate 1-Methylethyl 4-methylbenzenesulfonate; Propan-2-yl 4-methylbenzenesulfonate; Isopropyl tosilate; $C_{10}H_{14}O_3S$ = 214.3 (*2307-69-9*)

Content: minimum 97.0 per cent.

Clear liquid.

mp: about 20 °C.

Isopulegol (−)-Isopulegol; (1*R*,2*S*,5*R*)-2-Isopropenyl-5-methylcyclohexanol; $C_{10}H_{18}O$ = 154.2 (*89-79-2*)

d_4^{20}: about 0.911.

n_D^{20}: about 1.472.

bp: about 210 °C.

Isopulegol used in gas chromatography complies with the following additional test.

Assay. Gas chromatography (*2.2.28*) as prescribed in the monograph *Mint oil, partly dementholised (1838)*.

Content: minimum 99 per cent, calculated by the normalisation procedure.

Isoquercitrin 2-(3,4-Dihydroxyphenyl)-3-(β-D-glucopyranosyloxy)-5,7-dihydroxy-4*H*-1-benzopyran-4-one; $C_{21}H_{20}O_{12}$ = 464.4 (*482-35-9*)

Isoquercitroside Isoquercitrin; 3,3′,4′,5,7-Pentahydroxyflavone-3-glucoside; $C_{21}H_{20}O_{12}$ = 464.4 (*21637-25-2*)

Isorhamnetin-3-*O*-neohesperidoside 3-[6-Deoxy-α-L-mannopyranosyl-(1→2)-β-D-glucopyranosyloxy]-5,7-dihydroxy-2-(4-hydroxy-3-methoxyphenyl)-4*H*-1-benzopyran-4-one; $C_{28}H_{32}O_{16}$ = 625 (*55033-90-4*)

Isorhamnetin-3-O-rutinoside 3-*O*-Methylquercetin-3-rutinoside; Narcissoside; $C_{28}H_{32}O_{16}$ = 625 (*604-80-8*)

Isorhynchophylline Methyl (16*E*)-17-methoxy-2-oxo-16,17-didehydro-20α-corynoxan-16-carboxylate; Methyl (16*E*)-16-(methoxymethylidene)-2-oxo-20α-corynoxan-17-oate; $C_{22}H_{28}N_2O_4$ = 384.5 (*6859-01-4*)

Isosilibinin 3,5,7-Trihydroxy-2-[2-(4-hydroxy-3-methoxyphenyl)-3-hydroxymethyl-2,3-dihydro-1,4-benzodioxin-6-yl]chroman-4-one; $C_{25}H_{22}O_{10}$ = 482.4 (*72581-71-6*)

White to yellowish powder, practically insoluble in water, soluble in acetone and in methanol.

Isovitexin 6-β-D-Glucopyranosyl-5,7-dihydroxy-2-(4-hydroxyphenyl)-4*H*-1-benzopyran-4-one; Apigenin 6-*C*-β-glucopyranoside; $C_{21}H_{20}O_{10}$ = 432.4 (*38953-85-4*)

Kaempferol 3,5,7-Trihydroxy-2-(4-hydroxyphenyl)-4*H*-1-benzopyran-4-one; $C_{15}H_{10}O_6$ = 286.2 (*520-18-3*)

Kaolin, Light (*1332-58-7*)

A purified native hydrated aluminium silicate. It contains a suitable dispersing agent.

Light, white or almost white powder free from gritty particles, unctuous to the touch, practically insoluble in water and in mineral acids.

Coarse particles: maximum 0.5 per cent.

Place 5.0 g in a ground-glass-stoppered cylinder about 160 mm long and 35 mm in diameter and add 60 mL of a 10 g/L solution of *sodium pyrophosphate R*. Shake vigorously and allow to stand for 5 min. Using a pipette, remove 50 mL of the liquid from a point about 5 cm below the surface. To the remaining liquid add 50 mL of *water R*, shake, allow to stand for 5 min and remove 50 mL as before. Repeat the operations until a total of 400 mL has been removed. Transfer the remaining suspension to an evaporating dish. Evaporate to dryness on a water-bath and dry the residue to constant mass at 100-105 °C. The residue weighs not more than 25 mg.

Fine particles. Disperse 5.0 g in 250 mL of *water R* by shaking vigorously for 2 min. Immediately pour into a glass cylinder 50 mm in diameter and, using a pipette, transfer 20 mL to a glass dish, evaporate to dryness on a water-bath and dry to constant mass at 100-105 °C. Allow the remainder of the suspension to stand at 20 °C for 4 h and, using a pipette with its tip exactly 5 cm below the surface, withdraw a further 20 mL without disturbing the sediment, place in a glass dish, evaporate to dryness on a water-bath and dry to constant mass at 100-105 °C. The mass of the second residue is not less than 70 per cent of that of the first residue.

Kerosene, Deodorised

General grade of commerce.

11-Keto-β-boswellic Acid 3α-Hydroxy-11-oxours-12-en-24-oic acid; (4β)-3α-Hydroxy-11-oxours-12-en-23-oic acid; $C_{30}H_{46}O_4$ = 470.7 (*17019-92-0*)

White or almost white powder, insoluble in water, soluble in acetone, in anhydrous ethanol and in methanol.

mp: 195 °C to 197 °C.*11-Keto-β-boswellic acid used in liquid chromatography complies with the following additional test.*

Assay. Liquid chromatography (*2.2.29*) as prescribed in the monograph *Indian frankincense (2310)*.

Content: minimum 90 per cent, calculated by the normalisation procedure.

Kieselguhr

Acid-purified grade of commerce.

Kieselguhr for Chromatography

White or yellowish-white, light powder, practically insoluble in water, in dilute acids and in organic solvents.

Filtration rate. Use a chromatography column 0.25 m long and 10 mm in internal diameter with a sintered-glass (100) plate and two marks at 0.10 m and 0.20 m above the plate. Place sufficient of the substance to be examined in the column to reach the first mark and fill to the second mark with *water R*. When the first drops begin to flow from the column, fill to the second mark again with *water R* and measure the time required for the first 5 mL to flow from the column. The flow rate is not less than 1 mL/min.

Appearance of the eluate. The eluate obtained in the test for filtration rate is colourless (*2.2.2, Method I*).

Acidity or alkalinity. To 1.00 g add 10 mL of *water R*, shake vigorously and allow to stand for 5 min. Filter the suspension on a filter previously washed with hot *water R* until the

washings are neutral. To 2.0 mL of the filtrate add 0.05 mL of *methyl red solution R*; the solution is yellow. To 2.0 mL of the filtrate add 0.05 mL of *phenolphthalein solution R1*; the solution is at most slightly pink.

Water-soluble substances. Place 10.0 g in a chromatography column 0.25 m long and 10 mm in internal diameter and elute with *water R*. Collect the first 20 mL of eluate, evaporate to dryness and dry the residue at 100 °C to 105 °C. The residue weighs not more than 10 mg.

Iron (*2.4.9*): maximum 200 ppm.

To 0.50 g add 10 mL of a mixture of equal volumes of *hydrochloric acid R1* and *water R*, shake vigorously, allow to stand for 5 min and filter. 1.0 mL of the filtrate complies with the test for iron.

Loss on ignition: maximum 0.5 per cent. During heating to red heat (600 ± 50 °C) the substance does not become brown or black.

Kieselguhr G

Consists of kieselguhr treated with hydrochloric acid and calcined, to which is added about 15 per cent of calcium sulfate hemihydrate.

A fine greyish-white powder; the grey colour becomes more pronounced on triturating with water. The average particle size is 10-40 µm.

Calcium sulfate content. Determine by the method prescribed for *silica gel G R*.

pH (*2.2.3*). Shake 1 g with 10 mL of *carbon dioxide-free water R* for 5 min. The pH of the suspension is 7 to 8.

Chromatographic separation. Thin-layer chromatography (*2.2.27*). Prepare plates using a slurry of the kieselguhr G with a 2.7 g/L solution of *sodium acetate R*. Apply 5 µL of a solution containing 0.1 g/L of lactose, sucrose, glucose and fructose in *pyridine R*. Develop over a path of 14 cm using a mixture of 12 volumes of *water R*, 23 volumes of *2-propanol R* and 65 volumes of *ethyl acetate R*.
The migration time of the solvent is about 40 min. Dry, spray onto the plate about 10 mL of *anisaldehyde solution R* and heat for 5-10 min at 100-105 °C. The chromatogram shows four well-defined spots without tailing and well separated from each other.

Lactic Acid (*50-21-5*)

See *Lactic acid (0458)*.

Lactic Reagent Lactic acid reagent

Solution A. To 60 mL of *lactic acid R* add 45 mL of previously filtered *lactic acid R* saturated without heating with *Sudan red G R*; as lactic acid saturates slowly without heating, an excess of colorant is always necessary.

Solution B. Prepare 10 mL of a saturated solution of *aniline R*. Filter.

Solution C. Dissolve 75 mg of *potassium iodide R* in water and dilute to 70 mL with the same solvent. Add 10 mL of *ethanol (96 per cent) R* and 0.1 g of *iodine R*. Shake.

Mix solutions A and B. Add solution C.

Lactobionic Acid $C_{12}H_{22}O_{12}$ = 358.3 (*96-82-2*)

White or almost white, crystalline powder, freely soluble in water, practically insoluble in ethanol (96 per cent).

mp: about 115 °C.

Lactose Lactose monohydrate; (*5989-81-1*)

See *Lactose monohydrate (0187)*.

β-Lactose β-D-Lactose; $C_{12}H_{22}O_{11}$ = 342.3 (*5965-66-2*)

White or slightly yellowish powder.

Content: minimum 99 per cent.

α-D-Lactose: not greater than 35 per cent.

Assay. Gas chromatography (*2.2.28*): use the normalisation procedure.

Column:
— *size*: *l* = 30 m, Ø = 0.25 mm;
— *stationary phase*: *cyanopropyl(3)phenyl(3)methyl(94) polysiloxane R* (film thickness 1 µm).

Carrier gas: *helium for chromatography R*.

Temperature:

	Time (min)	Temperature (°C)
Column	0 - 32.5	20 → 280
Injection port		250
Detector		250

Detection: flame ionisation.

Injection: an appropriate derivatised sample.

α-Lactose Monohydrate α-D-Lactose monohydrate; $C_{12}H_{22}O_{11},H_2O$ = 360.3 (*5989-81-1*)

White or almost white powder.

Content: minimum 97 per cent.

β-D-Lactose: less than 3 per cent.

Assay. Gas chromatography (*2.2.28*): use the normalisation procedure.

Column:
— *size*: *l* = 30 m, Ø = 0.25 mm;
— *stationary phase*: *methylpolysiloxane R* (film thickness 1 µm).

Carrier gas: *helium for chromatography R*.

Temperature:

	Time (min)	Temperature (°C)
Column	0 - 12.5	230 → 280
Injection port		250
Detector		280

Detection: flame ionisation.

Injection: an appropriate derivatised sample.

Lactulose (*4618-18-2*)

See *Lactulose (1230)*.

Lanatoside C 3β-[(β-D-Glucopyranosyl-(1→4)-3-*O*-acetyl-2,6-dideoxy-β-D-*ribo*-hexopyranosyl-(1→4)-2,6-dideoxy-β-D-*ribo*-hexopyranosyl-(1→4)-2,6-dideoxy-β-D-*ribo*-hexopyranosyl)oxy]-12β,14-dihydroxy-5β-card-20(22)-enolide; $C_{49}H_{76}O_{20}$ = 985 (*17575-22-3*)

Long, flat prisms obtained after recrystallisation in ethanol (96 per cent), freely soluble in pyridine and in dioxan.

Lanthanum Chloride Heptahydrate
$LaCl_3,7H_2O$ = 371.4

White or almost white powder or colourless crystals, freely soluble in water.

Lanthanum Chloride Solution

To 58.65 g of *lanthanum trioxide R* slowly add 100 mL of *hydrochloric acid R*. Heat to boiling. Allow to cool and dilute to 1000.0 mL with *water R*.

Lanthanum Nitrate Lanthanum trinitrate hexahydrate; $La(NO_3)_3,6H_2O$ = 433.0 (*10277-43-7*)

Colourless crystals, deliquescent, freely soluble in water.

Storage: in an airtight container.

Lanthanum Nitrate Solution

A 50 g/L solution of *lanthanum nitrate R*.

Lanthanum Trioxide Lanthanum oxide; La_2O_3 = 325.8 (*1312-81-8*)

An almost white, amorphous powder, practically insoluble in *water R*. It dissolves in dilute solutions of mineral acids and absorbs atmospheric carbon dioxide.

Calcium: maximum 5 ppm.

Lauric Acid Dodecanoic acid; $C_{12}H_{24}O_2$ = 200.3 (*143-07-7*)

White or almost white, crystalline powder, practically insoluble in water, freely soluble in ethanol (96 per cent).

mp: about 44 °C.

Lauric acid used in the assay of total fatty acids in Saw palmetto fruit (1848) complies with the following additional test.

Assay. Gas chromatography (*2.2.28*) as prescribed in the monograph *Saw palmetto fruit (1848)*.

Content: minimum 98 per cent, calculated by the normalisation procedure.

Lavandulol 2-Isopropenyl-5-methylhex-4-en-1-ol; $C_{10}H_{18}O$ = 154.2 (*498-16-8*)

Oily liquid with a characteristic odour.

Lavandulol used in gas chromatography complies with the following additional test.

Assay. Gas chromatography (*2.2.28*) as prescribed in the monograph *Lavender oil (1338)*.

Test solution. The substance to be examined.

Content: minimum 90.0 per cent, calculated by the normalisation procedure.

Lavandulyl Acetate 2-Isopropenyl-5-methylhex-4-en-1-yl acetate; $C_{12}H_{20}O_2$ = 196.3 (*25905-14-0*)

Colourless liquid with a characteristic odour.

Lavandulyl acetate used in gas chromatography complies with the following additional test.

Assay. Gas chromatography (*2.2.28*) as prescribed in the monograph *Lavender oil (1338)*.

Test solution. The substance to be examined.

Content: minimum 93.0 per cent, calculated by the normalisation procedure.

Lead(II) Acetate Lead di-acetate; Lead acetate; $C_4H_6O_4Pb,3H_2O$ = 379.3 (*6080-56-4*)

Colourless crystals, efflorescent, freely soluble in water, soluble in ethanol (96 per cent).

Lead Acetate Cotton

Immerse absorbent cotton in a mixture of 1 volume of *dilute acetic acid R* and 10 volumes of *lead acetate solution R*. Drain off the excess of liquid, without squeezing the cotton, by placing it on several layers of filter paper. Allow to dry in air.

Storage: in an airtight container.

Lead Acetate Paper

Immerse filter paper (about 80 g/m^2) in a mixture of 1 volume of *dilute acetic acid R* and 10 volumes of *lead acetate solution R*. Dry, then cut the paper into strips 15 mm by 40 mm.

Lead Acetate Solution

A 95 g/L solution of *lead acetate R* in *carbon dioxide-free water R*.

Lead(II) Nitrate Lead dinitrate; Lead nitrate; $Pb(NO_3)_2$ = 331.2 (*10099-74-8*)

White or almost white, crystalline powder or colourless crystals, freely soluble in water.

Lead Nitrate Solution

A 33 g/L solution of *lead nitrate R*.

Lead(IV) Oxide Lead dioxide; PbO_2 = 239.2 (*1309-60-0*)

Dark brown powder, evolving oxygen when heated, practically insoluble in water, soluble in hydrochloric acid with evolution of chlorine, soluble in dilute nitric acid in the presence of hydrogen peroxide, oxalic acid or other reducing agents, soluble in hot, concentrated alkali hydroxide solutions.

Lead Subacetate Solution Basic lead acetate solution; (*1335-32-6*)

Content: 16.7 per cent *m/m* to 17.4 per cent *m/m* of Pb (A_r 207.2) in a form corresponding approximately to the formula $C_8H_{14}O_{10}Pb_3$.

Dissolve 40.0 g of *lead acetate R* in 90 mL of *carbon dioxide-free water R*. Adjust the pH to 7.5 with *strong sodium hydroxide solution R*. Centrifuge and use the clear colourless supernatant solution.

The solution remains clear when stored in a well-closed container.

L-α-Lecithin from egg yolk L-α-Phosphatidylcholine; (*8002-43-5*)

General reagent grade of commerce.

Leiocarposide 2-(β-D-Glucopyranosyloxy)benzyl 3-(β-D-glucopyranosyloxy)-6-hydroxy-2-methoxybenzoate; 2-[[[3-(β-D-Glucopyranosyloxy)-6-hydroxy-2-methoxybenzoyl]oxy]methyl]phenyl-β-D-glucopyranoside; $C_{27}H_{34}O_{16}$ = 614.5 (*71953-77-0*)

White or almost white powder, soluble in water, freely soluble in methanol, slightly soluble in ethanol (96 per cent).

mp: 190 °C to 193 °C.

Lemon Oil See *Lemon oil (0620)*.

L-Leucine Leucine; (*61-90-5*)

See *Leucine (0771)*.

Levodopa (*59-92-7*)

See *Levodopa (0038)*.

(*Z*)-Ligustilide (3*Z*)-3-Butylidene-1,3,4,5-tetrahydroisobenzofuran-1-one; $C_{12}H_{14}O_2$ = 190.2 (*81944-09-4*)

Limonene D-Limonene; (+)-*p*-Mentha-1,8-diene; (*R*)-4-Isopropenyl-1-methylcyclohex-1-ene; $C_{10}H_{16}$ = 136.2 (*5989-27-5*)

Colourless liquid, practically insoluble in water, soluble in ethanol (96 per cent).

d_{20}^{20}: about 0.84.

n_D^{20}: 1.471 to 1.474.

$[\alpha]_D^{20}$: about + 124.

bp: 175 °C to 177 °C.

Limonene used in gas chromatography complies with the following additional test.

Assay. Gas chromatography (*2.2.28*) as prescribed in the monograph *Peppermint oil (0405)*.

Test solution. The substance to be examined.

Content: minimum 99.0 per cent, calculated by the normalisation procedure.

Linalool (*RS*)-3,7-Dimethylocta-1,6-dien-3-ol; Linalol; $C_{10}H_{18}O$ = 154.2 (*78-70-6*)

Mixture of two stereoisomers (licareol and coriandrol).

Liquid, practically insoluble in water.

d_{20}^{20}: about 0.860.

n_D^{20}: about 1.462.

bp: about 200 °C.

Linalol used in gas chromatography complies with the following test.

Assay. Gas chromatography (*2.2.28*) as prescribed in the monograph *Anise oil (0804)*.

Test solution. The substance to be examined.

Content: minimum 98.0 per cent, calculated by the normalisation procedure.

Linalyl Acetate (*RS*)-1,5-Dimethyl-1-vinylhex-4-enyl acetate; $C_{12}H_{20}O_2$ = 196.3 (*115-95-7*)

Colourless or slightly yellow liquid with a strong odour of bergamot and lavender.

d_{25}^{25}: 0.895 to 0.912.

n_D^{20}: 1.448 to 1.451.

bp: about 215 °C.

Linalyl acetate used in gas chromatography complies with the following additional test.

Assay. Gas chromatography (*2.2.28*) as prescribed in the monograph *Bitter-orange-flower oil (1175)*.

Test solution. The substance to be examined.

Content: minimum 95.0 per cent, calculated by the normalisation procedure.

Lindane γ-Hexachlorocyclohexane; $C_6H_6Cl_6$ = 290.8 (*58-89-9*)

For the monograph *Wool fat (0134)*, a suitable certified reference solution (10 ng/µL in cyclohexane) may be used.

Linoleic Acid (9*Z*,12*Z*)-Octadeca-9,12-dienoic acid; $C_{18}H_{32}O_2$ = 280.5 (*60-33-3*)

Colourless, oily liquid.

d_4^{20}: about 0.903.

n_D^{20}: about 1.470.

Linoleic acid used in the assay of total fatty acids in Saw palmetto fruit (1848) complies with the following additional test.

Assay. Gas chromatography (*2.2.28*) as prescribed in the monograph *Saw palmetto fruit (1848)*.

Content: minimum 98 per cent, calculated by the normalisation procedure.

Linolenic Acid (9*Z*,12*Z*,15*Z*)-Octadeca-9,12,15-trienoic acid; α-Linolenic acid; $C_{18}H_{30}O_2$ = 278.4 (*463-40-1*)

Colourless liquid, practically insoluble in water, soluble in organic solvents.

d_4^{20}: about 0.915.

n_D^{20}: about 1.480.

Linolenic acid used in the assay of total fatty acids in Saw palmetto fruit (1848) complies with the following additional test.

Assay. Gas chromatography (*2.2.28*) as prescribed in the monograph *Saw palmetto fruit (1848)*.

Content: minimum 98 per cent, calculated by the normalisation procedure.

Linolenyl Alcohol $C_{18}H_{32}O$ = 264.4 (*506-44-5*)

(9*Z*,12*Z*,15*Z*)-Octadeca-9,12,15-trien-1-ol. α-Linolenyl alcohol.

Content: minimum 96 per cent.

Linoleyl Alcohol (9*Z*,12*Z*)-Octadeca-9,12-dien-1-ol; $C_{18}H_{34}O$ = 266.5 (*506-43-4*)

Relative density: 0.830.

Content: minimum 85 per cent.

Linsidomine Hydrochloride 3-(Morpholin-4-yl) sydnonimine hydrochloride; 3-(Morpholin-4-yl)-1,2,3-oxadiazol-3-ium-5-aminide hydrochloride; $C_6H_{11}ClN_4O_2$ = 206.6 (*16142-27-1*)

White or almost white powder.

Lipase Solvent Maleate buffer solution pH 7.0

Dissolve 10.0 g of *sodium chloride R*, 6.06 g of *tris(hydroxymethyl)aminomethane R* and 4.90 g of *maleic anhydride R* in 900 mL of *water R*. Adjust the pH using a 170 g/L solution of *sodium hydroxide R*. Dilute to 1000.0 mL with *water R*.

Storage: at 2 °C to 8 °C; use within 3 days.

Liquid Scintillation Cocktail

Commercially available solution for the determination of radioactivity by liquid scintillation counting. It contains one or more fluorescent agents and mostly one or more emulsifying agents in a suitable organic solvent or mixture of organic solvents.

Liquid Scintillation Cocktail R1

To 1000 mL of *dioxan R*, add 0.3 g of *methylphenyloxazolylbenzene R*, 7 g of *diphenyloxazole R* and 100 g of *naphthalene R*.

Lithium Li = 6.94 (*7439-93-2*)

A soft metal whose freshly cut surface is silvery-grey. It rapidly tarnishes in contact with air. It reacts violently with water, yielding hydrogen and giving a solution of lithium hydroxide; soluble in methanol, yielding hydrogen and a solution of lithium methoxide; practically insoluble in light petroleum.

Storage: under light petroleum or liquid paraffin.

Lithium Carbonate Dilithium carbonate; Li_2CO_3 = 73.9 (*554-13-2*)

White or almost white, light powder, sparingly soluble in water, very slightly soluble in ethanol (96 per cent). A saturated solution at 20 °C contains about 13 g/L of Li_2CO_3.

Lithium Chloride LiCl = 42.39 (*7447-41-8*)

Crystalline powder or granules or cubic crystals, deliquescent, freely soluble in water, soluble in acetone and in ethanol (96 per cent). Aqueous solutions are neutral or slightly alkaline.

Storage: in an airtight container.

Lithium Hydroxide Lithium hydroxide monohydrate; $LiOH,H_2O$ = 41.96 (*1310-66-3*)

White or almost white, granular powder, strongly alkaline, it rapidly absorbs water and carbon dioxide, soluble in water, sparingly soluble in ethanol (96 per cent).

Storage: in an airtight container.

Lithium Metaborate, Anhydrous $LiBO_2$ = 49.75 (*13453-69-5*)

Lithium Sulfate Lithium sulphate; Li_2SO_4,H_2O = 128.0 (*10102-25-7*)

Colourless crystals, freely soluble in water, practically insoluble in ethanol (96 per cent).

Lithium Trifluoromethanesulfonate Lithium trifluoromethanesulphonate; CF_3LiO_3S = 156.0 (*33454-82-9*)

Litmus (*1393-92-6*)

Schultz No. 1386

Indigo-blue fragments prepared from various species of Rocella, Lecanora or other lichens, soluble in water, practically insoluble in ethanol (96 per cent).

Colour change: pH 5 (red) to pH 8 (blue).

Litmus Paper

Use *red litmus paper* or *blue litmus paper*, as appropriate.

Litmus Paper, Blue

Boil 10 parts of coarsely powdered *litmus R* for 1 h with 100 parts of *ethanol (96 per cent) R*. Decant the alcohol and add to the residue a mixture of 45 parts of *ethanol (96 per cent) R* and 55 parts of *water R*. After 2 days decant the clear liquid. Impregnate strips of filter paper with the solution and allow to dry.

Test for sensitivity. Immerse a strip measuring 10 mm by 60 mm in a mixture of 10 mL of *0.02 M hydrochloric acid* and 90 mL of *water R*. On shaking the paper turns red within 45 s.

Litmus Paper, Red

To the blue litmus extract, add *dilute hydrochloric acid R* dropwise until the blue colour becomes red. Impregnate strips of filter paper with the solution and allow to dry.

Test for sensitivity. Immerse a strip measuring 10 mm by 60 mm in a mixture of 10 mL of *0.02 M sodium hydroxide* and 90 mL of *water R*. On shaking the paper turns blue within 45 s.

Litmus Solution

Boil 25 g of coarsely powdered *litmus* with 100 mL of *ethanol (90%)* under a reflux condenser for 1 hour, discard the clear liquid and repeat this operation using two 75-mL quantities of *ethanol (90%)*. Digest the extracted litmus with 250 mL of *water* and filter.

Loganin Methyl (1*S*,4a*S*,6*S*,7*R*,7a*S*)-1-(β-D-glucopyranosyloxy)-6-hydroxy-7-methyl-1,4a,5,6,7,7a-hexahydrocyclopenta[*c*]pyran-4-carboxylate; $C_{17}H_{26}O_{10}$ = 390.4 (*18524-94-2*)

mp: 220 °C to 221 °C.

Longifolene (1*S*,3a*R*,4*S*,8a*S*)-4,8,8-Trimethyl-9-methylenedecahydro-1,4-methanoazulene; $C_{15}H_{24}$ = 204.4 (*475-20-7*)

Oily, colourless liquid, practically insoluble in water, miscible with ethanol (96 per cent).

d_4^{18}: 0.9319.

n_D^{20}: 1.5050.

$[\alpha]_D^{20}$: + 42.7.

bp: 254 °C to 256 °C.

Longifolene used in gas chromatography complies with the following additional test.

Assay. Gas chromatography (*2.2.28*) as prescribed in the monograph *Turpentine oil, Pinus pinaster type (1627)*.

Content: minimum 98.0 per cent, calculated by the normalisation procedure.

Lumiflavine 7,8,10-Trimethylbenzo[*g*]pteridine-2,4(3*H*,10*H*)-dione; $C_{13}H_{12}N_4O_2$ = 256.3 (*1088-56-8*)

Yellow powder or orange crystals, very slightly soluble in water, freely soluble in methylene chloride.

Luteolin 2-(3,4-Dihydroxyphenyl)-5,7-dihydroxy-4*H*-1-benzopyran-4-one; $C_{15}H_{10}O_6$ = 286.2 (*491-70-3*)

Luteolin-7-glucoside 2-(3,4-Dihydroxyphenyl)-7-(β-D-glucopyranosyloxy)-5-hydroxy-4*H*-1-benzopyran-4-one; $C_{21}H_{20}O_{11}$ = 448.4 (*5373-11-5*)

Yellow powder.

Absorbance (*2.2.25*). A solution in *methanol R* shows absorption maxima at 255 nm, 267 nm and 350 nm.

mp: about 247 °C.

Lysine Hydrochloride (2*S*)-2,6-Diaminohexanoic acid hydrochloride; $C_6H_{15}ClN_2O_2$ = 182.7 (*657-27-2*)

White or almost white, crystalline powder or colourless crystals, freely soluble in water, slightly soluble in ethanol (96 per cent).

Lysyl Endopeptidase (*78642-25-8*)

Achromobacter endoproteinase I. Lysyl bond specific proteinase (EC 3.4.21.50).

It belongs to the serine endopeptidase family. Initially isolated from *Achromobacter lyticus*. Enzymes with similar specificity are produced by *Lysobacter enzymogenes* (endoproteinase Lys-C) and *Pseudomonas aeruginosa* (Ps-1). It cleaves peptide bonds at the carboxy-terminal of both lysine residues and *S*-aminoethylcysteine residues with a high degree of specificity. 1 amidase unit (U) is the amount of enzyme that will produce 1 micromole of *p*-nitroaniline from *N*-benzoyl-DL-lysine-*p*-nitroaniline per minute at 30 °C at pH 9.5.

Lutetium Chloride Hexahydrate $LuCl_3,6H_2O$ = 389.4 (*15230-79-2*)

White to yellow, crystalline powder, freely soluble in water.

Macrogol 23 Lauryl Ether See *Macrogol lauryl ether (1124)*, the number of moles of ethylene oxide reacted per mole of lauryl alcohol being 23 (nominal value).

Macrogol 600 Polyethyleneglycol 600; (*25322-68-3*)

See *Macrogols (1444)*.

Macrogol 4000 Polyethyleneglycol 4000; (*25322-68-3*)

See *Macrogols (1444)*.

Macrogol 6000 Polyethyleneglycol 6000; (*25322-68-3*)

White or almost white solid with a waxy or paraffin-like appearance, very soluble in water and in methylene chloride, practically insoluble in ethanol (96 per cent), in fatty oils and in mineral oils.

Macrogol 20,000 2-Nitroterephthalate Polyethylene Glycol 20,000 2-Nitroterephthalate

Polyethyleneglycol 20 000 with embedded 2-nitroterephthalate groups.

Macrogol, Base-deactivated

Base-deactivated polyethyleneglycol.

Macrogol Cetostearyl Ether See *Macrogol cetostearyl ether (1123)*.

Macrogol, Polar-deactivated

Polar-deactivated polyethyleneglycol.

Magnesium Mg = 24.30 (*7439-95-4*)

Silver-white ribbon, turnings or wire, or a grey powder.

Magnesium Acetate Magnesium diacetate tetrahydrate; $C_4H_6MgO_4,4H_2O$ = 214.5 (*16674-78-5*)

Colourless crystals, deliquescent, freely soluble in water and in ethanol (96 per cent).

Storage: in an airtight container.

Magnesium Chloride (*7791-18-6*)

See *Magnesium chloride hexahydrate (0402)*.

Magnesium Nitrate Magnesium nitrate hexahydrate; $Mg(NO_3)_2,6H_2O$ = 256.4 (*13446-18-9*)

Colourless, clear crystals, deliquescent, very soluble in water, freely soluble in ethanol (96 per cent).

Storage: in an airtight container.

Magnesium Nitrate Solution

Dissolve 17.3 g of *magnesium nitrate R* in 5 mL of *water R* warming gently and add 80 mL of *ethanol (96 per cent) R*. Cool and dilute to 100.0 mL with the same solvent.

Magnesium Oxide (*1309-48-4*)

See *Light magnesium oxide (0040)*.

Magnesium Oxide, Heavy (*1309-48-4*)

See *Heavy magnesium oxide (0041)*.

Magnesium Oxide R1

Complies with the requirements prescribed for *magnesium oxide R* with the following modifications.

Arsenic (*2.4.2, Method A*): maximum 2 ppm.

Dissolve 0.5 g in a mixture of 5 mL of *water R* and 5 mL of *hydrochloric acid R1*.

Heavy metals (*2.4.8*): maximum 10 ppm.

Dissolve 1.0 g in a mixture of 3 mL of *water R* and 7 mL of *hydrochloric acid R1*. Add 0.05 mL of *phenolphthalein solution R* and *concentrated ammonia R* until a pink colour is obtained. Neutralise the excess of ammonia by the addition of *glacial acetic acid R*. Add 0.5 mL in excess and dilute to 20 mL with *water R*. Filter, if necessary. 12 mL of the solution complies with test A. Prepare the reference solution using a mixture of 5 mL of *lead standard solution (1 ppm Pb) R* and 5 mL of *water R*.

Iron (*2.4.9*): maximum 50 ppm.

Dissolve 0.2 g in 6 mL of *dilute hydrochloric acid R* and dilute to 10 mL with *water R*.

Magnesium Silicate for Pesticide Residue Analysis (*1343-88-0*)

Magnesium silicate for chromatography (60-100 mesh).

Magnesium Sulfate Magnesium sulphate; (*10034-99-8*)

See *Magnesium sulfate heptahydrate (0044)*.

Magneson Azo violet; 4-(4-nitrophenylazo)resorcinol; $C_{12}H_9N_3O_4$ = 259.2 (*74-39-5*)

Indicator grade of commerce.

When used for titration in non-aqueous media, it changes from orange (acidic) through pink (neutral) to blue (basic).

Magneson Reagent

A 0.1% w/v solution of *magneson* in a 1% w/v solution of *sodium hydroxide*.

Magneson Solution

A 0.2% w/v solution of *magneson* in *toluene*.

Magnolin $C_{23}H_{28}O_7$ = 416.5 (*31008-18-1*)

(3*S*,3a*R*,6*S*,6a*R*)-3-(3,4-Dimethoxyphenyl)-6-(3,4,5-trimethoxyphenyl)-1,3,3a,4,6,6a-hexahydrofuro[3,4-*c*]furan.

Magnolol 5,5′-Di(prop-2-enyl)biphenyl-2,2′-diol; 5,5′-Diallyl-2,2′-dihydroxybiphenyl; 5,5′-Di-2-propenyl-[1,1′-biphenyl]-2,2′-diol; $C_{18}H_{18}O_2$ = 266.3 (*528-43-8*)

Maize Oil See *Maize oil, refined (1342)*.

Makisterone A (22*R*)-2β,3β,14,20,22,25-Hexahydroxy-5β-ergost-7-en-6-one; $C_{28}H_{46}O_7$ = 494.7 (*20137-14-8*)

Malachite Green Basic green 4; $C_{23}H_{25}ClN_2$ = 364.9 (*123333-61-9*)

Schultz No. 754

Colour Index No. 42000

Green crystals with a metallic lustre, very soluble in water giving a bluish-green solution, soluble in ethanol (96 per cent) and in methanol.

Absorbance (*2.2.25*). A 0.01 g/L solution in *ethanol (96 per cent) R* shows an absorption maximum at 617 nm.

Malachite Green Solution

A 5 g/L solution of *malachite green R* in *anhydrous acetic acid R*.

Malathion $C_{10}H_{19}O_6PS_2$ = 330.3 (*121-75-5*)

bp: about 156 °C.

A suitable certified reference solution (10 ng/µL in iso-octane) may be used.

Maleic Acid (*Z*)-But-2-ene-1,4-dioic acid; (*110-16-7*)

See *Maleic acid (0365)*.

Maleic Anhydride Furan-2,5-dione; $C_4H_2O_3$ = 98.1 (*108-31-6*)

White or almost white crystals, soluble in water forming maleic acid, very soluble in acetone and in ethyl acetate, freely soluble in toluene, soluble in ethanol (96 per cent) with ester formation, very slightly soluble in light petroleum.

mp: about 52 °C.

Any residue insoluble in toluene does not exceed 5 per cent (maleic acid).

Maleic Anhydride Solution

Dissolve 5 g of *maleic anhydride R* in *toluene R* and dilute to 100 mL with the same solvent. Use within one month. If the solution becomes turbid, filter.

Malic Acid (*6915-15-7*)

See *Malic acid (2080)*.

Maltitol (*585-88-6*)

See *Maltitol (1235)*.

Maltol 3-Hydroxy-2-methyl-4*H*-pyran-4-one; $C_6H_6O_3$ = 126.1 (*118-71-8*)

White or almost white crystalline powder, soluble in hot water.

mp: 161 °C to 162 °C.

Maltose Monohydrate 4-*O*-α-D-glucopyranosyl-D-glucopyranose monohydrate; $C_{12}H_{22}O_{11},H_2O$ = 360.3 (*6363-53-7*)

Maltotriose α-D-Glucopyranosyl-(1→4)-α-D-glucopyranosyl-(1→4)-D-glucose; $C_{18}H_{32}O_{16}$ = 504.4 (*1109-28-0*)

White or almost white, crystalline powder, very soluble in water.

mp: about 134 °C.

Mandelic Acid 2-Hydroxy-2-phenylacetic acid; $C_8H_8O_3$ = 152.1 (*90-64-2*)

White crystalline flakes, soluble in water.

mp: 118 to 121 °C.

Manganese(IV) Oxide, Pre-washed Manganese(IV) Oxide Activated; MnO_2 = 86.9 (*1313-13-9*)

mp: about 535°.

General reagent grade of commerce.

Manganese(II) Sulfate Manganese sulphate; Manganese(II) sulphate; Manganese sulfate; $MnSO_4$, H_2O = 169.0 (*10034-96-5*)

Pale-pink, crystalline powder or crystals, freely soluble in water, practically insoluble in ethanol (96 per cent).

Loss on ignition: 10.0 per cent to 12.0 per cent, determined on 1.000 g at 500 ± 50 °C.

D-Mannitol Mannitol; (*69-65-8*)

See *Mannitol (0559)*.

D-Mannose Mannose; $C_6H_{12}O_6$ = 180.2 (*3458-28-4*)

white or almost white, crystalline powder or small crystals, very soluble in water, slightly soluble in anhydrous ethanol.

$[\alpha]_D^{20}$: + 13.7 + 14.7, determined on a 200 g/L solution in *water R* containing about 0.05 per cent of NH_3.

mp: about 132 °C, with decomposition.

Marrubiin (2a*S*,5a*S*,6*R*,7*R*,8a*R*,8b*R*)-6-[2-(Furan-3-yl) ethyl]-6-hydroxy-2a,5a,7-trimethyldecahydro-2*H*-naphtho [1,8-*bc*]furan-2-one; $C_{20}H_{28}O_4$ = 332.4 (*465-92-9*)

Colourless, microcrystalline powder.

Marrubiin used in liquid chromatography complies with the following additional test.

Assay. Liquid chromatography (*2.2.29*) as prescribed in the monograph *White horehound (1835)*.

Content: minimum 95.0 per cent, calculated by the normalisation procedure.

Mebendazole $C_{16}H_{13}N_3O_3$ = 295.3 (*31431-39-7*)

General reagent grade of commerce.

Meclozine Hydrochloride Meclozine dihydrochloride; (*1104-22-9*)

See *Meclozine dihydrochloride (0622)*.

Medronic Acid (*1984-15-2*)

See *Medronic acid for radiopharmaceutical preparations (2350)*.

Melamine 1,3,5-Triazine-2,4,6-triamine; $C_3H_6N_6$ = 126.1 (*108-78-1*)

A white or almost white, amorphous powder, very slightly soluble in water and in ethanol (96 per cent).

Menthofuran 3,9-Epoxy-*p*-mentha-3,8-diene; 3,6-Dimethyl-4,5,6,7-tetrahydro-benzofuran; $C_{10}H_{14}O$ = 150.2 (*17957-94-7*)

Slightly bluish liquid, very slightly soluble in water, soluble in ethanol (96 per cent).

d_{15}^{20}: about 0.965.

n_D^{20}: about 1.480.

$[\alpha]_D^{20}$: about + 93.

bp: 196 °C.

Menthofuran used in gas chromatography complies with the following additional test.

Assay. Gas chromatography (*2.2.28*) as prescribed in the monograph *Peppermint oil (0405)*.

Test solution. The substance to be examined.

Content: minimum 97.0 per cent, calculated by the normalisation procedure.

Menthol (–)-*p*-Menthan-3-ol (2216-51-5); (±)-*p*-menthan-3-ol; (*2216-51-5*)

See *Levomenthol (0619)* and *Racemic menthol (0623)*.

Menthol used in gas chromatography complies with the following additional test.

Assay. Gas chromatography (*2.2.28*) as prescribed in the related substances test included in the monograph *Racemic menthol (0623)*.

Content: minimum 98.0 per cent, calculated by the normalisation procedure.

Menthone (2*S*,5*R*)-2-Isopropyl-5-methylcyclohexanone; (–)-*trans*-*p*-Menthan-3-one; $C_{10}H_{18}O$ = 154.2 (*14073-97-3*)

Contains variable amounts of isomenthone.

Colourless liquid, very slightly soluble in water, very soluble in ethanol (96 per cent).

d_{20}^{20}: about 0.897.

n_D^{20}: about 1.450.

Menthone used in gas chromatography complies with the following additional test.

Assay. Gas chromatography (*2.2.28*) as prescribed in the monograph *Peppermint oil (0405)*.

Test solution. The substance to be examined.

Content: minimum 90.0 per cent, calculated by the normalisation procedure.

Menthyl Acetate (1*R*,2*S*,5*R*)-5-Methyl-2-(propan-2-yl) cyclohexyl acetate; $C_{12}H_{22}O_2$ = 198.3 (*2623-23-6*)

Colourless liquid, slightly soluble in water, miscible with ethanol (96 per cent).

d_{20}^{20}: about 0.92.

n_D^{20}: about 1.447.

bp: about 228 °C.

Menthyl acetate used in gas chromatography complies with the following additional test.

Assay. Gas chromatography (*2.2.28*) as prescribed in the monograph *Peppermint oil (0405)*.

Test solution. The substance to be examined.

Content: minimum 97.0 per cent, calculated by the normalisation procedure.

Mercaptoacetic Acid Thioglycollic acid; $C_2H_4O_2S$ = 92.1 (*68-11-1*)

Colourless liquid, miscible with water, soluble in ethanol (96 per cent).

2-Mercaptobenzimidazole 1*H*-benzimidazole-2-thiol; $C_7H_6N_2S$ = 150.2 (*583-39-1*)

mp: about 302 °C.

2-Mercaptoethanol C_2H_6OS = 78.1 (*60-24-2*)

Liquid, miscible with water.

d_{20}^{20}: about 1.116.

bp: about 157 °C.

6-Mercaptopurine Purine-6-thiol; Mercaptopurine; (*6112-76-1*)

See *Mercaptopurine monohydrate (0096)*.

Mercuric Oxide Yellow mercury(II) oxide; Mercury(II) Oxide, Yellow; HgO = 216.6 (*21908-53-2*)

A yellow to orange-yellow powder, practically insoluble in water and in ethanol (96 per cent).

Storage: protected from light.

Mercury Hg = 200.6 (*7439-97-6*)

d_{20}^{20}: about 13.5.

bp: about 357°.

Silver-white liquid, breaking into spherical globules which do not leave a metallic trace when rubbed on paper.

Mercury(II) Acetate Mercury diacetate; Mercuric acetate; $C_4H_6HgO_4$ = 318.7 (*1600-27-7*)

White or almost white crystals, freely soluble in water, soluble in ethanol (96 per cent).

Mercury(II) Acetate Solution Mercuric acetate solution

Dissolve 3.19 g of *mercuric acetate R* in *anhydrous acetic acid R* and dilute to 100 mL with the same acid. If necessary, neutralise the solution with *0.1 M perchloric acid* using 0.05 mL of *crystal violet solution R* as indicator.

Mercury(II) Chloride Mercuric chloride; (*7487-94-7*)

See *Mercuric chloride (0120)*.

Mercury(II) Chloride Solution Mercuric chloride solution

A 54 g/L solution of *mercuric chloride R*.

Mercury(II) Iodide Mercury di-iodide; Mercuric iodide; HgI_2 = 454.4 (*7774-29-0*)

Dense, scarlet, crystalline powder, slightly soluble in water, sparingly soluble in acetone and in ethanol (96 per cent), soluble in an excess of *potassium iodide solution R*.

Storage: protected from light.

Mercury(II) Nitrate Mercury dinitrate monohydrate; Mercuric nitrate; $Hg(NO_3)_2,H_2O$ = 342.6 (*7783-34-8*)

Colourless or slightly coloured crystals, hygroscopic, soluble in water in the presence of a small quantity of nitric acid.

Storage: in an airtight container, protected from light.

Mercury, Nitric Acid Solution of

Carefully dissolve 3 mL of *mercury* in 27 mL of *fuming nitric acid*. Dilute the solution with an equal volume of *water*.

Store protected from light, use within 2 months.

Mercury(II) Sulfate Solution Mercuric sulphate solution; Mercury(II) sulphate solution; Mercuric sulfate solution; (*7783-35-9*)

Dissolve 1 g of *mercuric oxide R* in a mixture of 20 mL of *water R* and 4 mL of *sulfuric acid R*.

Mercury(II) Thiocyanate Mercury di(thiocyanate); Mercuric thiocyanate; $Hg(SCN)_2$ = 316.7 (*592-85-8*)

White or almost white, crystalline powder, very slightly soluble in water, slightly soluble in ethanol (96 per cent), soluble in solutions of sodium chloride.

Mercury(II) Thiocyanate Solution Mercuric thiocyanate solution

Dissolve 0.3 g of *mercuric thiocyanate R* in *anhydrous ethanol R* and dilute to 100 mL with the same solvent.

Storage: use within 1 week.

Mesalazine (*89-57-6*)

See *Mesalazine (1699)*.

Mesityl Oxide 4-Methylpent-3-en-2-one; $C_6H_{10}O$ = 98.1 (*141-79-7*)

Colourless, oily liquid, soluble in 30 parts of water, miscible with most organic solvents.

d_{20}^{20}: about 0.858.

bp: 129 °C to 130 °C.

Metanil Yellow 4′-anilinoazobenzene-3-sulfonic acid sodium salt; $C_{18}H_{14}N_3NaO_3S$ = 375.4 (*587-98-4*)

Schultz No. 169

Colour Index No. 13065

A brownish-yellow powder, soluble in water and in ethanol (96 per cent).

Metanil Yellow Solution

A 1 g/L solution of *metanil yellow R* in *methanol R*.

Test for sensitivity. To 50 mL of *anhydrous acetic acid R* add 0.1 mL of the metanil yellow solution. Add 0.05 mL of *0.1 M perchloric acid*; the colour changes from pinkish-red to violet.

Colour change: pH 1.2 (red) to pH 2.3 (orange-yellow).

Metaphosphoric Acid $(HPO_3)_x$ (*37267-86-0*)

Glassy lumps or sticks containing a proportion of sodium metaphosphate, hygroscopic, very soluble in water.

Nitrates. Boil 1.0 g with 10 mL of *water R*, cool, add 1 mL of *indigo carmine solution R*, 10 mL of *nitrogen-free sulfuric acid R* and heat to boiling. The blue colour is not entirely discharged.

Reducing substances: maximum 0.01 per cent, calculated as H_3PO_3.

Dissolve 35.0 g in 50 mL of *water R*. Add 5 mL of a 200 g/L solution of *sulfuric acid R*, 50 mg of *potassium bromide R* and 5.0 mL of *0.02 M potassium bromate* and heat on a water-bath for 30 min. Allow to cool and add 0.5 g of *potassium iodide R*. Titrate the liberated iodine with *0.1 M sodium thiosulfate*, using 1 mL of *starch solution R* as indicator. Carry out a blank test.

1 mL of *0.02 M potassium bromate* is equivalent to 4.10 mg of H_3PO_3.

Storage: in an airtight container.

Methacrylic Acid 2-Methylprop-2-enoic acid; $C_4H_6O_2$ = 86.1 (*79-41-4*)

Colourless liquid.

n_D^{20}: about 1.431.

bp: about 160 °C.

mp: about 16 °C.

Methane CH_4 = 16 (*74-82-8*)

Content: minimum 99.0 per cent *V/V*.

Methane R1 CH_4 = 16 (*74-82-8*)

Content: minimum 99.995 per cent *V/V*.

Methanesulfonic Acid Methanesulphonic acid; CH_4O_3S = 96.1 (*75-75-2*)

Clear, colourless liquid, solidifying at about 20 °C, miscible with water, slightly soluble in toluene, practically insoluble in hexane.

d_{20}^{20}: about 1.48.

n_D^{20}: about 1.430.

Methanesulfonic Acid, Methanolic

Methanesulphonic acid, methanolic

Solutions of the requisite molarity may be obtained by dissolving the appropriate quantity of *methanesulfonic acid* in *methanol*.

Methanesulfonyl Chloride Methanesulphonyl chloride; CH_3ClO_2S = 114.6 (*124-63-0*)

Clear, colourless or slightly yellow liquid.

Content: minimum 99.0 per cent.

Density: 1.48 g/cm^3.

n_D^{20}: about 1.452.

bp: about 161 °C.

Methanol Methyl alcohol; CH_4O = 32.04 (*67-56-1*)

Clear, colourless, flammable liquid, miscible with water and with ethanol (96 per cent).

d_{20}^{20}: 0.791 to 0.793.

bp: 64 °C to 65 °C.When 'methanol' is followed by a percentage figure, an instruction to use methanol diluted with water to produce the specified percentage v/v of methanol is implied.

Methanol, Acidified

To 900 mL of *methanol* add 18 mL of *glacial acetic acid* and dilute to 1000 mL with *water*.

Methanol, Aldehyde-free

Dissolve 25 g of *iodine R* in 1 L of *methanol R* and pour the solution, with constant stirring, into 400 mL of *1 M sodium hydroxide*. Add 150 mL of *water R* and allow to stand for 16 h. Filter. Boil under a reflux condenser until the odour of

iodoform disappears. Distil the solution by fractional distillation.

Aldehydes and ketones: maximum 0.001 per cent.

Methanol, Anhydrous (*67-56-1*)

Treat 1000 mL of *methanol R* with 5 g of *magnesium R*. If necessary initiate the reaction by adding 0.1 mL of *mercuric chloride solution R*. When the evolution of gas has ceased, distil the liquid and collect the distillate in a dry container protected from moisture.

Water (*2.5.12*): maximum 0.3 g/L.

Methanol, Hydrochloric

Dilute 1.0 mL of *hydrochloric acid R1* to 100.0 mL with *methanol R*.

Methanol R1

Complies with the requirements prescribed for *methanol R* with the following additional requirement.

Absorbance (*2.2.25*): maximum 0.70 at 210 nm, 0.30 at 220 nm, 0.13 at 230 nm, 0.02 at 250 nm, 0.01 at 260 nm and higher wavelengths, determined using *water R* as compensation liquid.

Methanol R2

Complies with the requirements prescribed for *methanol R* and the following additional requirements.

Content: minimum 99.8 per cent.

Absorbance (2.2.25): maximum 0.17, determined at 225 nm using *water R* as the compensation liquid.

Methimazole 1-Methyl-1H-imidazole-2-thiol; Thiamazole; $C_4H_6N_2S$ = 114.2 (*60-56-0*)

White or almost white, crystalline powder, freely soluble in water, soluble in ethanol (96 per cent) and in methylene chloride.

mp: about 145 °C.

DL-Methionine (*59-51-8*)

See *DL-Methionine (0624)*.

L-Methionine (*63-68-3*)

See *Methionine (1027)*.

L-Methionine Sulfoxide (2*S*)-2-Amino-4-[(*RS*)-methylsulfinyl]butanoic acid; $C_5H_{11}NO_3S$ = 165.2 (*3226-65-1*)

(*RS*)-Methotrexate (*RS*)-2-[4-[[(2,4-diaminopteridin-6-yl)methyl]methylamino]benzoylamino]pentanedioic acid; $C_{20}H_{22}N_8O_5$ (*60388-53-6*)

Content: minimum 96.0 per cent.

mp: about 195 °C.

Methoxyazobenzene $C_{13}H_{12}NO$ = 212.2 (*2396-60-3*)

mp: about 55°.

Use a grade of commerce suitable for chromatographic separations.

Methoxychlor 1,1-(2,2,2-Trichloroethylidene)-bis(4-methoxybenzene); $C_{16}H_{15}Cl_3O_2$ = 345.7 (*72-43-5*)

Practically insoluble in water, freely soluble in most organic solvents.

bp: about 346 °C.

mp: 78 °C to 86 °C.

A suitable certified reference solution (10 ng/µL in iso-octane) may be used.

***trans*-2-Methoxycinnamaldehyde** $C_{10}H_{10}O_2$ = 162.2 (*60125-24-8*)

mp: 44 °C to 46 °C.

trans-2-Methoxycinnamaldehyde used in gas chromatography complies with the following additional test.

Assay. Gas chromatography (*2.2.28*) as prescribed in the monograph *Cassia oil (1496)*.

Content: minimum 96.0 per cent, calculated by the normalisation procedure.

2-Methoxyethanol Ethylene glycol monomethyl ether; $C_3H_8O_2$ = 76.1 (*109-86-4*)

Content: minimum 99.0 per cent.

Clear, colourless liquid, miscible with water, with acetone and with ethanol (96 per cent).

d_{20}^{20}: about 0.97.

n_D^{20}: about 1.403.

bp: about 125 °C.

(1*RS*)-1-(6-Methoxynaphthalen-2-yl)ethanol 6-Methoxy-α-methyl-2-naphthalenemethanol; $C_{13}H_{14}O_2$ = 202.3 (*77301-42-9*)

White or almost white powder.

mp: about 113 °C.

1-(6-Methoxynaphthalen-2-yl)ethanone 6′-Methoxy-2′-acetonaphthone; $C_{13}H_{12}O_2$ = 200.2 (*3900-45-6*)

White or almost white powder.

mp: about 108 °C.

6-Methoxy-2-naphthoic Acid 6-Methoxynaphthalene-2-carboxylic acid; $C_{12}H_{10}O_3$ = 202.2 (*2471-70-7*)

White or almost white, crystalline powder.

mp: 201 °C to 206 °C.

Methoxyphenylacetic Acid (*RS*)-2-Methoxy-2-phenylacetic acid; $C_9H_{10}O_3$ = 166.2 (*7021-09-2*)

White, crystalline powder or white or almost white crystals, sparingly soluble in water, freely soluble in ethanol (96 per cent).

mp: about 70 °C.

Methoxyphenylacetic Reagent

Dissolve 2.7 g of *methoxyphenylacetic acid R* in 6 mL of *tetramethylammonium hydroxide solution R* and add 20 mL of *anhydrous ethanol R*.

Storage: in a polyethylene container.

3-Methoxy-L-tyrosine $C_{10}H_{13}NO_4H_2O$ = 229.2 (*200630-46-2*)

Off-white or yellow powder.

Methyl Acetate $C_3H_6O_2$ = 74.1 (*79-20-9*)

Clear, colourless liquid, soluble in water, miscible with ethanol (96 per cent).

d_{20}^{20}: about 0.933.

n_D^{20}: about 1.361.

bp: 56 °C to 58 °C.

Methyl 4-acetylbenzoate $C_{10}H_{10}O_3$ = 178.2 (*3609-53-8*)

mp: about 94 °C.

Methyl 4-acetylbenzoate Reagent

Dissolve 0.25 g of *methyl 4-acetylbenzoate R* in a mixture of 5 mL of *sulfuric acid R* and 85 mL of cooled *methanol R*.

Methyl Acrylate Methyl prop-2-enoate; $C_4H_6O_2$ = 86.1 (*96-33-3*)

Clear, colourless liquid.

bp: about 80 °C.

Methylal Dimethoxymethane; Dioxapentane; Formaldehyde dimethyl acetal; Methylene dimethyl ether; $C_3H_8O_2$ = 76.1 (*109-87-5*)

Clear, colourless, volatile, flammable liquid, soluble in water and miscible with ethanol (96 per cent).

d_{20}^{20}: about 0.860.

n_D^{20}: about 1.354.

bp: about 41 °C.

Methylal used in gas chromatography complies with the following additional test.

Content: minimum 99.5 per cent, determined by gas chromatography.

Methylamine Hydrochloride Methanamine hydrochloride; CH_6ClN = 67.5 (*593-51-1*)

White or almost white powder.

Content: minimum 98.0 per cent.

Methyl 4-aminobenzoate $C_8H_9NO_2$ = 151.2 (*619-45-4*)

mp: 110 °C to 113 °C.

4-Methylaminophenol Sulfate $C_{14}H_{20}N_2O_6S$ = 344.4 (*55-55-0*)

Colourless crystals, very soluble in water, slightly soluble in ethanol (96 per cent).

mp: about 260 °C.

Methylaminophenol-Sulfite Reagent

Methylaminophenol-sulfite reagent

Dissolve 0.1 g of *4-methylaminophenol sulfate*, 20 g of *sodium metabisulfite* and 0.5 g of *anhydrous sodium sulfite* in sufficient *water* to produce 100 mL.

3-(Methylamino)-1-phenylpropan-1-ol $C_{10}H_{15}NO$ = 165.2 (*42142-52-9*)

White or almost white powder.

mp: 59 °C to 64 °C.

Methylamphetamine Hydrochloride Methamphetamine hydrochloride; $C_{10}H_{16}ClN$ = 185.7

General reagent grade of commerce.

Methyl Anthranilate Methyl 2-aminobenzoate; $C_8H_9NO_2$ = 151.2 (*134-20-3*)

Colourless crystals or a colourless or yellowish liquid, soluble in water, freely soluble in ethanol (96 per cent).

mp: 24 °C to 25 °C.

Methyl anthranilate used in gas chromatography complies with the following additional test.

Assay. Gas chromatography (*2.2.28*) as prescribed in the monograph *Bitter-orange-flower oil (1175)*.

Test solution. The substance to be examined.

Content: minimum 95.0 per cent, calculated by the normalisation procedure.

Methyl Arachidate Methyl eicosanoate; $C_{21}H_{42}O_2$ = 326.6 (*1120-28-1*)

Content: minimum 98.0 per cent, determined by gas chromatography (*2.4.22*).

White or yellow, crystalline mass, soluble in ethanol (96 per cent) and in light petroleum.

mp: about 46 °C.

Methyl Benzenesulfonate Ethyl benzenesulfonate; $C_7H_8O_3S$ = 172.2 (*80-18-2*)

Content: minimum 98.0 per cent.

Clear, colourless liquid.

bp: about 148 °C.

Methyl Benzoate Benzoic acid, methyl ester; $C_8H_8O_2$ = 136.2 (*93-58-3*)

Colourless liquid.

d_4^{20}: 1.088.

bp: about 200 °C.

3-Methylbenzothiazolin-2-one Hydrazone Hydrochloride 3-Methylbenzothiazol-2(3H)-one hydrazone hydrochloride monohydrate; Methylbenzothiazolone hydrazone hydrochloride; $C_8H_{10}ClN_3S,H_2O$ = 233.7 (*38894-11-0*)

Almost white or yellowish, crystalline powder.

mp: about 270 °C.

Suitability for determination of aldehydes. To 2 mL of *aldehyde-free methanol R* add 60 µL of a 1 g/L solution of *propionaldehyde R* in *aldehyde-free methanol R* and 5 mL of a 4 g/L solution of methylbenzothiazolone hydrazone hydrochloride. Mix. Allow to stand for 30 min. Prepare a blank omitting the propionaldehyde solution. Add 25.0 mL of a 2 g/L solution of *ferric chloride R* to the test solution and to the blank, dilute to 100.0 mL with *acetone R* and mix. The absorbance (*2.2.25*) of the test solution, measured at 660 nm using the blank as compensation liquid, is not less than 0.62.

(R)-(+)-α-Methylbenzyl Isocyanate (+)-(*R*)-α-Methylbenzyl isocyanate; (+)-[(1*R*)-1-Isocyanatoethyl] benzene; (+)-(1*R*)-1-Phenylethyl isocyanate; C_9H_9NO = 147.2 (*33375-06-3*)

Content: minimum 99.0 per cent.

Colourless liquid.

d_{20}^{20}: about 1.045.

n_D^{20}: about 1.513.

bp: 55 °C to 56 °C at 2.5 mm Hg.

Enantiomeric purity: minimum 99.5.

Storage: at a temperature of 2 °C to 8 °C.

(S)-(-)-α-Methylbenzyl Isocyanate (−)-(*S*)-α-Methylbenzyl isocyanate; (−)-[(1*S*)-1-Isocyanatoethyl] benzene; (−)-(1*S*)-1-Phenylethyl isocyanate; C_9H_9NO = 147.2 (*14649-03-7*)

Content: minimum 99.0 per cent.

Colourless liquid.

d_{20}^{20}: about 1.045.

n_D^{20}: about 1.514.

bp: 55 °C to 56 °C at 2.5 mm Hg.

Enantiomeric purity: minimum 99.5 per cent.

Storage: at a temperature of 2 °C to 8 °C.

NOTE: do not use the reagent if it is coloured.

1-Methylbiguanide Hydrochloride $C_3H_9N_5$ = 151.6 (*1674-62-0*)

2-Methylbutane Isopentane; C_5H_{12} = 72.2 (*78-78-4*)

Content: minimum 99.5 per cent of C_5H_{12}.

Very flammable colourless liquid.

d_{20}^{20}: about 0.621.

n_D^{20}: about 1.354.

bp: about 29 °C.

Water (2.5.12): maximum 0.02 per cent.

Residue on evaporation: maximum 0.0003 per cent.

Absorbance (2.2.25): maximum 0.30 at 210 nm, 0.07 at 220 nm, 0.01 at 240 nm and higher wavelengths, determined using *water R* as compensation liquid.

2-Methylbut-2-ene C_5H_{10} = 70.1 (*513-35-9*)

Very flammable liquid, practically insoluble in water, miscible with ethanol (96 per cent).

bp: 37.5 °C to 38.5 °C.

Methyl 4-(butylamino)benzoate $C_{12}H_{17}NO_2$ = 207.3 (*71839-12-8*)

White or almost white solid.

Content: minimum 99.9 per cent.

Methyl Caprate Methyl Decanoate

See *Methyl decanoate R*.

Methyl Caproate Methyl hexanoate; $C_7H_{14}O_2$ = 130.2 (*106-70-7*)

d_{20}^{20}: about 0.885.

n_D^{20}: about 1.405.

bp: 150 °C to 151 °C.

Methyl Caprylate Methyl octanoate; $C_9H_{18}O_2$ = 158.2 (*111-11-5*)

d_{20}^{20}: about 0.876.

n_D^{20}: about 1.417.

bp: 193 °C to 194 °C.

Methylcellulose 450 (*9004-67-5*)

See *Methylcellulose (0345)*.

Nominal viscosity: 450 mPa·s.

Methyl Cinnamate $C_{10}H_{10}O_2$ = 162.2 (*103-26-4*)

Colourless crystals practically insoluble in water, soluble in ethanol (96 per cent).

n_D^{20}: about 1.56.

bp: about 260 °C.

mp: 34 °C to 36 °C.

Methylcodeine $C_{19}H_{23}NO_3$ = 313.4 g/mol (*2859-16-7*)

Analytical reagent grade of commerce.

Content: minimum 95.0 per cent

Methylcyclohexane C_7H_{14} = 98.2 (*108-87-2*)

Methyl Decanoate Methyl Caprate; $C_{11}H_{22}O_2$ = 186.3 (*110-42-9*)

Content: minimum 99.0 per cent.

Clear, colourless or yellow liquid, soluble in light petroleum.

d_{20}^{20}: 0.871 to 0.876.

n_D^{20}: 1.425 to 1.426.

Methyl Docosanoate Methyl behenate; $C_{23}H_{46}O_2$ = 354.6 (*929-77-1*)

mp: 54 °C to 55 °C.

Methyldopa, Racemic $C_{10}H_{13}NO_4$,1½H_2O = 238.2

Mixture of equal volumes of (2*S*)- and (2*R*)-2-amino-3-(3,4-dihydroxyphenyl)-2-methylpropanoic acids.

3-*O*-Methyldopamine Hydrochloride 4-(2-Aminoethyl)-2-methoxyphenol hydrochloride; $C_9H_{14}ClNO_2$ = 203.7 (*1477-68-5*)

mp: 213 °C to 215 °C.

4-*O*-Methyldopamine Hydrochloride 5-(2-Aminoethyl)-2-methoxyphenol hydrochloride; $C_9H_{14}ClNO_2$ = 203.7 (*645-33-0*)

mp: 207 °C to 208 °C.

Methyl Eicosenoate Methyl (11*Z*)-eicos-11-enoate; $C_{21}H_{40}O_2$ = 324.5 (*2390-09-2*)

Methylenebisacrylamide *N,N'*-Methylenebispropenamide; $C_7H_{10}N_2O_2$ = 154.2 (*110-26-9*)

Fine, white or almost white powder, slightly soluble in water, soluble in ethanol (96 per cent).

mp: 300 °C, with decomposition.

4,4'-Methylenebis-N,N-dimethylaniline Tetramethyldiaminodiphenylmethane; $C_{17}H_{22}N_2$ = 254.4 (*101-61-1*)

White or bluish-white crystals or leaflets, practically insoluble in water, slightly soluble in ethanol (96 per cent), soluble in mineral acids.

mp: about 90 °C.

4,4'-Methylenebis-N,N-dimethylaniline Reagent Tetramethyldiaminodiphenylmethane reagent

Solution A. Dissolve 2.5 g of *tetramethyldiaminodiphenylmethane R* in 10 mL of *glacial acetic acid R* and add 50 mL of *water R*.

Solution B. Dissolve 5 g of *potassium iodide R* in 100 mL of *water R*.

Solution C. Dissolve 0.30 g of *ninhydrin R* in 10 mL of *glacial acetic acid R* and add 90 mL of *water R*.

Mix solution A, solution B and 1.5 mL of solution C.

Methylene Blue 3,7-Dimethylaminophenothiazin-5-ium chloride; It occurs in different hydrated forms and may contain up to 22 per cent of water; $C_{16}H_{18}ClN_3S$, xH_2O = 319.9 for the anhydrous substance (*122965-43-9*)

Schultz No. 1038

Colour Index No. 52015

Dark-green or bronze, crystalline powder, freely soluble in water, soluble in ethanol (96 per cent).

Methylene Blue Solution

Dissolve 3 mg of *methylene blue R*, 1.2 g of *sulfuric acid R* and 5.0 g of *anhydrous sodium sulfate R* in 100 mL of *water R*.

Methyl Erucate Methyl cis-13-docosenoate; $C_{23}H_{44}O_2$ = 352.6 (*1120-34-9*)

d_{20}^{20}: about 0.871.

n_D^{20}: about 1.456.

3-*O*-Methylestrone 3-Methoxy-1,3,5(10)-estratrien-17-one; $C_{19}H_{24}O_2$ = 284.4 (*1624-62-0*)

White to yellowish-white powder.

$[\alpha]_D^{20}$: about + 157.

mp: about 173 °C.

Methyl Ethyl Ketone Ethyl methyl ketone; 2-Butanone; C_4H_8O = 72.1 (*78-93-3*)

Clear, colourless, flammable liquid, very soluble in water, miscible with ethanol (96 per cent).

d_{20}^{20}: about 0.81.

bp: 79 °C to 80 °C.

Methyleugenol 1,2-Dimethoxy-4-prop-2-enylbenzene; $C_{11}H_{14}O_2$ = 178.2 (*93-15-2*)

Methyleugenol used in gas chromatography complies with the following additional test.

Assay. Gas chromatography (*2.2.28*) as prescribed in the monograph *Niaouli oil, cineole type (2468)*.

Content: minimum 97.0 per cent, calculated by the normalisation procedure.

***N*-Methylglucamine** Meglumine; $C_7H_{17}NO_5$ = 195.2 (*6284-40-8*)

mp: about 130°.

General reagent grade of commerce.

Methyl Green CI 42585; basic blue 20, 4-[[4-(Dimethylamino)phenyl][4-(dimethyliminio)cyclohexa-2,5-dienylidene]-methylphenyl]trimethylammonium dichloride; $C_{26}H_{33}Cl_2N_3$ = 458.5 (*7114-03-6*)

Green powder, soluble in water, soluble in sulfuric acid giving a yellow solution turning green on dilution with water.

Methyl Green-Iodomercurate Paper

Immerse thin strips of suitable filter paper in a 4% w/v solution of *methyl green* and allow to dry in air. Immerse the strips for 1 hour in a solution containing 14% w/v of *potassium iodide* and 20% w/v of *mercuric iodide*. Wash with *distilled water* until the washings are practically colourless and allow to dry in air.

Store protected from light; use within 48 hours.

Methyl 4-Hydroxybenzoate *(99-76-3)*

See *Methyl parahydroxybenzoate R*.

1-Methylimidazole 1-Methyl-1*H*-imidazole; $C_4H_6N_2$ = 82.1 *(616-47-7)*

Colourless or slightly yellowish liquid.

n_D^{20}: about 1.495.

bp: 195 °C to 197 °C.

Storage: in an airtight container, protected from light.

1-Methylimidazole R1

Complies with the requirements prescribed for *1-methylimidazole R* with the following additional requirement.

Content: minimum 95.0 per cent.

2-Methylimidazole $C_4H_6N_2$ = 82.1 *(693-98-1)*

White or almost white, crystalline powder.

mp: about 145 °C.

Methyl Isobutyl Ketone, Water-saturated

Shake *methyl isobutyl ketone R* with *water R* prior to use.

Methyl Laurate Methyl dodecanoate; $C_{13}H_{26}O_2$ = 214.4 *(111-82-0)*

Content: minimum 98.0 per cent, determined by gas chromatography *(2.4.22)*.

Colourless or yellow liquid, soluble in ethanol (96 per cent) and in light petroleum.

d_{20}^{20}: about 0.87.

n_D^{20}: about 1.431.

mp: about 5 °C.

Methyl Lignocerate Methyl tetracosanoate; $C_{25}H_{50}O_2$ = 382.7 *(2442-49-1)*

Flakes.

mp: about 58 °C.

Methyl Linoleate Methyl (9*Z*,12*Z*)-octadeca-9,12-dienoate; $C_{19}H_{34}O_2$ = 294.5 *(112-63-0)*

d_{20}^{20}: about 0.888.

n_D^{20}: about 1.466.

bp: 207 °C to 208 °C.

Methyl Linolenate Methyl (9*Z*,12*Z*,15*Z*)-octadeca-9,12,15-trienoate; Methyl α-linolenate; $C_{19}H_{32}O_2$ = 292.5 *(301-00-8)*

d_{20}^{20}: about 0.901.

n_D^{20}: about 1.471.

bp: about 207 °C.

Methyl γ-linolenate Methyl (6*Z*,9*Z*,12*Z*)-octadeca-6,9,12-trienoate; $C_{19}H_{32}O_2$ = 292.5 *(16326-32-2)*

Content: minimum 99.0 per cent, determined by gas chromatography.

Methyl Margarate Methyl heptadecanoate; $C_{18}H_{36}O_2$ = 284.5 *(1731-92-6)*

White or almost white powder.

mp: 32 °C to 34 °C.

Methyl margarate used in the assay of total fatty acids in Saw palmetto fruit (1848) complies with the following additional test.

Assay. Gas chromatography *(2.2.28)* as prescribed in the monograph *Saw palmetto fruit (1848)*.

Content: minimum 97 per cent, calculated by the normalisation procedure.

Methyl Methacrylate Methyl 2-methylprop-2-enoate; $C_5H_8O_2$ = 100.1 *(80-62-6)*

Colourless liquid.

n_D^{20}: about 1.414.

bp: about 100 °C.

mp: about −48 °C.

It contains a suitable stabilising reagent.

Methyl Methanesulfonate $C_2H_6O_3S$ = 110.1 *(66-27-3)*

Clear, colourless or slightly yellow liquid.

Content: minimum 99.0 per cent.

Density: about 1.3 g/cm^3 (25 °C).

n_D^{20}: about 1.414.

bp: about 202 °C.

Methyl 2-Methoxybenzoate $C_9H_{10}O_3$ = 166.2 *(606-45-1)*

Colourless liquid.

Methyl 4-Methoxybenzoate $C_9H_{10}O_3$ = 166.2 *(121-98-2)*

White or almost white powder.

Methyl *N*-methylanthranilate Methyl 2-(methylamino) benzoate; $C_9H_{11}NO_2$ = 165.2 *(85-91-6)*

Pale yellow liquid.

d_4^{20}: about 1.128.

n_D^{20}: about 1.579.

bp: 255 °C to 258 °C.

Methyl N-methylanthranilate used in gas chromatography complies with the following additional test.

Assay. Gas chromatography *(2.2.28)* as prescribed in the monograph *Mandarin oil (2355)*.

Test solution. The substance to be examined.

Content: minimum 97 per cent, calculated by the normalisation procedure.

Methyl Myristate Methyl tetradecanoate; $C_{15}H_{30}O_2$ = 242.4 *(124-10-7)*

Content: minimum 98.0 per cent, determined by gas chromatography *(2.4.22)*.

Colourless or slightly yellow liquid, soluble in ethanol (96 per cent) and in light petroleum.

d_{20}^{20}: about 0.87.

n_D^{20}: about 1.437.

mp: about 20 °C.

2-Methyl-1,4-naphthoquinone Menadione; *(58-27-5)*

See *Menadione (0507)*.

Methyl Nervonate *(2733-88-2)*

See *Tetracos-15-enoic acid methyl ester R*.

2-Methyl-5-nitroimidazole $C_4H_5N_3O_2$ = 127.1 *(88054-22-2)*

mp: 252° to 254°.

White to light yellow powder.

Content, minimum 98.0%.

2-Methyl-2-nitropropane-1,3-diol 2-Nitro-2-methyl-1,3-propanediol; $C_4H_9NO_4$ = 135.1 (*77-49-6*)

mp: about 148°.

General reagent grade of commerce.

Methyl Oleate Methyl (9*Z*)-octadec-9-enoate; $C_{19}H_{36}O_2$ = 296.4 (*112-62-9*)

Content: minimum 98.0 per cent, determined by gas chromatography (*2.4.22*).

Colourless or slightly yellow liquid, soluble in ethanol (96 per cent) and in light petroleum.

d_{20}^{20}: about 0.88.

n_D^{20}: about 1.452.

Methylophiopogonanone A (3*R*)-3-[(1,3-Benzodioxol-5-yl)methyl]-2,3-dihydro-5,7-dihydroxy-6,8-dimethyl-4*H*-1-benzopyran-4-one; $C_{19}H_{18}O_6$ = 342.3 (*74805-92-8*)

Methyl Orange Sodium 4′-(dimethylamino)azobenzene-4-sulfonate; $C_{14}H_{14}N_3NaO_3S$ = 327.3 (*547-58-0*)

Schultz No. 176

Colour Index No. 13025

Orange-yellow, crystalline powder, slightly soluble in water, practically insoluble in ethanol (96 per cent).

Methyl Orange Mixed Solution

Dissolve 20 mg of *methyl orange R* and 0.1 g of *bromocresol green R* in 1 mL of *0.2 M sodium hydroxide* and dilute to 100 mL with *water R*.

Colour change: pH 3.0 (orange) to pH 4.4 (olive-green).

Methyl Orange Solution

Dissolve 0.1 g of *methyl orange R* in 80 mL of *water R* and dilute to 100 mL with *ethanol (96 per cent) R*.

Test for sensitivity. A mixture of 0.1 mL of the methyl orange solution and 100 mL of *carbon dioxide-free water R* is yellow. Not more than 0.1 mL of *1 M hydrochloric acid* is required to change the colour to red.

Colour change: pH 3.0 (red) to pH 4.4 (yellow).

Methyl Orange-Xylene Cyanol FF Solution

Dissolve 0.1 g of *methyl orange* and 0.26 g of *xylene cyanol FF* in 50 mL of *ethanol (96%)* and add sufficient *water* to produce 100 mL.

Methyl Palmitate Methyl hexadecanoate; $C_{17}H_{34}O_2$ = 270.5 (*112-39-0*)

Content: minimum 98.0 per cent, determined by gas chromatography (*2.4.22*).

White or yellow, crystalline mass, soluble in ethanol (96 per cent) and in light petroleum.

mp: about 30 °C.

Methyl Palmitoleate Methyl cis-9-hexadecenoate; $C_{17}H_{32}O_2$ = 268.4 (*1120-25-8*)

d_{20}^{20}: about 0.876.

n_D^{20}: about 1.451.

Methyl Parahydroxybenzoate (*99-76-3*)

See *Methyl parahydroxybenzoate (0409)*.

Methyl Pelargonate Methyl nonanoate; $C_{10}H_{20}O_2$ = 172.3 (*1731-84-6*)

Clear, colourless liquid.

d_4^{20}: about 0.873.

n_D^{20}: about 1.422.

bp: 91 °C to 92 °C.

Methyl pelargonate used in the assay of total fatty acids in Saw palmetto fruit (1848) complies with the following additional test.

Assay. Gas chromatography (*2.2.28*) as prescribed in the monograph *Saw palmetto fruit (1848)*.

Content: minimum 98 per cent, calculated by the normalisation procedure.

2-Methylpentane C_6H_{14} = 86.2 (*107-83-5*)

Isohexane.

d_{20}^{20}: about 0.653.

bp: about 60.0 °C.

Colourless, flammable liquid, practically insoluble in water, miscible with anhydrous ethanol.

3-Methylpentan-2-one sec-Butyl methyl ketone; $C_6H_{12}O$ = 100.2 (*565-61-7*)

Colourless, flammable liquid.

d_{20}^{20}: about 0.815.

n_D^{20}: about 1.400.

bp: about 118 °C

4-Methylpentan-2-ol 4-Methyl-2-pentanol; $C_6H_{14}O$ = 102.2 (*108-11-2*)

Clear, colourless, volatile liquid.

d_4^{20}: about 0.802.

n_D^{20}: about 1.411.

bp: about 132 °C.

4-Methylpentan-2-one Methyl isobutyl ketone; $C_6H_{12}O$ = 100.2 (*108-10-1*)

Clear, colourless liquid, slightly soluble in water, miscible with most organic solvents.

d_{20}^{20}: about 0.80.

bp: about 115 °C.

Distillation range (*2.2.11*). Distil 100 mL. The range of temperature of distillation from 1 mL to 95 mL of distillate does not exceed 4.0 °C.

Residue on evaporation: maximum 0.01 per cent, determined by evaporating on a water-bath and drying at 100-105 °C.

4-Methylpentan-2-one R1 Methyl isobutyl ketone R1

Shake 50 mL of freshly distilled *methyl isobutyl ketone R* with 0.5 mL of *hydrochloric acid R1* for 1 min. Allow the phases to separate and discard the lower phase. Prepare immediately before use.

4-Methylpentan-2-one R3 Methyl isobutyl ketone R3

Complies with the requirements for *methyl isobutyl ketone R* and with the following limits.

Cr: maximum 0.02 ppm.

Cu: maximum 0.02 ppm.

Pb: maximum 0.1 ppm.

Ni: maximum 0.02 ppm.

Sn: maximum 0.1 ppm.

4-Methylphenazone 1,5-Dimethyl-2-(4-methylphenyl)-1,2-dihydro-3*H*-pyrazol-3-one; $C_{12}H_{14}N_2O$ = 202.3 (*56430-08-1*)

Methylphenyloxazolylbenzene 1,4-Bis[2-(4-methyl-5-phenyl)oxazolyl]benzene; $C_{26}H_{20}N_2O_2$ = 392.5 (*3073-87-8*)

Fine, greenish-yellow powder with a blue fluorescence or small crystals, soluble in ethanol (96 per cent), sparingly soluble in xylene.

mp: about 233 °C.

Methylphenyloxazolylbenzene used for liquid scintillation is of a suitable analytical grade.

1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine MPTP; $C_{12}H_{15}N$ = 173.3 (*28289-54-5*)

White or almost white, crystalline powder, slightly soluble in water.

mp: about 41 °C.

N-Methylpiperazine 1-Methylpiperazine; Methylpiperazine; $C_5H_{12}N_2$ = 100.2 (*109-01-3*)

Colourless liquid, miscible with water and with ethanol (96 per cent).

d_{20}^{20}: about 0.90.

n_D^{20}: about 1.466.

bp: about 138 °C.

4-(4-Methylpiperidin-1-yl)pyridine 4-(4-Methylpiperidino)pyridine; $C_{11}H_{16}N_2$ = 176.3 (*80965-30-6*)

Clear liquid.

n_D^{20}: about 1.565.

Methylpolysiloxane

Polysiloxane substituted with 100 per cent of methyl groups.

Methylprednisolone 11β,17,21-Trihydroxy-6α-methylpregna-1,4-diene-3,20-dione; $C_{22}H_{30}O_5$ = 374.5 (*83-43-2*)

White or almost white, crystalline powder.

2-Methylpropan-1-ol Isobutyl alcohol; 2-Methylpropanol; $C_4H_{10}O$ = 74.1 (*78-83-1*)

Clear colourless liquid, soluble in water, miscible with ethanol (96 per cent).

d_{20}^{20}: about 0.80.

n_D^{15}: 1.397 to 1.399.

bp: about 107 °C.

Distillation range (*2.2.11*). Not less than 96 per cent distils between 107 °C and 109 °C.

2-Methylpropan-2-ol *tert*-Butyl alcohol; 1,1-Dimethyl ethyl alcohol; 2-Methyl-2-propanol; $C_4H_{10}O$ = 74.1 (*75-65-0*)

Clear, colourless liquid or crystalline mass, soluble in water, miscible with ethanol (96 per cent).

Freezing point (*2.2.18*): about 25 °C.

Distillation range (*2.2.11*). Not less than 95 per cent distils between 81 °C and 83 °C.

(15R)-15-Methylprostaglandin $F_{2\alpha}$ (5*Z*)-7-[(1*R*,2*R*,3*R*,5*S*)-3,5-Dihydroxy-2-[(1*E*)-(3*R*)-3-hydroxy-3-methyloct-1-enyl]cyclopentyl]hept-5-enoic acid; $C_{21}H_{36}O_5$ = 368.5 (*35864-81-4*)

Available as a 10 g/L solution in *methyl acetate R*.

Storage: at a temperature below −15 °C.

2-Methylpyridine C_6H_7N = 93.1 (*109-06-8*)

Colourless or light yellow liquid.

Content: minimum 97.5 per cent.

5-Methylpyridin-2-amine 6-Amino-3-picoline; $C_6H_8N_2$ = 108.1 (*1603-41-4*)

White or yellow crystals or crystalline powder.

mp: about 76 °C.

5-Methylpyridin-2(1*H*)-one C_6H_7NO = 109.1 (*1003-68-5*)

White or almost white powder, soluble in anhydrous ethanol and in methanol.

mp: about 181 °C.

Storage: at a temperature of 2 °C to 8 °C.

***N*-Methylpyrrolidine** $C_5H_{11}N$ = 85.2 (*120-94-5*)

Content: minimum 97.0 per cent.

bp: about 80 °C.

***N*-Methylpyrrolidone** 1-Methylpyrrolidin-2-one; C_5H_9NO = 99.1 (*872-50-4*)

d_{20}^{20}: about 1.028.

bp: about 202 °C.

mp: about −24 °C.

Methyl Red 2-(4-Dimethylamino-phenylazo)benzoic acid; $C_{15}H_{15}N_3O_2$ = 269.3 (*493-52-7*)

Schultz No. 250

Colour Index No. 13020

Dark-red powder or violet crystals, practically insoluble in water, soluble in ethanol (96 per cent).

Methyl Red Mixed Solution

Dissolve 0.1 g of *methyl red R* and 50 mg of *methylene blue R* in 100 mL of *ethanol (96 per cent) R*.

Colour change: pH 5.2 (red-violet) to pH 5.6 (green).

Methyl Red Solution

Dissolve 50 mg of *methyl red R* in a mixture of 1.86 mL of *0.1 M sodium hydroxide* and 50 mL of *ethanol (96 per cent) R* and dilute to 100 mL with *water R*.

Test for sensitivity. To 0.1 mL of the methyl red solution add 100 mL of *carbon dioxide-free water R* and 0.05 mL of *0.02 M hydrochloric acid*. The solution is red. Not more than 0.1 mL of *0.02 M sodium hydroxide* is required to change the colour to yellow.

Colour change: pH 4.4 (red) to pH 6.0 (yellow).

Methyl Salicylate (*119-36-8*)

See *Methyl salicylate (0230)*

When used in the Assay of Methyl Salicylate preparations, use a grade containing not less than 99.0% of $C_8H_8O_3$.

Methyl Stearate Methyl octadecanoate; $C_{19}H_{38}O_2$ = 298.5 (*112-61-8*)

Content: minimum 98.0 per cent, determined by gas chromatography (*2.4.22*).

White or yellow, crystalline mass, soluble in ethanol (96 per cent) and in light petroleum.

mp: about 38 °C.

Methyl Thymol Blue Tetrasodium 2,2′,2″,2‴-[3H-2,1-benzoxathiol-3-ylidenebis[[6-hydroxy-2-methyl-5-(1-methylethyl)-3,1-phenylene]methylenenitrilo]]tetraacetate S,S-dioxide; Methylthymol blue; $C_{37}H_{40}N_2Na_4O_{13}S$ = 845 (*1945-77-3*)

Produces a blue colour with calcium in alkaline solution.

Methylthymol Blue Mixture

A mixture of 1 part of *methylthymol blue R* and 100 parts of *potassium nitrate R*.

Methyl Toluenesulfonate Methyl 4-methylbenzenesulfonate; Methyl tosilate; $C_8H_{10}O_3S$ = 186.2 (*80-48-8*)

Content: minimum 97.0 per cent.

Density: about 1.234 g/mL (25 °C).

bp: about 292 °C.

mp: 25 °C to 28 °C.

***N*-Methyl-*m*-toluidine** *N*,3-Dimethylaniline; *N*,3-Dimethylbenzenamine; Methyl-*m*-tolylamine; $C_8H_{11}N$ = 121.2 (*696-44-6*)

Content: minimum 97 per cent.

Methyl Tricosanoate Tricosanoic acid methyl ester; $C_{24}H_{48}O_2$ = 368.6 (*2433-97-8*)

Content: minimum 99.0 per cent.

White or almost white crystals, practically insoluble in water, soluble in hexane.

mp: 55 °C to 56 °C.

Methyl Tridecanoate $C_{14}H_{28}O_2$ = 228.4 (*1731-88-0*)

Colourless or slightly yellow liquid, soluble in ethanol (96 per cent) and in light petroleum.

d_{20}^{20}: about 0.86.

n_D^{20}: about 1.441.

mp: about 6 °C.

Methyl 3,4,5-Trimethoxybenzoate $C_{11}H_{14}O_5$ = 226.23 (*1916-07-0*)

***N*-Methyltrimethylsilyl-trifluoroacetamide** 2,2,2-Trifluoro-*N*-methyl-*N*-(trimethylsilyl)acetamide; $C_6H_{12}F_3NOSi$ = 199.3 (*24589-78-4*)

n_D^{20}: about 1.380.

bp: 130 °C to 132 °C.

Minocycline Hydrochloride

See *Minocycline hydrochloride (1030)*.

Molecular Sieve (*70955-01-0*)

Molecular sieve composed of sodium aluminosilicate. It is available as beads or powder with a pore size of 0.4 nm.

When reused, it is recommended that the molecular sieve be regenerated according to the manufacturer's instructions.

Molecular Sieve for Chromatography

Molecular sieve composed of sodium aluminosilicate. The pore size is indicated after the name of the reagent in the tests where it is used. If necessary, the particle size is also indicated.

Molybdenum(VI) Oxide Molybdenum trioxide; MoO_3 = 143.9 (*1313-27-5*)

Analytical reagent grade of commerce.

Molybdovanadic Reagent

In a 150 mL beaker, mix 4 g of finely powdered *ammonium molybdate R* and 0.1 g of finely powdered *ammonium vanadate R*. Add 70 mL of *water R* and grind the particles using a glass rod. A clear solution is obtained within a few minutes. Add 20 mL of *nitric acid R* and dilute to 100 mL with *water R*.

Monodocosahexaenoin Monoglyceride of docosahexaenoic acid (C22:6); Glycerol monodocosahexaenoate; (*all-Z*)-Docosa-4,7,10,13,16,19-hexaenoic acid, monoester with propane-1,2,3-triol; $C_{25}H_{38}O_4$ = 402.6 (*124516-13-8*)

The reagent from Nu-Chek Prep, Inc. has been found suitable.

Mordant Black 11 Eriochrome black T; Solochrome black; $C_{20}H_{12}N_3NaO_7S$ = 461.4 (*1787-61-7*)

Schultz No. 241

Colour Index No. 14645

Brownish-black powder, soluble in water and in ethanol (96 per cent).

Storage: in an airtight container, protected from light.

Mordant Black 11 Mixed Triturate

A mixture of 1 g of *mordant black 11*, 0.4 g of *methyl orange* and 100 g of *sodium chloride*.

Complies with the following test.

Sensitivity to magnesium Dissolve 50 mg in 100 mL of *water*; a brown colour is produced. Add 0.3 mL of 6M *ammonia*; the colour changes to pale green. Add 0.1 mL of a 1.0% w/v solution of *magnesium sulfate*; the colour changes to red.

Mordant Black 11 Solution

A 0.1% w/v solution of *mordant black 11* in *ethanol (96%)*.

Mordant Black 11 Triturate

Mix 1 g of *mordant black 11 R* with 99 g of *sodium chloride R*.

Test for sensitivity. Dissolve 50 mg in 100 mL of *water R*. The solution is brownish-violet. On addition of 0.3 mL of *dilute ammonia R1* the solution turns blue. On the subsequent addition of 0.1 mL of a 10 g/L solution of *magnesium sulfate R*, it turns violet.

Storage: in an airtight container, protected from light.

Mordant Black 11 Triturate R1

Mix 1.0 g of *mordant black 11 R*, 0.4 g of *methyl orange R* and 100 g of *sodium chloride R*.

Mordant Blue 3 Chromoxane cyanine; (*3564-18-9*)

Colour Index No. 43820

General reagent grade of commerce.

A dark red or reddish brown powder.

Produces an intense purple colour with aluminium in weakly acidic solutions. When metal ions are absent, for example, in the presence of an excess of disodium edetate, the solution is pale pink.

Morphine, Anhydrous

Add 5M *ammonia*, in slight excess, to a solution of *morphine sulfate* in *water*, wash the precipitated morphine with *water* until free from ammonium salts and dry at 110°.

Morphine Hydrochloride See *Morphine hydrochloride (0097)*.

Morpholine Tetrahydro-1,4-oxazine; C_4H_9NO = 87.1 (*110-91-8*)

Colourless, hygroscopic liquid, flammable, soluble in water and in ethanol (96 per cent).

d_{20}^{20}: about 1.01.

Distillation range (*2.2.11*). Not less than 95 per cent distils between 126 °C and 130 °C.

Storage: in an airtight container.

Morpholine for Chromatography

Complies with the requirements prescribed for *morpholine R* with the following additional requirement.

Content: minimum 99.5 per cent.

2-[N-Morpholino]ethanesulfonic Acid 2-(Morpholin-4-yl)sulfonic acid; MES; $C_6H_{13}NO_4S$ = 195.2 (*4432-31-9*)

White or almost white, crystalline powder, soluble in water.

mp: about 300 °C.

Murexide 5,5′-Nitrilobis(pyrimidine-2,4,6(1*H*,3*H*,5*H*)-trione) monoammonium salt; $C_8H_8N_6O_6,H_2O$ = 302.2

Brownish-red crystalline powder, sparingly soluble in cold water, soluble in hot water, practically insoluble in ethanol (96 per cent), soluble in solutions of potassium hydroxide or sodium hydroxide giving a blue colour.

Myosmine 3-(4,5-Dihydro-3*H*-pyrrol-2-yl)pyridine; $C_9H_{10}N_2$ = 146.2 (*532-12-7*)

Colourless crystals.

mp: about 45 °C.

β-Myrcene 7-Methyl-3-methylenocta-1,6-diene; $C_{10}H_{16}$ = 136.2 (*123-35-3*)

Oily liquid with a pleasant odour, practically insoluble in water, miscible with ethanol (96 per cent), soluble in glacial acetic acid. It dissolves in solutions of alkali hydroxides.

d_4^{20}: about 0.794.

n_D^{20}: about 1.470.

β-Myrcene used in gas chromatography complies with the following additional test.

Assay. Gas chromatography (*2.2.28*) as prescribed in the monograph *Peppermint oil (0405)*.

Test solution. The substance to be examined.

Content: minimum 90.0 per cent, calculated by the normalisation procedure.

Myristic Acid Tetradecanoic acid; $C_{14}H_{28}O_2$ = 228.4 (*544-63-8*)

Colourless or white or almost white flakes.

mp: about 58.5 °C.

Myristic acid used in the assay of total fatty acids inSaw palmetto fruit (1848) complies with the following additional test.

Assay. Gas chromatography (*2.2.28*) as prescribed in the monograph *Saw palmetto fruit (1848)*.

Content: minimum 97 per cent, calculated by the normalisation procedure.

Myristicine 5-Allyl-1-methoxy-2,3-methylenedioxybenzene; 4-Methoxy-6-(prop-2-enyl)-1,3-benzodioxole; $C_{11}H_{12}O_3$ = 192.2 (*607-91-0*)

Oily colourless liquid, practically insoluble in water, slightly soluble in anhydrous ethanol, miscible with toluene and with xylene.

d_{20}^{20}: about 1.144.

n_D^{20}: about 1.540.

bp: 276 °C to 277 °C.

mp: about 173 °C.

Chromatography. Thin-layer chromatography (*2.2.27*) as prescribed in the monograph *Star anise (1153)*; the chromatogram shows only one principal spot.

Myristicine used in gas chromatography complies with the following additional test.

Assay. Gas chromatography (*2.2.28*) as prescribed in the monograph *Nutmeg oil (1552)*.

Content: minimum 95.0 per cent, calculated by the normalisation procedure.

Storage: protected from light.

Myristyl Alcohol Tetradecan-1-ol; $C_{14}H_{30}O$ = 214.4 (*112-72-1*)

d_{20}^{20}: about 0.823.

mp: 38 °C to 40 °C.

Myrtillin Delphinidin 3-*O*-glucoside chloride; $C_{21}H_{21}ClO_{12}$ = 500.8 (*6906-38-3*)

Nalorphine Hydrochloride $C_{19}H_{21}NO_3$, HCl = 347.8 (*57-29-4*)

General reagent grade of commerce.

Naphthalene $C_{10}H_8$ = 128.2 (*91-20-3*)

White or almost white crystals, practically insoluble in water, soluble in ethanol (96 per cent).

mp: about 80 °C.

Naphthalene used for liquid scintillation is of a suitable analytical grade.

Naphthalene-1,3-diol 1,3-Dihydroxynaphthalene; Naphthoresorcinol; Dihydroxynaphthalene; (*132-86-5*)

See *1,3-dihydroxynaphthalene R*.

Naphthalene-2,7-diol 2,7-Dihydroxynaphthalene; $C_{10}H_8O_2$ = 160.2 (*582-17-2*)

Needles, soluble in water and in ethanol (96 per cent).

mp: about 190 °C.

2,3-Naphthalenediamine Naphthalene-2,3-diamine; 2,3-Diaminonaphthalene; $C_{10}H_{10}N_2$ = 158.2 (*771-97-1*)

Brownish-yellow crystalline powder, slightly soluble in ethanol (96 per cent), practically insoluble in acetone.

mp: 195 °C to 198 °C.

Naphthalenediol Reagent Solution

Dissolve 2.5 mg of *naphthalene-2,7-diol* in 90 mL of *methanol* and add 10 mg of *potassium hexacyanoferrate(III)* and 50 mg of *potassium cyanide* dissolved in 10 mL of *water*. Allow to stand for 30 minutes and add 100 mL of 0.05M *sodium hydroxide*.

Naphthalenediol Solution

2,7-Dihydroxynaphthalene solution

Dissolve 10 mg of *2,7-dihydroxynaphthalene R* in 100 mL of *sulfuric acid R* and allow to stand until decolorised.

Storage: use within 2 days.

Naphtharson Thorin; Disodium 4-[(2-arsonophenyl)azo]-3-hydroxynaphthalene-2,7-disulfonate; $C_{16}H_{11}AsN_2Na_2O_{10}S_2$ = 576.3 (*3688-92-4*)

Red powder, soluble in water.

Naphtharson Solution

A 0.58 g/L solution of *naphtharson R*.

Test for sensitivity. To 50 mL of *ethanol (96 per cent) R*, add 20 mL of *water R*, 1 mL of *dilute sulfuric acid R1* and 1 mL of the naphtharson solution. Titrate with *0.025 M barium perchlorate*; the colour changes from orange-yellow to orange-pink.

Storage: protected from light; use within 1 week.

Naphtharson Solution R1

A 1 g/L solution in *deionised distilled water R*.

Test for sensitivity. To 50 mL of *ethanol (96 per cent) R*, add 20 mL of *water R*, 1 mL of *dilute sulfuric acid R1* and 1 mL of *naphtharson solution R1*. Titrate with *0.025 M barium perchlorate*; the colour changes from orange-yellow to orange-pink.

Storage: protected from light; use within 1 week.

β-Naphthol Solution R1

Dissolve 3.0 mg of *β-naphthol R* in 50 mL of *sulfuric acid R* and dilute to 100.0 mL with the same acid. Use the recently prepared solution.

1-Naphthol α-Naphthol; $C_{10}H_8O$ = 144.2 (*90-15-3*)

White or almost white, crystalline powder or colourless or white or almost white crystals, darkening on exposure to light, slightly soluble in water, freely soluble in ethanol (96 per cent).

mp: about 95 °C.

Storage: protected from light.

2-Naphthol β-Naphthol; $C_{10}H_8O$ = 144.2 (*135-19-3*)

White or slightly pink plates or crystals, very slightly soluble in water, very soluble in ethanol (96 per cent).

mp: about 122 °C.

Storage: protected from light.

1-Naphthol Solution α-Naphthol solution

Dissolve 0.10 g of *α-naphthol R* in 3 mL of a 150 g/L solution of *sodium hydroxide R* and dilute to 100 mL with *water R*. Prepare immediately before use.

1-Naphthol Solution, Strong

Dissolve 1 g of *1-naphthol* in a solution of 6 g of *sodium hydroxide* and 16 g of *anhydrous sodium carbonate* in 100 mL of *water*.

2-Naphthol Solution β-Naphthol solution

Dissolve 5 g of freshly recrystallised *β-naphthol R* in 40 mL of *dilute sodium hydroxide solution R* and dilute to 100 mL with *water R*. Prepare immediately before use.

1-Naphtholbenzein Phenylbis(4-hydroxynaphthyl) methanol; Naphtholbenzein; $C_{27}H_{18}O_2$ = 374.4 (*145-50-6*)

Brownish-red powder or shiny brownish-black crystals, practically insoluble in water, soluble in ethanol (96 per cent) and in glacial acetic acid.

1-Naphtholbenzein Solution Naphtholbenzein solution

A 2 g/L solution of *naphtholbenzein R* in *anhydrous acetic acid R*.

Test for sensitivity. To 50 mL of *glacial acetic acid R* add 0.25 mL of the naphtholbenzein solution. The solution is brownish-yellow. Not more than 0.05 mL of *0.1 M perchloric acid* is required to change the colour to green.

Naphthol Yellow 2,4-Dinitro-1-naphthol, sodium salt; $C_{10}H_5N_2NaO_5$ = 256.2

Orange-yellow powder or crystals, freely soluble in water, slightly soluble in ethanol (96 per cent).

Naphthol Yellow S 8-Hydroxy-5,7-dinitro-2-naphthalenesulfonic acid disodium salt; Disodium 5,7-dinitro-8-oxidonaphthalene-2-sulfonate; $C_{10}H_4N_2Na_2O_8S$ = 358.2 (*846-70-8*)

Colour Index No. 10316

Yellow or orange-yellow powder, freely soluble in water.

1-Naphthylacetic Acid (Naphthalen-1-yl)acetic acid; $C_{12}H_{10}O_2$ = 186.2 (*86-87-3*)

White or yellow crystalline powder, very slightly soluble in water, freely soluble in acetone.

mp: about 135 °C.

2-Naphthylacetic Acid 2-Naphthaleneacetic acid; $C_{12}H_{10}O_2$ = 186.2 (*581-96-4*)

mp: about 142°.

General reagent grade of commerce.

Light yellow to light brown crystals.

1-Naphthylamine 1-Aminonaphthalene; Naphthylamine; $C_{10}H_9N$ = 143.2 (*134-32-7*)

White or almost white, crystalline powder, turning pink on exposure to light and air, slightly soluble in water, freely soluble in ethanol (96 per cent).

mp: about 51 °C.

Storage: protected from light.

***N*-(1-Naphthyl)ethylenediamine Dihydrochloride** *N*-(1-Naphthyl)ethane-diamine dihydrochloride; Naphthylethylenediamine dihydrochloride; $C_{12}H_{16}Cl_2N_2$ = 259.2 (*1465-25-4*)

It may contain methanol of crystallisation.

White or yellowish-white powder, soluble in water, slightly soluble in ethanol (96 per cent).

Naphthylethylenediamine Dihydrochloride Solution

Dissolve 0.1 g of *naphthylethylenediamine dihydrochloride R* in *water R* and dilute to 100 mL with the same solvent. Prepare immediately before use.

Naringin 7-[[2-*O*-(6-Deoxy-α-L-mannopyranosyl)-β-D-glucopyranosyl]oxy]-5-hydroxy-2-(4-hydroxyphenyl)-2,3-dihydro-4*H*-chromen-4-one; $C_{27}H_{32}O_{14}$ = 580.5 (*10236-47-2*)

White or almost white crystalline powder, slightly soluble in water, soluble in methanol and in dimethylformamide.

mp: about 171 °C.

Absorbance (*2.2.25*). Naringin dissolved in a 5 g/L solution of *dimethylformamide R* in *methanol R* shows an absorption maximum at 283 nm.

Neohesperidin Hesperetin-7-neohesperidoside; (2*S*)-7-[[2-*O*-(6-Deoxy-α-L-mannopyranosyl)-β-D-glucopyranosyl]oxy]-5-hydroxy-2-(3-hydroxy-4-methoxyphenyl)-2,3-dihydro-4*H*-1-benzopyran-4-one; $C_{28}H_{34}O_{15}$ = 610.6 (*13241-33-3*)

***trans*-Nerolidol** 3,7,11-Trimethyldodeca-1,6,10-trien-3-ol; $C_{15}H_{26}O$ = 222.4 (*40716-66-3*)

Slightly yellow liquid, slight odour of lily and lily of the valley, practically insoluble in water and in glycerol, miscible with ethanol (96 per cent).

d_{20}^{20}: about 0.876.

n_D^{20}: about 1.479.

bp_{12}: 145 °C to 146 °C.

trans-Nerolidol used in gas chromatography complies with the following additional test.

Assay. Gas chromatography (*2.2.28*) as prescribed in the monograph *Bitter-orange-flower oil (1175)*.

Test solution. The substance to be examined.

Content: minimum 90.0 per cent, calculated by the normalisation procedure.

Neryl Acetate (*Z*)-3,7-Dimethylocta-2,6-dienyl acetate; $C_{12}H_{20}O_2$ = 196.3 (*141-12-8*)

Colourless, oily liquid.

d_{20}^{20}: about 0.907.

n_D^{20}: about 1.460.

bp_{25}: 134 °C.

Neryl acetate used in gas chromatography complies with the following additional test.

Assay. Gas chromatography (*2.2.28*) as prescribed in the monograph *Bitter-orange-flower oil (1175)*.

Test solution. The substance to be examined.

Content: minimum 93.0 per cent, calculated by the normalisation procedure.

Neutral Red CI 50040; basic red 5; $C_{15}H_{17}ClN_4$ = 288.8 (*553-24-2*)

Produces a red colour with acids and an orange colour with alkalis.

Neutral Red Solution

A 0.1% w/v solution of *neutral red* in *ethanol (50%)* (pH range, 6.8 to 8.0).

Nickel-Aluminium Alloy Raney nickel catalyst

Contains 48 per cent to 52 per cent of aluminium (Al; A_r 26.98) and 48 per cent to 52 per cent of nickel (Ni; A_r 58.70).

Before use, reduce to a fine powder (180) (*2.9.12*).

It is practically insoluble in water and soluble in mineral acids.

Nickel-Aluminium Alloy (Halogen-free)

Contains 48 per cent to 52 per cent of aluminium (Al; A_r 26.98) and 48 per cent to 52 per cent of nickel (Ni; A_r 58.71).

Fine, grey powder, practically insoluble in water, soluble in mineral acids with formation of salts.

Chlorides: maximum 10 ppm.

Dissolve 0.400 g in 40 mL of a mixture of 67 volumes of *sulfuric acid R* and 33 volumes of *dilute nitric acid R*. Evaporate the solution nearly to dryness, dissolve the residue in *water R* and dilute to 20.0 mL with the same solvent. To one half-aliquot of the solution, add 1.0 mL of *0.1 M silver nitrate*. Filter after 15 min and add 0.2 mL of sodium chloride solution (containing 10 µg of chlorides per millilitre) to the filtrate. After 5 min the solution is more opalescent than a mixture of the second half-aliquot of the solution with 1.0 mL of *0.1 M silver nitrate*.

Nickel(II) Chloride Anhydrous nickel chloride; Nickel chloride; $NiCl_2$ = 129.6 (*7718-54-9*)

Yellow, crystalline powder, very soluble in water, soluble in ethanol (96 per cent). It sublimes in the absence of air and readily absorbs ammonia. The aqueous solution is acid.

Nickel(II) Chloride Hexahydrate $NiCl_2,6H_2O$ = 237.7 (*7791-20-0*)

Analytical reagent grade of commerce.

Nickel Nitrate Hexahydrate $Ni(NO_3)_2,6H_2O$ = 290.8 (*13478-00-7*)

Nickel(II) Sulfate Nickel(II) sulphate; Nickel sulfate heptahydrate; Nickel sulfate; $NiSO_4,7H_2O$ = 280.9 (*10101-98-1*)

Green, crystalline powder or crystals, freely soluble in water, slightly soluble in ethanol (96 per cent).

Nicotinamide-adenine Dinucleotide NAD^+; $C_{21}H_{27}N_7O_{14}P_2$ = 663 (*53-84-9*)

White or almost white powder, very hygroscopic, freely soluble in water.

Nicotinamide-adenine Dinucleotide Solution

Dissolve 40 mg of *nicotinamide-adenine dinucleotide R* in *water R* and dilute to 10 mL with the same solvent. Prepare immediately before use.

Nicotinic Acid (*59-67-6*)

See *Nicotinic acid (0459)*.

Nicotinoyl Hydrazide Pyridine-3-carbohydrazide; $C_6H_7N_3O$ = 137.1 (*553-53-7*)

White or almost white powder or crystalline powder, soluble in water.

mp: about 160 °C.

Nile Blue A 5-Amino-9-(diethylamino)benzo[*a*] phenoxazinylium hydrogen sulfate; $C_{20}H_{21}N_3O_5S$ = 415.5 (*3625-57-8*)

Schultz No. 1029

Colour Index No. 51180

Green, crystalline powder with a bronze lustre, sparingly soluble in ethanol (96 per cent), in glacial acetic acid and in pyridine.

Absorbance (*2.2.25*). A 0.005 g/L solution in *ethanol (50 per cent V/V) R* shows an absorption maximum at 640 nm.

Nile Blue A Solution

A 10 g/L solution of *Nile blue A R* in *anhydrous acetic acid R*.

Test for sensitivity. To 50 mL of *anhydrous acetic acid R* add 0.25 mL of the Nile blue A solution. The solution is blue. On the addition of 0.1 mL of *0.1 M perchloric acid*, the colour changes to blue-green.

Colour change: pH 9.0 (blue) to pH 13.0 (red).

Aqueous Nile Blue A Solution

Dissolve 40 mg of *Nile Blue A* in 200 mL of *water*, shake with 100 mL of n-*heptane* in a 500 mL separating funnel and discard the heptane layer. Repeat the extraction with four 100 mL quantities of n-*heptane* and mix 20 mL of the aqueous solution with 180 mL of *ethanol*.

Ninhydrin Indane-1,2,3-trione; $C_9H_4O_3,H_2O$ = 178.1 (*485-47-2*)

White or very pale yellow, crystalline powder, soluble in water and in ethanol (96 per cent).

Storage: protected from light.

Ninhydrin and Stannous Chloride Reagent

Dissolve 0.2 g of *ninhydrin R* in 4 mL of hot *water R*, add 5 mL of a 1.6 g/L solution of *stannous chloride R*, allow to stand for 30 min, then filter and store at a temperature of 2 °C to 8 °C. Immediately before use dilute 2.5 mL of the solution with 5 mL of *water R* and 45 mL of *2-propanol R*.

Ninhydrin and Stannous Chloride Reagent R1

Dissolve 4 g of *ninhydrin* in 100 mL of *ethylene glycol monomethyl ether*. Shake gently with 1 g of *cation exchange resin* (300 µm to 840 µm) and filter (solution A). Dissolve 0.16 g of *stannous chloride* in 100 mL of *buffer solution pH 5.5* (solution B). Immediately before use, mix equal volumes of each solution.

Ninhydrin Reagent I Transfer 10 litres of *2-methoxyethanol* to a clean, dry 20-litre bottle and purge with *oxygen-free nitrogen* for 2 to 3 minutes. Continue the nitrogen flow and add 10 g of *hydrindantin* and 100 g of *ninhydrin*and allow to dissolve

Transfer 10 litres of *2-methoxyethanol* to a clean, dry 20-litre bottle and purge with *oxygen-free nitrogen* for 2 to 3 minutes. Continue the nitrogen flow and add 10 g of *hydrindantin* and 100 g of *ninhydrin* and allow to dissolve; the solution will be straw coloured when dissolution is complete. Add 2 litres of a solution prepared by dissolving 5.444 kg of *sodium acetate* in 5 litres of *water*, adding 1 litre of *glacial acetic acid*, adjusting the pH to 5.5 with *glacial acetic acid* and adding sufficient *water* to produce 10 litres. Add 6.5 litres of *water* and 20 mL of a 20% w/v solution of *polyoxyethylene 23 lauryl ether*.

The deep red reagent is stable for at least 8 weeks when stored under nitrogen and protected from light; avoid exposure to ammonia.

Ninhydrin Solution

A 2 g/L solution of *Ninhydrin R* in a mixture of 5 volumes of *dilute acetic acid R* and 95 volumes of *butanol R*.

Ninhydrin Solution R1

Dissolve 1.0 g of *ninhydrin R* in 50 mL of *ethanol (96 per cent) R* and add 10 mL of *glacial acetic acid R*.

Ninhydrin Solution R2

Dissolve 3 g of *ninhydrin R* in 100 mL of a 45.5 g/L solution of *sodium metabisulfite R*.

Ninhydrin Solution R3

A 4 g/L solution in a mixture of 5 volumes of *anhydrous acetic acid R* and 95 volumes of *butanol R*.

Ninhydrin Solution R4

A 3 g/L solution of *ninhydrin R* in a mixture of 5 volumes of *glacial acetic acid R* and 95 volumes of *2-propanol R*.

Nitrazepam (*146-22-5*)

See *Nitrazepam (0415)*.

Nitrazepam Impurity A 3-Amino-6-nitro-4-phenylquinol-2(1*H*)-one; *H*)-Quinolinone,-3-amino-6-nitro-4-phenyl; $C_{15}H_{11}N_3O_3$ = 281.3 (*36020-93-6*)

General reagent grade of commenrce.

Nitric Acid HNO_3 = 63.0 (*7697-37-2*)

Content: 63.0 per cent *m/m* to 70.0 per cent *m/m*.

Clear, colourless or almost colourless liquid, miscible with water.

d_{20}^{20}: 1.384 to 1.416.

A 10 g/L solution is strongly acid and gives the reaction of nitrates (*2.3.1*).

Appearance. Nitric acid is clear (*2.2.1*) and not more intensely coloured than reference solution Y_6 (*2.2.2, Method II*).

Chlorides (*2.4.4*): maximum 0.5 ppm.

To 5 g add 10 mL of *water R* and 0.3 mL of *silver nitrate solution R2* and allow to stand for 2 min protected from light. Any opalescence is not more intense than that of a standard prepared in the same manner using 13 mL of *water R*, 0.5 mL of *nitric acid R*, 0.5 mL of *chloride standard solution (5 ppm Cl) R* and 0.3 mL of *silver nitrate solution R2*.

Sulfates (*2.4.13*): maximum 2 ppm.

Evaporate 10 g to dryness with 0.2 g of *sodium carbonate R*. Dissolve the residue in 15 mL of *distilled water R*. Prepare the standard using a mixture of 2 mL of *sulfate standard solution (10 ppm SO_4) R* and 13 mL of *distilled water R*.

Arsenic (*2.4.2, Method A*): maximum 0.02 ppm.

Gently heat 50 g with 0.5 mL of *sulfuric acid R* until white fumes begin to evolve. To the residue add 1 mL of a 100 g/L solution of *hydroxylamine hydrochloride R* and dilute to 2 mL with *water R*. Prepare the standard using 1.0 mL of *arsenic standard solution (1 ppm As) R*.

Iron (*2.4.9*): maximum 1 ppm.

Dissolve the residue from the determination of sulfated ash in 1 mL of *dilute hydrochloric acid R* and dilute to 50 mL with *water R*. Dilute 5 mL of this solution to 10 mL with *water R*.

Heavy metals (*2.4.8*): maximum 2 ppm.

Dilute 10 mL of the solution prepared for the limit test for iron to 20 mL with *water R*. 12 mL of the solution complies with test A. Prepare the reference solution using *lead standard solution (2 ppm Pb) R*.

Sulfated ash: maximum 0.001 per cent.

Carefully evaporate 100 g to dryness. Moisten the residue with a few drops of *sulfuric acid R* and heat to dull red.

Assay. To 1.50 g add about 50 mL of *water R* and titrate with *1 M sodium hydroxide*, using 0.1 mL of *methyl red solution R* as indicator.

1 mL of *1 M sodium hydroxide* is equivalent to 63.0 mg of HNO_3.

Storage: protected from light.

Nitric Acid, Cadmium- and Lead-free

Complies with the requirements prescribed for *nitric acid R* and with the following additional test.

Test solution. To 100 g add 0.1 g of *anhydrous sodium carbonate R* and evaporate to dryness. Dissolve the residue in *water R* heating slightly, and dilute to 50.0 mL with the same solvent.

Cadmium: maximum 0.1 ppm.

Atomic absorption spectrometry (*2.2.23, Method II*).

Source: cadmium hollow-cathode lamp.

Wavelength: 228.8 nm.

Atomisation device: air-acetylene or air-propane flame.

Lead: maximum 0.1 ppm.

Atomic absorption spectrometry (*2.2.23, Method II*).

Source: lead hollow-cathode lamp.

Wavelength: 283.3 nm or 217.0 nm.

Atomisation device: air-acetylene flame.

Nitric Acid, Dilute

Contains about 125 g/L of HNO_3 (M_r 63.0).

Dilute 20 g of *nitric acid R* to 100 mL with *water R*.

Nitric Acid, Dilute R1

Dilute 40 g of *nitric acid R* to 100 mL with *water R*.

Nitric Acid, Dilute R2

Dilute 30 g of *nitric acid R* to 100 mL with *water R*.

Nitric Acid, Fuming (*7697-37-2*)

Clear, slightly yellowish liquid, fuming on contact with air.

d_{20}^{20}: about 1.5.

Nitric Acid, Heavy Metal-free

Complies with the requirements prescribed for *nitric acid R* with the following maximum contents of heavy metals.

As: 0.005 ppm.

Cd: 0.005 ppm.

Cu: 0.001 ppm.

Fe: 0.02 ppm.

Hg: 0.002 ppm.

Ni: 0.005 ppm.

Pb: 0.001 ppm.

Zn: 0.01 ppm.

Nitric Acid, Dilute, Heavy Metal-free

Complies with the requirements prescribed for *dilute nitric acid R* with the following maximum contents of heavy metals.

As: 0.005 ppm.

Cd: 0.005 ppm.

Cu: 0.001 ppm.

Fe: 0.02 ppm.

Hg: 0.002 ppm.

Ni: 0.005 ppm.

Pb: 0.001 ppm.

Zn: 0.01 ppm.

Nitric Acid, Lead-free, Dilute

Dilute 5 g of *lead-free nitric acid R1* to 100 mL with *deionised distilled water R*.

Nitric Acid, Lead-free

Complies with the requirements prescribed for *Nitric acid R* with the following additional test.

Lead: maximum 0.1 ppm.

Atomic absorption spectrometry (*2.2.23, Method II*).

Test solution. To 100 g add 0.1 g of *anhydrous sodium carbonate R* and evaporate to dryness. Dissolve the residue in *water R*, heating slightly, and dilute to 50.0 mL with the same solvent.

Source: lead hollow-cathode lamp.

Wavelength: 283.3 nm or 217.0 nm.

Atomisation device: air-acetylene flame.

Nitric Acid, Lead-free R1

Nitric acid R containing not more than 1 µg/kg of lead.

Nitric Acid, Nickel-free

Complies with the requirements prescribed for *nitric acid R* with the following additional requirement.

Nickel: maximum 0.005 ppm.

Nitrilotriacetic Acid $C_6H_9NO_6$ = 191.1 (*139-13-9*)

White or almost white crystalline powder, practically insoluble in water and in most organic solvents.

mp: about 240 °C, with decomposition.

4-Nitroaniline Nitroaniline; $C_6H_6N_2O_2$ = 138.1 (*100-01-6*)

Bright yellow, crystalline powder, very slightly soluble in water, sparingly soluble in boiling water, soluble in ethanol (96 per cent), forms water-soluble salts with strong mineral acids.

mp: about 147 °C.

Nitroaniline Solution, Diazotised

Dissolve 0.4 g of *4-nitroaniline* in 60 mL of 1M *hydrochloric acid* with the aid of heat, cool to 15° and add a 10% w/v solution of *sodium nitrite* until one drop of the mixture turns *starch iodide paper* blue.

Prepare immediately before use.

2-Nitrobenzaldehyde Nitrobenzaldehyde; $C_7H_5NO_3$ = 151.1 (*552-89-6*)

Yellow needles, slightly soluble in water, freely soluble in ethanol (96 per cent), volatile in steam.

mp: about 42 °C.

4-Nitrobenzaldehyde $C_7H_5NO_3$ = 151.1 (*555-16-8*)

Nitrobenzaldehyde Paper

Dissolve 0.2 g of *nitrobenzaldehyde R* in 10 mL of a 200 g/L solution of *sodium hydroxide R*. Use the solution within 1 h. Immerse the lower half of a slow filter paper strip 10 cm long and 0.8-1 cm wide. Absorb the excess reagent between two sheets of filter paper. Use within a few minutes of preparation.

Nitrobenzaldehyde Solution

Add 0.12 g of powdered *nitrobenzaldehyde R* to 10 mL of *dilute sodium hydroxide solution R*; allow to stand for 10 min shaking frequently and filter. Prepare immediately before use.

Nitrobenzene $C_6H_5NO_2$ = 123.1 (*98-95-3*)

Colourless or very slightly yellow liquid, practically insoluble in water, miscible with ethanol (96 per cent).

bp: about 211 °C.

Dinitrobenzene. To 0.1 mL add 5 mL of *acetone R*, 5 mL of *water R* and 5 mL of *strong sodium hydroxide solution R*. Shake and allow to stand. The upper layer is almost colourless.

4-Nitrobenzoic Acid $C_7H_5NO_4$ = 167.1 (*62-23-7*)

Yellow crystals.

mp: about 240 °C.

4-Nitrobenzyl Bromide $C_7H_6BrNO_2$ = 216.0 (*100-11-8*)

mp: about 99°.

General reagent grade of commerce.

Pale yellow crystals with a lachrymatory vapour.

Nitrobenzoyl Chloride 4-Nitrobenzoyl chloride; $C_7H_4ClNO_3$ = 185.6 (*122-04-3*)

Yellow crystals or a crystalline mass, decomposing in moist air, completely soluble in sodium hydroxide solution giving a yellowish-orange colour.

mp: about 72 °C.

4-Nitrobenzyl Chloride Nitrobenzyl chloride; $C_7H_6ClNO_2$ = 171.6 (*100-14-1*)

Pale-yellow crystals, lachrymatory, practically insoluble in water, very soluble in ethanol (96 per cent).

4-(4-Nitrobenzyl)pyridine $C_{12}H_{10}N_2O_2$ = 214.2 (*1083-48-3*)

Yellow powder.

mp: about 70 °C.

Nitrochromic Reagent

Dissolve 0.7 g of *potassium dichromate* in *nitric acid* and dilute to 100 mL with the same acid.

Nitroethane $C_2H_5NO_2$ = 75.1 (*79-24-3*)

Clear, oily, colourless liquid.

bp: about 114 °C.

2-Nitroethanol $C_2H_5NO_3$ = 91.1 (*625-48-9*)

bp: about 205°.

General reagent of commerce.

Nitrofurantoin (*67-20-9*)

See *Nitrofurantoin (0101)*.

(5-Nitro-2-furyl)methylene Diacetate Nitrofurfural diacetate; 5-nitrofurfurylidene diacetate; $C_9H_9NO_7$ = 243.2 (*92-55-7*)

mp: about 90°.

Yellow crystals.

Nitrogen N_2 = 28.01 (*7727-37-9*)

Nitrogen, washed and dried.

Nitrogen Dioxide NO_2 = 46.01 (*10102-44-0*)

Content: minimum 98.0 per cent *V/V*.

Nitrogen for Chromatography N_2 = 28.01 (*7727-37-9*)

Content: minimum 99.95 per cent *V/V*.

Nitrogen Gas Mixture Nitric oxide

Nitrogen R containing 1 per cent *V/V* of each of the following gases: *carbon dioxide R2*, *carbon monoxide R1* and *oxygen R1*.

Nitrogen Monoxide NO = 30.01

Content: minimum 98.0 per cent *V/V*.

Nitrogen, Oxygen-free

Nitrogen R which has been freed from oxygen by passing it through *alkaline pyrogallol solution R*.

Nitrogen R1 N_2 = 28.01 (*7727-37-9*)

Content: minimum 99.999 per cent *V/V*.

Carbon monoxide: less than 5 ppm.

Oxygen: less than 5 ppm.

Nitromethane CH_3NO_2 = 61.0 (*75-52-5*)

Clear, colourless, oily liquid, slightly soluble in water, miscible with ethanol (96 per cent).

d_{20}^{20}: 1.132 to 1.134.

n_D^{20}: 1.381 to 1.383.

Distillation range (*2.2.11*). Not less than 95 per cent distils between 100 °C and 103 °C.

4-Nitrophenol *p*-Nitrophenol; $C_6H_5NO_3$ = 139.1 (*100-02-7*)

Content: minimum 95 per cent.

Colourless or slightly yellow powder, sparingly soluble in water and in methanol.

mp: about 114 °C.

3-Nitrosalicylic Acid 2-Hydroxy-3-nitrobenzoic acid; $C_7H_5NO_5$ = 183.1 (*85-38-1*)

Yellowish crystals, slightly soluble in water, freely soluble in ethanol (96 per cent).

mp: 142 °C to 147 °C.

5-Nitrosalicylic Acid 2-Hydroxy-5-nitrobenzoic acid; $C_7H_5NO_5$ = 183 (*96-97-9*)

General reagent grade of commerce.

***N*-Nitrosodiethanolamine** 2,2′-(Nitrosoimino)diethanol; $C_4H_{10}N_2O_3$ = 134.1 (*1116-54-7*)

Yellow liquid, miscible with anhydrous ethanol.

n_D^{20}: about 1.485.

bp: about 125 °C.

***N*-Nitrosodiisopropanolamine** 1,1′-(Nitrosoimino) bispropan-2-ol; $C_6H_{14}N_2O_3$ = 162.2 (*53609-64-6*)

bp: 122-124 °C.

Nitrosodipropylamine Dipropylnitrosamine; $C_6H_{14}N_2O$ = 130.2 (*621-64-7*)

Liquid, soluble in anhydrous ethanol and in strong acids.

d_{20}^{20}: about 0.915.

bp: about 78 °C.

Appropriate grade for chemiluminescence determination.

Nitrosodipropylamine Solution

Inject 78.62 g of *anhydrous ethanol R* through the septum of a vial containing *nitrosodipropylamine R*. Dilute 1/100 in *anhydrous ethanol R* and place 0.5 mL aliquots in crimp-sealed vials.

Storage: in the dark at 5 °C.

Nitrotetrazolium Blue 3,3′-(3,3′-Dimethoxy-4,4′-diphenylene)di[2-(4-nitrophenyl)-5-phenyl-2*H*-tetrazolium] dichloride; *p*-Nitro-tetrazolium blue; $C_{40}H_{30}Cl_2N_{10}O_6$ = 818 (*298-83-9*)

Crystals, soluble in methanol, giving a clear, yellow solution.

mp: about 189 °C, with decomposition.

Nitrous Oxide N_2O = 44.01

Content: minimum 99.99 per cent *V/V*.

Nitrogen monoxide: less than 1 ppm.

Carbon monoxide: less than 1 ppm.

Nitro-vanado-molybdic Reagent Nitro-molybdovanadic reagent

Solution A. Dissolve 10 g of *ammonium molybdate R* in *water R*, add 1 mL of *ammonia R* and dilute to 100 mL with *water R*.

Solution B. Dissolve 2.5 g of *ammonium vanadate R* in hot *water R*, add 14 mL of *nitric acid R* and dilute to 500 mL with *water R*.

To 96 mL of *nitric acid R* add 100 mL of solution A and 100 mL of solution B and dilute to 500 mL with *water R*.

Nonivamide *N*-[(4-Hydroxy-3-methoxyphenyl)methyl] nonanamide; $C_{17}H_{27}NO_3$ = 293.4 (*2444-46-4*)

White or almost white, crystalline powder, practically insoluble in cold water, freely soluble in anhydrous ethanol.

Nonivamide used in the test for nonivamide in the monograph Capsicum (1859) complies with the following additional test.

Assay. Liquid chromatography (*2.2.29*) as prescribed in the monograph *Capsicum (1859)*.

Content: minimum 98.0 per cent, calculated by the normalisation procedure.

Nonylamine Nonan-1-amine; 1-Aminononane; $C_9H_{21}N$ = 143.3 (*112-20-9*)

Corrosive, colourless, clear liquid.

d_4^{20}: about 0.788.

n_D^{20}: about 1.433.

Noradrenaline Acid Tartrate Noradrenaline bitartrate; $C_8H_{11}NO_3,C_4H_6O_6$ = 319.3 (*69815-49-2*)

mp: about 102°.

General reagent grade of commerce.

A white, crystalline powder.

Nordazepam 7-Chloro-2,3-dihydro-5-phenyl-1*H*-1,4-benzodiazepin-2-one; $C_{15}H_{11}ClN_2O$ = 270.7 (*1088-11-5*)

White or pale yellow, crystalline powder, practically insoluble in water, slightly soluble in ethanol (96 per cent).

mp: about 216 °C.

DL-Norleucine (*RS*)-2-Aminohexanoic acid; $C_6H_{13}NO_2$ = 131.2 (*616-06-8*)

Shiny crystals, sparingly soluble in water and in ethanol (96 per cent), soluble in acids.

Noroxymorphone $C_{16}H_{17}NO_4$ = 287.3 (*33522-95-1*)

General reagent grade of commerce.

Norpseudoephedrine Hydrochloride $C_9H_{13}NO$, HCl = 187.7 (*53643-20-2*)

mp: 180° to 181°.

General reagent grade of commerce.

A crystalline powder.

Noscapine Hydrochloride (*912-60-7*)

See *Noscapine hydrochloride (0515)*.

Nystose $C_{24}H_{42}O_{21}$ = 666.6 (*13133-07-8*)

β-D-Fructofuranosyl-(2→1)-β-D-fructofuranosyl-(2→1)-β-D-fructofuranosyl α-D-glucopyranoside.

Ochratoxin A Solution

50 µg/mL solution of (2*S*)-2-([[(3*R*)-5-chloro-8-hydroxy-3-methyl-1-oxo-3,4-dihydro-1*H*-2-benzopyran-7-yl]carbonyl] amino)-3-phenylpropanoic acid (ochratoxin A) in a mixture of 1 volume of *acetic acid R* and 99 volumes of *benzene R*.

Octadecan-1-ol Stearyl alcohol; $C_{18}H_{38}O$ = 270.5 (*112-92-5*)

mp: about 60 °C.

Content: minimum 95 per cent.

Octadecyl [3-[3,5-bis(1,1-dimethylethyl)-4-hydroxyphenyl]-propionate] Octadecyl 3-(3,5-di-*tert*-butyl-4-hydroxyphenyl)propionate; $C_{35}H_{62}O_3$ = 530.9 (*2082-79-3*)

White or slightly yellowish, crystalline powder, practically insoluble in water, very soluble in acetone and in hexane, slightly soluble in methanol.

mp: 49 °C to 55 °C.

Octanal Octyl aldehyde; $C_8H_{16}O$ = 128.2 (*124-13-0*)

Oily, colourless liquid. Practically insoluble in water.

Octanal used in gas chromatography complies with the following additional test.

Assay. Gas chromatography (*2.2.28*) as prescribed in the monograph *Sweet orange oil (1811)*.

Content: minimum 99 per cent, calculated by the normalisation procedure.

***N*-Octane** Octane; C_8H_{18} = 114.2 (*111-65-9*)

Content: minimum 99 per cent.

Octanoic Acid Caprylic acid; $C_8H_{16}O_2$ = 144.2 (*124-07-2*)

Slightly yellow, oily liquid.

d_4^{20}: about 0.910.

n_D^{20}: about 1.428.

bp: about 239.7 °C.

mp: about 16.7 °C.

Caprylic acid used in the assay of total fatty acids in Saw palmetto fruit (1848) complies with the following additional test.

Assay. Gas chromatography (*2.2.28*) as prescribed in the monograph *Saw palmetto fruit (1848)*.

Content: minimum 98 per cent, calculated by the normalisation procedure.

Octan-1-ol Caprylic alcohol; 1-Octanol; Octanol; $C_8H_{18}O$ = 130.2 (*111-87-5*)

Colourless liquid, practically insoluble in water, miscible with ethanol (96 per cent).

d_{20}^{20}: about 0.828.

bp: about 195 °C.

Octan-2-ol *sec*-Octyl alcohol; $C_8H_{18}O$ = 130.2 (*6169-06-8*)

bp: about 178°.

General reagent grade of commerce.

An oily liquid; weight per mL, about 0.82 g.

3-Octanone Octan-3-one; Ethylpentylketone; $C_8H_{16}O$ = 128.2 (*106-68-3*)

Colourless liquid with a characteristic odour.

d_{20}^{20}: about 0.822.

n_D^{20}: about 1.415.

bp: about 167 °C.

3-Octanone used in gas chromatography complies with the following additional test.

Assay. Gas chromatography (*2.2.28*) as prescribed in the monograph *Lavender oil (1338)*.

Test solution. The substance to be examined.

Content: minimum 98.0 per cent, calculated by the normalisation procedure.

Octoxinol 10 α-[4-(1,1,3,3-Tetramethylbutyl)phenyl]-ω-hydroxypoly-(oxyethylene); $C_{34}H_{62}O_{11}$ (average) = 647 (*9002-93-1*)

Clear, pale-yellow, viscous liquid, miscible with water, with acetone and with ethanol (96 per cent), soluble in toluene.

Storage: in an airtight container.

Octreotide Acetate (Acetate-free peptide: M_r 1019; [83150-76-9]); D-Phenylalanyl-L-cysteinyl-L-phenylalanyl-D-tryptophyl-L-lysyl-L-threonyl-*N*-[(1*R*,2*R*)-2-hydroxy-1-(hydroxymethyl)propyl]-L-cysteinamide cyclic (2→7)-disulfide acetate; It contains a variable quantity of acetic acid; White or almost white powder, freely soluble in water and acetic acid; $C_{49}H_{66}N_{10}O_{10}S_2.xC_2H_4O_2$ (*79517-01-4*)

Content: minimum 96.0 per cent.

Octylamine 1-Amino-octane; *N*-Octylamine; $C_8H_{19}N$ = 129.2 (*111-86-4*)

Colourless liquid.

d_{20}^{20}: about 0.782.

bp: 175 °C to 179 °C.

Oleamide (9*Z*)-Octadec-9-enoamide; $C_{18}H_{35}NO$ = 281.5

Yellowish or white powder or granules, practically insoluble in water, very soluble in methylene chloride, soluble in anhydrous ethanol.

mp: about 80 °C.

Oleanolic Acid 3β-Hydroxyolean-12-en-28-oic acid; Astrantiagenin C; $C_{30}H_{48}O_3$ = 456.7 (*508-02-1*)

Oleic Acid (9*Z*)-Octadec-9-enoic acid; $C_{18}H_{34}O_2$ = 282.5 (*112-80-1*)

Clear, colourless liquid, practically insoluble in water.

d_4^{20}: about 0.891.

n_D^{20}: about 1.459.

mp: 13 °C to 14 °C.

Oleic acid used in the assay of total fatty acids in the monograph Saw palmetto fruit (1848) complies with the following additional test.

Assay. Gas chromatography (*2.2.28*) as prescribed in the monograph *Saw palmetto fruit (1848)*.

Content: minimum 98 per cent, calculated by the normalisation procedure.

Oleuropein 2-(3,4-Dihydroxyphenyl)ethyl[(2*S*,3*E*,4*S*)-3-ethylidene-2-(β-D-glucopyranosyloxy)-5-(methoxycarbonyl)-3,4-dihydro-2*H*-pyran-4-yl]acetate; $C_{25}H_{32}O_{13}$ = 540.5 (*32619-42-4*)

Powder, soluble in methanol.

Oleuropein used in Olive leaf (1878) complies with the following test.

Assay. Liquid chromatography (*2.2.29*) as prescribed in the monograph *Olive leaf (1878)*.

Content: minimum 80 per cent, calculated by the normalisation procedure.

Oleyl Alcohol (9*Z*)-Octadec-9-en-1-ol; $C_{18}H_{36}O$ = 268.5 (*143-28-2*)

bp: about 207 °C.

n_D^{20}: 1.460.

Content: minimum 85 per cent.

Olive Oil (*8001-25-0*)

See *Olive oil, virgin (0518)*.

Olive Oil Substrate Emulsion

Homogenise 40 mL of *olive oil*, 330 mL of *acacia solution* and 30 mL of *water* in an 800-mL beaker placed in a vessel containing a mixture of ice and ethanol as cooling mixture. Emulsify using a mixer at an average speed of 1000 to 2000 revolutions per minute. Cool to 5° to 10°. Increase the mixing speed to 8000 revolutions per minute. Mix for 30 minutes keeping the temperature below 25° by the continuous addition of crushed ice into the cooling mixture (a mixture of calcium chloride and crushed ice is also suitable). Store this preparation (the stock emulsion) in a refrigerator and use within 14 days. The emulsion must not separate into two distinct layers. Check the diameter of the globules of the emulsion under a microscope. At least 90% have a diameter below 3 µm and none has a diameter greater than 10 µm. Shake the emulsion thoroughly before preparing the substrate emulsion.

For 10 determinations mix the following solutions in the order indicated: 100 mL of the stock emulsion, 80 mL of *tris-chloride buffer solution*, 20 mL of a freshly prepared 8% w/v solution of *sodium taurocholate EPBRP* and 95 mL of *water*.

Use on the day of preparation.

Oracet Blue B

Solvent blue 19

A mixture of 1-methylamino-4-anilinoanthraquinone ($C_{21}H_{16}N_2O_2$) and 1-amino-4-anilinoanthraquinone ($C_{20}H_{14}N_2O_2$). When used for titration in non-aqueous media, it changes from blue (basic) through purple (neutral) to pink (acidic).

Oracet Blue B Solution

A 0.5% w/v solution of *oracet blue B* in *anhydrous acetic acid*.

Oracet Blue 2R 1-Amino-4-(phenylamino)anthracene-9,10-dione; CI 61110; $C_{20}H_{14}N_2O_2$ = 314.3 (*4395-65-7*)

mp: about 194°.

Oracet Blue 2R Solution

A 0.5% w/v solution of *oracet blue 2R* in *anhydrous acetic acid*.

Orcinol 5-Methylbenzene-1,3-diol monohydrate; $C_7H_8O_2$, H_2O = 142.2 (*6153-39-5*)

Crystalline powder, sensitive to light.

bp: about 290 °C.

mp: 58 °C to 61 °C.

Organosilica Polymer, Amorphous, Octadecylsilyl

Synthetic, spherical hybrid particles, containing both inorganic (silica) and organic (organosiloxanes) components, chemically modified at the surface by trifunctionally bonded octadecylsilyl groups.

Organosilica Polymer, Amorphous, Octadecylsilyl, End-capped

Synthetic, spherical hybrid particles, containing both inorganic (silica) and organic (organosiloxanes) components, chemically modified at the surface by trifunctionally bonded octadecylsilyl groups. To minimise any interaction with basic compounds, it is carefully end-capped to cover most of the remaining silanol groups. The particle size is indicated after the name of the reagent in the tests where it is used.

Organosilica Polymer, Amorphous, Polar-embedded Octadecylsilyl, End-capped

Synthetic, spherical hybrid particles containing both inorganic (silica) and organic (organosiloxanes) components, chemically modified at the surface by the bonding of polar-embedded octadecylsilyl groups. To minimise any interaction with basic compounds, they are carefully end-capped to cover most of the remaining silanol groups.

Organosilica Polymer, Amorphous, Propyl-2-phenylsilyl, End-capped

Synthetic, spherical hybrid particles containing both inorganic (silica) and organic (organosiloxanes) components, chemically modified at the surface by the bonding of propyl-2-phenylsilyl groups. To minimise any interaction with basic compounds, they are carefully end-capped to cover most of the remaining silanol groups.

Organosilica Polymer Compatible with 100% Aqueous Mobile Phases, Octadecylsilyl, Solid Core, End-capped

Silica gel with spherical silica particles containing a solid non-porous silica core surrounded by a thin outer organosilica polymer coating with octadecylsilyl groups, suitable for use with highly aqueous mobile phases including 100 per cent aqueous phases. To minimise any interaction with basic compounds, it is carefully end-capped to cover most of the remaining silanol groups.

Organosilica Polymer for Mass Spectrometry, Amorphous, Octadecylsilyl, End-capped Organosilica Polymer for Chromatography, Amorphous, Octadecylsilyl, End-capped

Synthetic, spherical hybrid particles containing both inorganic (silica) and organic (organosiloxanes) components, chemically modified at the surface by the bonding of octadecylsilyl groups. To minimise any interaction with basic compounds, they are carefully end-capped to cover most of the remaining silanol groups.

Organosilica Polymer, Multi-layered, Octadecylsilyl, End-capped

Synthetic, spherical hybrid particles, multi-layered, containing both inorganic (silica) and organic (organosiloxanes) components, chemically modified at the surface by the bonding of octadecylsilyl groups. To minimise any interaction with basic compounds, they are carefully end-capped to cover most of the remaining silanol groups.

Orientin 2-(3,4-Dihydroxyphenyl)-8-β-D-glucopyranosyl-5,7-dihydroxy-4*H*-1-benzopyran-4-one; 8-β-D-Glucopyranosyl-3′,4′,5,7-tetrahydroxyflavone; Luteolin-8-*C*-β-D-glucopyranoside; Luteolin-8-glucoside; $C_{21}H_{20}O_{11}$ = 448.4 (*28608-75-5*)

Orthophosphoric Acid Phosphoric acid; (*7664-38-2*)

See *Concentrated phosphoric acid (0004)*.

Orthophosphorous Acid Phosphorous acid; H_3PO_3 = 82.0 (*13598-36-2*)

White or almost white, very hygroscopic and deliquescent crystalline mass; slowly oxidised by oxygen (air) to H_3PO_4.

Unstable, orthorhombic crystals, soluble in water, in ethanol (96 per cent) and in a mixture of 3 volumes of ether and 1 volume of ethanol (96 per cent).

d_4^{21}: 1.651.

mp: about 73 °C.

Osthole 7-Methoxy-8-(3-methylbut-2-enyl)-2*H*-1-benzopyran-2-one; 7-Methoxy-8-isopentenylcoumarin; $C_{15}H_{16}O_3$ = 244.3 (*484-12-8*)

Oxalic Acid Ethanedioic acid dihydrate; $C_2H_2O_4,2H_2O$ = 126.1 (*6153-56-6*)

White or almost white crystals, soluble in water, freely soluble in ethanol (96 per cent).

Oxalic Acid and Sulfuric Acid Solution Oxalic acid and sulphuric acid solution

A 50 g/L solution of *oxalic acid R* in a cooled mixture of equal volumes of *sulfuric acid R* and *water R*.

Oxazepam (*604-75-1*)

See *Oxazepam (0778)*.

Ox Brain, Acetone-dried

Cut into small pieces a fresh ox brain previously freed from vascular and connective tissue. Place in *acetone R* for preliminary dehydration. Complete the dehydration by pounding in a mortar 30 g of this material with successive quantities, each of 75 mL, of *acetone R* until a dry powder is obtained after filtration. Dry at 37 °C for 2 h or until the odour of acetone is no longer present.

2,2′-Oxybis(N,N-dimethylethylamine) Bis(2-dimethylaminoethyl) ether; $C_8H_{20}N_2O$ = 160.3 (*3033-62-3*)

Colourless, corrosive liquid.

d_{20}^{20}: about 0.85.

n_D^{20}: about 1.430.

Oxygen O_2 = 32.00

Content: minimum 99.99 per cent *V/V*.

Nitrogen and argon: less than 100 ppm.

Carbon dioxide: less than 10 ppm.

Carbon monoxide: less than 5 ppm.

Oxygen R1 O_2 = 32.00

Content: minimum 99 per cent *V/V*.

Oxytetracycline Hydrochloride

See *Oxytetracycline hydrochloride (0198)*.

Paeoniflorin [(1*R*,2*S*,3*R*,5*R*,6*R*,8*S*)-3-(β-D-Glucopyranosyloxy)6-hydroxy-8-methyl-9,10-dioxatetracyclo [4.3.1.$0^{2,5}$.$0^{3,8}$]decan-2-yl]methyl benzoate; $C_{23}H_{28}O_{11}$ = 480.5 (*23180-57-6*)

Paeonol 1-(2-Hydroxy-4-methoxyphenyl)ethan-1-one; 2′-Hydroxy-4′-methoxyacetophenone; $C_9H_{10}O_3$ = 166.2 (*552-41-0*)

Palladium Pd = 106.4 (*7440-05-3*)

Grey white metal, soluble in hydrochloric acid.

Palladium(II) Chloride Palladium chloride; $PdCl_2$ = 177.3 (*7647-10-1*)

Red crystals.

mp: 678 °C to 680 °C.

Palladium Chloride Solution

Dissolve 1 g of *palladium chloride R* in 10 mL of warm *hydrochloric acid R*. Dilute the solution to 250 mL with a mixture of equal volumes of *dilute hydrochloric acid R* and *water R*. Dilute this solution immediately before use with 2 volumes of *water R*.

Palmatine 2,3,9,10-Tetramethoxy-5,6-dihydro-7λ^5-isoquinolino[3,2-*a*]isoquinolin-7-ylium; 7,8,13,13a-Tetradehydro-2,3,9,10-tetramethoxyberbinium; $C_{21}H_{22}NO_4^+$ = 352.4 (*3486-67-7*)

Palmatine Chloride Berbericine Chloride; $C_{21}H_{22}NO_4$, Cl = 387.86 (*10605-02-4*)

General reagent grade of commerce.

Palmitic Acid Hexadecanoic acid; $C_{16}H_{32}O_2$ = 256.4 (*57-10-3*)

White or almost white, crystalline scales, practically insoluble in water, freely soluble in hot ethanol (96 per cent).

mp: about 63 °C.

Chromatography. Thin-layer chromatography (*2.2.27*) as prescribed in the monograph *Chloramphenicol palmitate (0473)*; the chromatogram shows only one principal spot.

Palmitic acid used in the assay of total fatty acids in the monograph Saw palmetto fruit (1848) complies with the following additional test.

Assay. Gas chromatography (*2.2.28*) as prescribed in the monograph *Saw palmetto fruit (1848)*.

Content: minimum 98 per cent, calculated by the normalisation procedure.

Palmitoleic Acid (9*Z*)-Hexadec-9-enoic acid; $C_{16}H_{30}O_2$ = 254.4 (*373-49-9*)

Clear, colourless liquid.

bp: about 162 °C.

Palmitoleic acid used in the assay of total fatty acids in Saw palmetto fruit (1848) complies with the following additional test.

Assay. Gas chromatography (*2.2.28*) as prescribed in the monograph *Saw palmetto fruit (1848)*.

Content: minimum 98 per cent, calculated by the normalisation procedure.

Palmityl Alcohol Hexadecan-1-ol; Cetyl alcohol; $C_{16}H_{34}O$ = 242.4 (*36653-82-4*)

mp: about 48 °C.

Content: minimum 96 per cent.

Pancreas Powder See *Pancreas powder (0350)*.

Papain (*9001-73-4*)

A proteolytic enzyme obtained from the latex of the green fruit and leaves of *Carica papaya* L.

Papaverine Hydrochloride (*61-25-6*)

See *Papaverine hydrochloride (0102)*.

Paper Chromatography Performance Test Solutions

Test solution (A). Sodium pertechnetate (^{99m}Tc) injection (fission) (0124) or *Sodium pertechnetate (^{99m}Tc) injection (non-fission) (0283)*.

Test solution (B). In a closed vial mix 100 µL of a 5 g/L solution of *stannous chloride R* in *0.05 M hydrochloric acid* and 100 MBq to 200 MBq of *Sodium pertechnetate (^{99m}Tc) injection (fission) (0124)* or *Sodium pertechnetate (^{99m}Tc) injection (non-fission) (0283)* in a volume not exceeding 2 mL.

Paper for Chromatography

Pure cellulose grade thin paper with a smooth surface and a thickness of about 0.2 mm.

Chromatographic separation. To 2 strips of *paper for chromatography R* apply separately 2-5 µL of test solution (a) and test solution (b) of *paper chromatography performance test solutions R*. Develop over a pathlength of 3/4 of the paper height, using a mixture of equal volumes of *methanol R* and *water R*. Allow to dry and determine the distribution of radioactivity using a suitable detector. The paper is not satisfactory, unless the chromatogram obtained with test solution (a) shows a single radioactivity spot with an R_F value in the range 0.8-1.0 and the chromatogram obtained with test solution (b) shows a single radioactivity spot at the application point (R_F value in the range 0.0-0.1).

Paracetamol (*103-90-2*)

See *Paracetamol (0049)*.

Paracetamol, 4-Aminophenol-free

Recrystallise *paracetamol R* from *water R* and dry *in vacuo* at 70 °C; repeat the procedure until the product complies with the following test: dissolve 5 g of the dried substance in a mixture of equal volumes of *methanol R* and *water R* and dilute to 100 mL with the same mixture of solvents. Add 1 mL of a freshly prepared solution containing 10 g/L of *sodium nitroprusside R* and 10 g/L of *anhydrous sodium carbonate R*, mix and allow to stand for 30 min protected from light. No blue or green colour is produced.

Paraffin, Liquid (*8042-47-5*)

See *Liquid paraffin (0239)*.

Paraffin, White Soft

A semi-liquid mixture of hydrocarbons obtained from petroleum and bleached, practically insoluble in water and in ethanol (96 per cent), soluble in *light petroleum R1*, the solution sometimes showing a slight opalescence.

Paraldehyde (*123-63-7*)

See *Paraldehyde (0351)*.

Pararosaniline Hydrochloride Pararosaniline chloride; Basic red 9; $C_{19}H_{18}ClN_3$ = 323.8 (*569-61-9*)

Schultz No. 779

Colour Index No. 42500

Bluish-red, crystalline powder, slightly soluble in water, soluble in anhydrous ethanol. Solutions in water and anhydrous ethanol are deep-red; solutions in sulfuric acid and in hydrochloric acid are yellow.

mp: about 270 °C, with decomposition.

Pararosaniline Solution, Decolorised

To 0.1 g of *pararosaniline hydrochloride R* in a ground-glass-stoppered flask add 60 mL of *water R* and a solution of 1.0 g of *anhydrous sodium sulfite R* or 2.0 g of *sodium sulfite heptahydrate R* or 0.75 g of *sodium metabisulfite R* in 10 mL of *water R*. Slowly and with stirring add 6 mL of *dilute*

hydrochloric acid R, stopper the flask and continue stirring until dissolution is complete. Dilute to 100 mL with *water R*. Allow to stand for 12 h before use.

Storage: protected from light.

Parthenolide (4*E*)-(1a*R*,7a*S*,10a*S*,10b*S*)-1a,5-Dimethyl-8-methylene-2,3,6,7,7a,8,10a,10b-octahydro-oxireno[9,10] cyclodeca[1,2-*b*]furan-9(1a*H*)-one; (*E*)-(5*S*,6*S*)-4,5-Epoxygermacra-1(10),11(13)-dieno-12(6)-lactone; $C_{15}H_{20}O_3$ = 248.3 (*20554-84-1*)

White or almost white, crystalline powder, very slightly soluble in water, very soluble in methylene chloride, soluble in methanol.

$[\alpha]_D^{22}$: −71.4, determined on a 2.2 g/L solution in *methylene chloride R*.

mp: 115 °C to 116 °C.

Absorbance (*2.2.25*). A 0.01 g/L solution in *ethanol (96 per cent) R* shows an absorption maximum at 214 nm.

Assay. Liquid chromatography (*2.2.29*) as prescribed in the monograph *Feverfew (1516)*, at the concentration of the reference solution.

Content: minimum 90 per cent, calculated by the normalisation procedure.

L-Penicillamine Coated Silica Gel For Chiral Separations

A very finely divided silica gel for chromatography coated with L-penicillamine.

Penicillinase Solution

Dissolve 10 g of casein hydrolysate, 2.72 g of *potassium dihydrogen phosphate R* and 5.88 g of *sodium citrate R* in 200 mL of *water R*, adjust to pH 7.2 with a 200 g/L solution of *sodium hydroxide R* and dilute to 1000 mL with *water R*. Dissolve 0.41 g of *magnesium sulfate R* in 5 mL of *water R* and add 1 mL of a 1.6 g/L solution of *ferrous ammonium sulfate R* and sufficient *water R* to produce 10 mL. Sterilise both solutions by heating in an autoclave, cool, mix, distribute in shallow layers in conical flasks and inoculate with *Bacillus cereus* (NCTC 9946). Allow the flasks to stand at 18 °C to 37 °C until growth is apparent and then maintain at 35 °C to 37 °C for 16 h, shaking constantly to ensure maximum aeration. Centrifuge and sterilise the supernatant by filtration through a membrane filter. 1.0 mL of penicillinase solution contains not less than 0.4 microkatals (corresponding to the hydrolysis of not less than 500 mg of benzylpenicillin to benzylpenicilloic acid per hour) at 30 °C and pH 7, provided that the concentration of benzylpenicillin does not fall below the level necessary for enzyme saturation.

The Michaelis constant for benzylpenicillin of the penicillinase in penicillinase solution is approximately 12 µg/mL.

Sterility (*2.6.1*). It complies with the test for sterility.

Storage: at a temperature between 0 °C and 2 °C for 2 to 3 days. When freeze-dried and kept in sealed ampoules, it may be stored for several months.

Pentaerythrityl Tetrakis[3-(3,5-di-*tert*-butyl-4-hydroxyphenyl)propionate] Pentaerythrityl tetrakis [3-(3,5-di(1,1-dimethylethyl)-4-hydroxyphenyl)propionate]; $C_{73}H_{108}O_{12}$ = 1178 (*6683-19-8*)

White or slightly yellow, crystalline powder, practically insoluble in water, very soluble in acetone, soluble in methanol, slightly soluble in hexane.

mp: 110 °C to 125 °C.

α-form: 120 °C to 125 °C.

β-form: 110 °C to 115 °C.

Pentafluoropropanoic Acid $C_3HF_5O_2$ = 164.0 (*422-64-0*)

Clear, colourless liquid.

d_{20}^{20}: about 1.561.

n_D^{20}: about 1.284.

bp: about 97 °C.

Pentafluoropropionic Anhydride Pentafluoropropanoic anhydride; $C_6F_{10}O_3$ = 310.0 (*356-42-3*)

***N*-Pentane** Pentane; C_5H_{12} = 72.2 (*109-66-0*)

Clear, colourless, flammable liquid, very slightly soluble in water, miscible with acetone and with anhydrous ethanol.

d_{20}^{20}: about 0.63.

n_D^{20}: about 1.359.

bp: about 36 °C.

Pentane used in spectrophotometry complies with the following additional test.

Absorbance (*2.2.25*): maximum 0.70 at 200 nm, 0.30 at 210 nm, 0.07 at 220 nm, 0.03 at 230 nm, 0.01 at 240 nm, determined using *water R* as compensation liquid.

1,2-Pentanediol (2*RS*)-Pentane-1,2-diol; $C_5H_{12}O_2$ = 104.2 (*5343-92-0*)

d_4^{20}: about 0.971.

n_D^{20}: about 1.439.

bp: about 201 °C.

3-Pentanone Pentan-3-one; Diethyl ketone; $C_5H_{10}O$ = 86.13 (*96-22-0*)

Pentan-1-ol *N*-Pentyl alcohol; Pentanol; $C_5H_{12}O$ = 88.1 (*71-41-0*)

Colourless liquid, sparingly soluble in water, miscible with ethanol (96 per cent).

n_D^{20}: about 1.410.

bp: about 137 °C.

Pentetic Acid [[(Carboxymethyl)imino] bis(ethylenenitrilo)]tetraacetic acid; White or almost white powder, slightly soluble in water; $C_{14}H_{23}N_3O_{10}$ = 393.3 (*67-43-6*)

mp: 219 °C to 220 °C, with decomposition.

***tert*-Pentyl Alcohol** *tert*-Amyl alcohol; 2-Methyl-2-butanol; $C_5H_{12}O$ = 88.1 (*75-85-4*)

Volatile, flammable liquid, freely soluble in water, miscible with ethanol (96 per cent) and with glycerol.

d_{20}^{20}: about 0.81.

Distillation range (*2.2.11*). Not less than 95 per cent distils between 100 °C and 104 °C.

Storage: protected from light.

Pepsin

A substance containing a proteolytic enzyme of the gastric secretion of animals, diluted, if necessary, by admixture with Lactose or Sucrose. Use a grade of commerce capable of digesting 2500 times its own weight of coagulated egg albumen.

Pepsin Powder (*9001-75-6*)

See *Pepsin powder (0682)*.

Peptide *N*-glycosidase F (*83534-39-8*)

Peptide-N^4-(*N*-acetyl-β-glucosaminyl)asparagine amidase (EC 3.5.1.52). PNGase F.

Perchloric Acid $HClO_4$ = 100.5 (*7601-90-3*)

Content: 70.0 per cent *m/m* to 73.0 per cent *m/m*.

Clear, colourless liquid, miscible with water.

d_{20}^{20}: about 1.7.

Assay. To 2.50 g add 50 mL of *water R* and titrate with *1 M sodium hydroxide*, using 0.1 mL of *methyl red solution R* as indicator.

1 mL of *1 M sodium hydroxide* is equivalent to 100.5 mg of $HClO_4$.

Perchloric Acid Solution

Dilute 8.5 mL of *perchloric acid R* to 100 mL with *water R*.

Perfluoroheptanoic Acid Tridecafluoroheptanoic acid; $C_7HF_{13}O_2$ = 364.1 (*375-85-9*)

Periodic Acetic Acid Solution

Dissolve 0.446 g of *sodium periodate R* in 2.5 mL of a 25 per cent *V/V* solution of *sulfuric acid R*. Dilute to 100.0 mL with *glacial acetic acid R*.

Periodic Acid H_5IO_6 = 227.9 (*10450-60-9*)

Crystals, freely soluble in water and soluble in ethanol (96 per cent).

mp: about 122 °C.

Periodic Acid Reagent

Dissolve 0.5 g of *sodium periodate* in 5 mL of *water*, add 1 mL of 2M *sulfuric acid* and dilute to 10 mL with *water*.

Prepare immediately before use.

Periodic Acid Solution

General reagent grade of commerce containing about 50% w/v of $HIO_4,2H_2O$.

Permethrin $C_{21}H_{20}Cl_2O_3$ = 391.3 (*52645-53-1*)

mp: 34 °C to 35 °C.

A suitable certified reference solution (10 ng/µL in cyclohexane) may be used.

Peroxide Test Strips

Use commercial test strips with a suitable scale in the range from 0 ppm to 25 ppm peroxide.

Peroxyacetic Acid Solution

Dilute 1 mL of *hydrogen peroxide (100 volumes)* to 100 mL with *glacial acetic acid*. Mix and allow to stand for 12 hours before use.

Discard 24 hours after preparation.

Perylene Dibenz[*de,kl*]anthracene; $C_{20}H_{12}$ = 252.3 (*198-55-0*)

Orange powder.

mp: about 279 °C.

Petroleum, Light Petroleum ether 50-70 °C; (*8032-32-4*)

Clear, colourless, flammable liquid without fluorescence, practically insoluble in water, miscible with ethanol (96 per cent).

d_{20}^{20}: 0.661 to 0.664.

Distillation range (*2.2.11*): 50 °C to 70 °C.

Petroleum R1, Light Petroleum ether 40-60 °C

Complies with the requirements prescribed for *light petroleum R*, with the following modifications.

d_{20}^{20}: 0.630 to 0.656.

Distillation range (*2.2.11*): 40 °C to 60 °C. It does not become cloudy at 0 °C.

Petroleum R2, Light Petroleum ether 30-40 °C

Complies with the requirements prescribed for *light petroleum R*, with the following modifications.

d_{20}^{20}: 0.620 to 0.630.

Distillation range (*2.2.11*): 30 °C to 40 °C. It does not become cloudy at 0 °C.

Petroleum R3, Light Petroleum ether 100-120 °C

Complies with the requirements prescribed for *light petroleum R*, with the following modifications.

d_{20}^{20}: about 0.720.

Distillation range (*2.2.11*): 100 °C to 120 °C.

Water (*2.5.12*): maximum 0.03 per cent.

Petroleum R4, Light Petroleum ether 80-100 °C

Complies with the requirements prescribed for *light petroleum R*, with the following modifications.

d_{20}^{20}: about 0.70.

Distillation range (*2.2.11*): 80 °C to 100 °C.

Petroleum Spirit Petroleum ether

Petroleum ether; light petroleum

Analytical reagent grades of commerce.

Colourless, volatile, highly flammable liquids obtained from petroleum, consisting of a mixture of the lower members of the paraffin series of hydrocarbons supplied in the following fractions:

boiling range, 30° to 40°; weight per mL, about 0.63 g,

boiling range, 40° to 60°; weight per mL, about 0.64 g,

boiling range, 50° to 70°; weight per mL, about 0.66 g,

boiling range, 60° to 80°; weight per mL, about 0.67 g,

boiling range, 80° to 100°; weight per mL, about 0.70 g,

boiling range, 100° to 120°; weight per mL, about 0.72 g,

boiling range, 120° to 160°; weight per mL, about 0.75 g.

Petroleum Spirit (boiling range, 40° to 60°), Aromatic-free

Petroleum spirit (boiling range, 40° to 60°) complying with the following additional test.

Light absorption *Absorbance* against air at 235 nm, not more than 0.7, Appendix II B.

pH Indicator Strip

Paper strip, or plastic strip containing multiple segments of different dye-impregnated papers, allowing visual determination of pH in the prescribed range, by comparison with the corresponding master chart.

α-Phellandrene (*R*)-5-Isopropyl-2-methyl-cyclohexa-1,3-diene; (–)-*p*-Mentha-1,5-diene; $C_{10}H_{16}$ = 136.2 (*4221-98-1*)

n_D^{20}: about 1.471.

bp: 171 °C to 174 °C.

α-Phellandrene used in gas chromatography complies with the following additional test.

Assay. Gas chromatography (*2.2.28*) as prescribed in the monograph *Eucalyptus oil (0390)*.

Test solution. The substance to be examined.

Content: 95.0 per cent, calculated by the normalisation procedure.

Phenacetin *p*-Ethoxyacetanilide; $C_{10}H_{13}NO_2$ = 179.2 (*62-44-2*)

mp: about 135°.

General reagent grade of commerce.

Phenanthrene $C_{14}H_{10}$ = 178.2 (*85-01-8*)

White or almost white crystals, practically insoluble in water, sparingly soluble in ethanol (96 per cent).

mp: about 100 °C.

Phenanthroline Hydrochloride 1,10-Phenanthroline hydrochloride monohydrate; $C_{12}H_9ClN_2,H_2O$ = 234.7 (*18851-33-7*)

White or almost white, crystalline powder, freely soluble in water, soluble in ethanol (96 per cent).

mp: about 215 °C, with decomposition.

Phenazone (*60-80-0*)

See *Phenazone (0421)*.

Phenol (*108-95-2*)

See *Phenol (0631)*.

Phenol, Liquefied

General reagent grade of commerce.

A solution of phenol in water containing about 80% w/w of C_6H_6O.

Phenol Red 4,4′-(3H-2,1-Benzoxathiol-3-ylidene) diphenol S,S-dioxide; Phenolsulfonphthalein; (*143-74-8*)

Bright red or dark red, crystalline powder, very slightly soluble in water, slightly soluble in ethanol (96 per cent).

Phenol Red Solution

Dissolve 0.1 g of *phenol red R* in a mixture of 2.82 mL of *0.1 M sodium hydroxide* and 20 mL of *ethanol (96 per cent) R* and dilute to 100 mL with *water R*.

Test for sensitivity. Add 0.1 mL of the phenol red solution to 100 mL of *carbon dioxide-free water R*. The solution is yellow. Not more than 0.1 mL of *0.02 M sodium hydroxide* is required to change the colour to reddish-violet.

Colour change: pH 6.8 (yellow) to pH 8.4 (reddish-violet).

Phenol Red Solution R1

Buffered phenol red solution

Dissolve 33 mg of *phenol red* in 1.5 mL of 2M *sodium hydroxide* and dilute to 100 mL with *water* (solution A). To 250 mL of 2M *sodium hydroxide* add 325 mL of 2M *acetic acid* and 575 mL of *water* (solution B). Mix 25 mL of solution A with 475 mL of solution B.

Phenol Red Solution R2

Solution A. Dissolve 33 mg of *phenol red R* in 1.5 mL of *dilute sodium hydroxide solution R* and dilute to 100 mL with *water R*.

Solution B. Dissolve 25 mg of *ammonium sulfate R* in 235 mL of *water R*; add 105 mL of *dilute sodium hydroxide solution R* and 135 mL of *dilute acetic acid R*.

Add 25 mL of solution A to solution B. If necessary, adjust the pH of the mixture to 4.7.

Phenol Red Solution R3

Solution A. Dissolve 33 mg of *phenol red R* in 1.5 mL of *dilute sodium hydroxide solution R* and dilute to 50 mL with *water R*.

Solution B. Dissolve 50 mg of *ammonium sulfate R* in 235 mL of *water R*; add 105 mL of *dilute sodium hydroxide solution R* and 135 mL of *dilute acetic acid R*.

Add 25 mL of solution A to solution B; if necessary, adjust the pH of the mixture to 4.7.

Phenoldisulfonic Acid Solution

Phenoldisulphonic acid solution

A clear liquid which may develop a pale brown colour on storage, prepared either by heating 3 g of *phenol* with 20 mL of *sulfuric acid* on a water bath for 6 hours and transferring the resulting liquid to a stoppered vessel, or by diluting a 25% w/v solution of commerce with *sulfuric acid* to contain 15% w/v of phenol. The solution complies with the following test.

Sensitivity to nitrate Evaporate a solution containing 0.1 mg of *potassium nitrate* to dryness in a porcelain dish on a water bath. To the cooled residue add 1 mL of the reagent and allow to stand for 10 minutes. Add 10 mL of *water*, cool, add 10 mL of 5M *ammonia* and dilute to 25 mL with *water*. A distinct yellow colour is produced when compared with a solution prepared in the same manner but omitting the potassium nitrate.

Phenolphthalein 3,3-Bis(4-hydroxyphenyl)-3*H*-isobenzofuran-1-one; $C_{20}H_{14}O_4$ = 318.3 (*77-09-8*)

White or yellowish-white powder, practically insoluble in water, soluble in ethanol (96 per cent).

Phenolphthalein Paper

Immerse strips of filter paper for a few minutes in *phenolphthalein solution R*. Allow to dry.

Phenolphthalein Solution

Dissolve 0.1 g of *phenolphthalein R* in 80 mL of *ethanol (96 per cent) R* and dilute to 100 mL with *water R*.

Test for sensitivity. To 0.1 mL of the phenolphthalein solution add 100 mL of *carbon dioxide-free water R*. The solution is colourless. Not more than 0.2 mL of *0.02 M sodium hydroxide* is required to change the colour to pink.

Colour change: pH 8.2 (colourless) to pH 10.0 (red).

Phenolphthalein Solution R1

A 10 g/L solution of *phenolphthalein R* in *ethanol (96 per cent) R*.

Phenolphthalein-Thymol Blue Solution

Dissolve 0.1 g of *thymol blue* in a mixture of 2.2 mL of 0.1M *sodium hydroxide* and 50 mL of *ethanol (96%)* and dilute to 100 mL with *water*. Mix 3 volumes of this solution with 2 volumes of *phenolphthalein solution*.

Phenoxyacetic Acid 2-Phenoxyethanoic acid; $C_8H_8O_3$ = 152.1 (*122-59-8*)

Almost white crystals, sparingly soluble in water, freely soluble in ethanol (96 per cent), and in glacial acetic acid.

mp: about 98 °C.

Chromatography. Thin-layer chromatography (*2.2.27*) as prescribed in the monograph *Phenoxymethylpenicillin (0148)*; the chromatogram shows only one principal spot.

2-Phenoxyaniline 2-Phenoxybenzenamine; 2-Aminophenyl phenyl ether; $C_{12}H_{11}NO$ = 185.2 (*2688-84-8*)**Phenoxybenzamine Hydrochloride** *N*-(2-Chloroethyl)-*N*-(1-methyl-2-phenoxyethyl)-benzylamine hydrochloride; $C_{18}H_{23}Cl_2NO$ = 340.3

Content: 97.0 per cent to 103.0 per cent (dried substance).

White or almost white, crystalline powder, sparingly soluble in water, freely soluble in ethanol (96 per cent).

mp: about 138 °C.

Loss on drying (*2.2.32*): maximum 0.5 per cent, determined by drying over *diphosphorus pentoxide R* at a pressure not exceeding 670 Pa for 24 h.

Assay. Dissolve 0.500 g in 50.0 mL of *ethanol-free chloroform R* and extract with three quantities, each of 20 mL, of *0.01 M hydrochloric acid*. Discard the acid extracts, filter the chloroform layer through cotton and dilute 5.0 mL of the filtrate to 500.0 mL with *ethanol-free chloroform R*. Measure the absorbance of the resulting solution in a closed cell at the maximum at 272 nm. Calculate the content of $C_{18}H_{23}Cl_2NO$, taking the specific absorbance to be 56.3.

Storage: protected from light.

2-Phenoxyethanol Phenoxyethanol; $C_8H_{10}O_2$ = 138.2 (*122-99-6*)

Clear, colourless, oily liquid, slightly soluble in water, freely soluble in ethanol (96 per cent).

d_{20}^{20}: about 1.11.

n_D^{20}: about 1.537.

Freezing point (*2.2.18*): minimum 12 °C.

Phenyl Benzoate $C_{13}H_{10}O_2$ = 198.2 (*93-99-2*)

mp: about 70°.

General reagent grade of commerce.

Phenyl Isothiocyanate C_7H_5NS = 135.2 (*103-72-0*)

Liquid, insoluble in water, soluble in ethanol (96 per cent).

d_{20}^{20}: about 1.13.

n_D^{20}: about 1.65.

bp: about 221 °C.

mp: about −21 °C.

Use a grade suitable for protein sequencing.

Phenyl(5)methyl(95)polysiloxane

Polysiloxane substituted with 5 per cent of phenyl groups and 95 per cent of methyl groups.

Phenyl(5)methyl(95)polysiloxane, Base-deactivated

Base-deactivated polysiloxane substituted with 5 per cent of phenyl groups and 95 per cent of methyl groups.

Phenyl(50)methyl(50)polysiloxane

Polysiloxane substituted with 50 per cent of phenyl groups and 50 per cent of methyl groups.

Phenylacetic Acid $C_8H_8O_2$ = 136.2 (*103-82-2*)

White or almost white powder, soluble in water.

bp: about 265 °C.

mp: about 75 °C.

L-Phenylalanine Phenylalanine; (*63-91-2*)

See *Phenylalanine (0782)*.

***o*-Phenylbenzoic Acid** 2-Biphenylcarboxylic acid; $C_{13}H_{10}O_2$ = 198 (*947-84-2*)

mp: about 113°.

General reagent grade of commerce.

2-Phenylbutanoic acid (2*RS*)-2-Phenylbutanoic acid (primidone impurity E); $C_{10}H_{12}O_2$ = 164.2 (*90-27-7*)

General reagent grade of commerce containing > 95.0% of $C_{10}H_{12}O_2$

(2-Phenylbutanoyl)urea *N*-Carbamoyl-2-phenylbutanamide; 2-Phenylbutyrylurea; Pheneturide; $C_{11}H_{14}N_2O_2$ = 206.2 (*90-49-3*)

General reagent grade of commerce containing > 95.0% of $C_{11}H_{14}N_2O_2$.

(*E*)-4-Phenylbut-3-en-2-one Benzalacetone; $C_{10}H_{10}O$ = 146.2 (*122-57-6*)

White or pale yellow mass.

Content: minimum 98.0 per cent.

bp: about 261 °C.

mp: about 39 °C.

***N*-Phenylcarbazole** $C_{18}H_{13}N$ = 243.3 (*1150-62-5*)

mp: about 96°.

General reagent grade of commerce.

A white to pale tan, crystalline powder.

***p*-Phenylenediamine Dihydrochloride** 1,4-Diaminobenzene dihydrochloride; $C_6H_{10}Cl_2N_2$ = 181.1 (*624-18-0*)

Crystalline powder or white or slightly coloured crystals, turning reddish on exposure to air, freely soluble in water, slightly soluble in ethanol (96 per cent).

D-Phenylglycine (2*R*)-2-Amino-2-phenylacetic acid; $C_8H_9NO_2$ = 151.2 (*875-74-1*)

Content: minimum 99 per cent.

White or almost white, crystalline powder.

DL-Phenylglycine 2-Amino-2-phenylacetic acid; α-Phenylglycine; $C_8H_9NO_2$ = 151.2 (*2835-06-5*)

Phenylhydrazine $C_6H_8N_2$ = 108.1 (*100-63-0*)

White or almost white, crystalline powder, becoming yellow or dark red on exposure to air, melting at room temperature giving an oily liquid, miscible with anhydrous ethanol, sparingly soluble in water.

bp: about 244 °C, with decomposition.

mp: about 20 °C.

Phenylhydrazine Hydrochloride Phenylhydrazinium chloride; $C_6H_9ClN_2$ = 144.6 (*59-88-1*)

White or almost white, crystalline powder, becoming brown on exposure to air, soluble in water and in ethanol (96 per cent).

mp: about 245 °C, with decomposition.

Storage: protected from light.

Phenylhydrazine Hydrochloride Solution Strong phenylhydrazine hydrochloride solution

Dissolve 0.9 g of *phenylhydrazine hydrochloride R* in 50 mL of *water R*. Decolorise with *activated charcoal R* and filter. To the filtrate add 30 mL of *hydrochloric acid R* and dilute to 250 mL with *water R*.

Phenylhydrazine-Sulfuric Acid Solution Phenylhydrazine-sulphuric acid solution

Dissolve 65 mg of *phenylhydrazine hydrochloride R*, previously recrystallised from *ethanol (85 per cent V/V) R*, in a mixture of 80 volumes of *water R* and 170 volumes of *sulfuric acid R* and dilute to 100 mL with the same mixture of solvents. Prepare immediately before use.

1-Phenylpiperazine $C_{10}H_{14}N_2$ = 162.2 (*92-54-6*)

Slightly viscous, yellow liquid, not miscible with water.

d_4^{20}: about 1.07.

n_D^{20}: about 1.588.

3-Phenylpropanoic Acid Dihydrocinnamic acid; $C_6H_5CH_2CH_2CO_2H$ = 150.17 (*501-52-0*)

mp: 47° - 50°.

White to off-white powder or crystals.

Analytical reagent grade of commerce.

1-Phenylpropan-2-ol (2*RS*)-1-Phenylpropan-2-ol; $C_9H_{12}O$ = 136.2 (*698-87-3*)

mp: 65 °C to 67 °C.

1-Phenyl-1,2,3,4-tetrahydroisoquinoline $C_{15}H_{15}N$ = 209.3 (*22990-19-8*)

Phloroglucide 2,3′,4,5′,6-Biphenylpentol; $C_{12}H_{10}O_5$ = 234.2 (*491-45-2*)

White or almost white powder, hygroscopic, light sensitive. Slowly discolours on exposure to light.

Phloroglucinol Benzene-1,3,5-triol; $C_6H_6O_3,2H_2O$ = 162.1 (*6099-90-7*)

White or yellowish crystals, slightly soluble in water, soluble in ethanol (96 per cent).

mp: about 223 °C (instantaneous method).

Phloroglucinol Solution

To 1 mL of a 100 g/L solution of *phloroglucinol R* in *ethanol (96 per cent) R*, add 9 mL of *hydrochloric acid R*.

Storage: protected from light.

Phosalone $C_{12}H_{15}ClNO_4PS_2$ = 367.8 (*2310-17-0*)

mp: 45 °C to 48 °C

A suitable certified reference solution (10 ng/µL in iso-octane) may be used.

Phospholipid

Wash a quantity of human or bovine brain freed from meninges and blood vessels and macerate in a suitable blender. Weigh 1000 to 1300 g of the macerate and measure its volume (*V* mL). Extract with three quantities, each of 4*V* mL, of *acetone*, filter by suction and dry the precipitate at 37° for 18 hours. Extract the dried precipitate with two quantities, each of 2*V mL*, of a mixture of 2 volumes of *petroleum spirit (boiling range, 30° to 40°)* and 3 volumes of *petroleum spirit (boiling range, 40° to 60°)*, filtering each extract through a filter paper previously washed with the petroleum spirit mixture. Combine the extracts and evaporate to dryness at 45° at a pressure not exceeding 0.7 kPa. Dissolve the residue in 0.2*V* mL of *ether* and allow to stand at 4° until a deposit is produced. Centrifuge and evaporate the clear supernatant liquid under reduced pressure until the volume is about 100 mL per kg of the original macerate. Allow to stand at 4° until a precipitate is produced (12 to 24 hours) and centrifuge. To the clear supernatant liquid add 5 volumes of *acetone*, centrifuge, discard the supernatant liquid, dry the precipitate and store protected from light in a vacuum desiccator.

Phosphomolybdic Acid Dodecamolybdophosphoric acid; $12MoO_3,H_3PO_4,xH_2O$ (*51429-74-4*)

Orange-yellow, fine crystals, freely soluble in water, soluble in ethanol (96 per cent).

Phosphomolybdic Acid Solution

Dissolve 4 g of *phosphomolybdic acid R* in *water R* and dilute to 40 mL with the same solvent. Add cautiously and with cooling 60 mL of *sulfuric acid R*. Prepare immediately before use.

Phosphomolybdic Acid Solution, Ethanolic

A 20% w/v solution of *phosphomolybdic acid* in *ethanol (96%)*.

Prepare immediately before use.

Phosphomolybdotungstic Reagent Lithium and sodium molybdotungstophosphate solution; Folin Ciocalteau phenol reagent of commerce

Dissolve 100 g of *sodium tungstate R* and 25 g of *sodium molybdate R* in 700 mL of *water R*. Add 100 mL of *hydrochloric acid R* and 50 mL of *phosphoric acid R*. Heat the mixture under a reflux condenser in a glass apparatus for 10 h. Add 150 g of *lithium sulfate R*, 50 mL of *water R* and a few drops of *bromine R*. Boil to remove the excess of bromine (15 min), allow to cool, dilute to 1000 mL with *water R* and filter. The reagent should be yellow in colour. If it acquires a greenish tint, it is unsatisfactory for use but may be regenerated by boiling with a few drops of *bromine R*. Care must be taken to remove the excess of bromine by boiling.

Storage: at 2 °C to 8 °C.

Phosphomolybdotungstic Reagent, Dilute

To 1 volume of *phosphomolybdotungstic reagent R* add 2 volumes of *water R*.

Phosphoric Acid, Dilute See *Dilute phosphoric acid (0005)*.

Phosphoric Acid, Dilute R1

Dilute 93 mL of *dilute phosphoric acid R* to 1000 mL with *water R*.

Phosphorus Pentoxide Diphosphorus pentoxide; P_2O_5 = 141.9 (*1314-56-3*)

White or almost white powder, amorphous, deliquescent. It is hydrated by water with the evolution of heat.

Storage: in an airtight container.

Phosphotungstic Acid Solution

Heat under a reflux condenser for 3 h, 10 g of *sodium tungstate R* with 8 mL of *phosphoric acid R* and 75 mL of *water R*. Allow to cool and dilute to 100 mL with *water R*.

Phthalaldehyde Benzene-1,2-dicarboxaldehyde; $C_8H_6O_2$ = 134.1 (*643-79-8*)

Yellow, crystalline powder.

mp: about 55 °C.

Storage: protected from light and air.

Phthalaldehyde Reagent

Dissolve 2.47 g of *boric acid R* in 75 mL of *water R*, adjust to pH 10.4 using a 450 g/L solution of *potassium hydroxide R* and dilute to 100 mL with *water R*. Dissolve 1.0 g of *phthalaldehyde R* in 5 mL of *methanol R*, add 95 mL of the boric acid solution and 2 mL of *thioglycollic acid R* and adjust to pH 10.4 with a 450 g/L solution of *potassium hydroxide R*.

Storage: protected from light; use within 3 days.

Phthalazine $C_8H_6N_2$ = 130.1 (*253-52-1*)

Pale yellow crystals, freely soluble in water, soluble in anhydrous ethanol, in ethyl acetate and in methanol.

mp: 89 °C to 92 °C.

Phthalein Purple Metalphthalein; 2,2′,2″,2‴-[*o*-Cresolphthalein-3′,3″-bis(methylenenitrilo)]tetra-acetic acid; (1,3-Dihydro-3-oxo-isobenzofuran-1-ylidene)bis[(6-hydroxy-5-methyl-3,1-phenylene)bis(methyleneimino)diacetic acid]; $C_{32}H_{32}N_2O_{12},xH_2O$ = 637, anhydrous substance (*2411-89-4*)

Yellowish-white or brownish powder, practically insoluble in water, soluble in ethanol (96 per cent). The product may be found in commerce in the form of the sodium salt: a yellowish-white to pink powder, soluble in water, practically insoluble in ethanol (96 per cent).

Test for sensitivity. Dissolve 10 mg in 1 mL of *concentrated ammonia R* and dilute to 100 mL with *water R*. To 5 mL of the solution add 95 mL of *water R*, 4 mL of *concentrated ammonia R*, 50 mL of *ethanol (96 per cent) R* and 0.1 mL of *0.1 M barium chloride*. The solution is blue-violet. Add 0.15 mL of *0.1 M sodium edetate*. The solution becomes colourless.

Phthalic Acid Benzene-1,2-dicarboxylic acid; $C_8H_6O_4$ = 166.1 (*88-99-3*)

White or almost white, crystalline powder, soluble in hot water and in ethanol (96 per cent).

Phthalic Anhydride Isobenzofuran-1,3-dione; $C_8H_4O_3$ = 148.1 (*85-44-9*)

Content: minimum 99.0 per cent.

White or almost white flakes.

mp: 130 °C to 132 °C.

Assay. Dissolve 2.000 g in 100 mL of *water R* and boil under a reflux condenser for 30 min. Cool and titrate with *1 M sodium hydroxide*, using *phenolphthalein solution R* as indicator.

1 mL of *1 M sodium hydroxide* is equivalent to 74.05 mg of $C_8H_4O_3$.

Phthalic Anhydride Solution

Dissolve 42 g of *phthalic anhydride R* in 300 mL of *anhydrous pyridine R*. Allow to stand for 16 h.

Storage: protected from light; use within 1 week.

Picein 1-[4-(β-D-Glucopyranosyloxy)phenyl]ethanone; *p*-(Acetylphenyl)-β-D-glucopyranoside; $C_{14}H_{18}O_7$ = 298.3 (*530-14-3*)

mp: 194 °C to 195 °C.

Picric Acid 2,4,6-Trinitrophenol; $C_6H_3N_3O_7$ = 229.1 (*88-89-1*)

Yellow prisms or plates, soluble in water and in ethanol (96 per cent).

Storage: moistened with *water R*.

Picric Acid Solution

A 10 g/L solution of *picric acid R*.

Picric Acid Solution R1

Prepare 100 mL of a saturated solution of *picric acid R* and add 0.25 mL of *strong sodium hydroxide solution R*.

Picrolonic Acid 3-Methyl-4-nitro-1-(4-nitrophenyl)-5-pyrazolone; $C_{10}H_8N_4O_5$ = 264.2 (*550-74-3*)

mp: about 116°.

General reagent grade of commerce.

Complies with the following test.

Sensitivity Dissolve 25 mg in 10 mL of warm *water* containing 0.1 mL of *glacial acetic acid*; to 1 mL of this solution add 1 mL of a 0.05% w/v solution of *calcium chloride* previously heated to 60°. A bulky precipitate is produced within 5 minutes.

A yellow or brownish yellow, crystalline powder.

Picrotin (1*R*,3*R*,5*S*,8*S*,9*R*,12*S*,13*R*,14*S*)-1-hydroxy-14-(2-hydroxypropan-2-yl)-13-methyl-4,7,10-trioxapentacyclo [6.4.1.1.^{9,12}.0^{3,5}.0^{5,13}]tetradecane-6,11-dione; $C_{15}H_{18}O_7$ = 310.3 (*21416-53-5*)

White or colourless crystalline powder or crystals, soluble in boiling water and in ethanol (96 per cent), practically insoluble in methylene chloride.

mp: 248 °C to 250 °C.

Picrotoxinin (1*R*,3*R*,5*S*,8*S*,9*R*,12*S*,13*R*,14*R*)-1-hydroxy-13-methyl-14-(prop-1-en-2-yl)-4,7,10-trioxapentacyclo [6.4.1.1^{9,12}.0^{3,5}.0^{5,13}]tetradecane-6,11-dione; $C_{15}H_{16}O_6$ = 292.2 (*17617-45-7*)

White or colourless crystalline powder or crystals, soluble in methylene chloride, in ethanol (96 per cent) and in alkaline solutions.

mp: 207 to 210 °C.

α-Pinene (1*R*,5*R*)-2,6,6-Trimethylbicyclo[3.1.1]hept-2-ene; $C_{10}H_{16}$ = 136.2 (*7785-70-8*)

Liquid not miscible with water.

d_{20}^{20}: about 0.859.

n_D^{20}: about 1.466.

bp: 154 °C to 156 °C.

α-Pinene used in gas chromatography complies with the following additional test.

Assay. Gas chromatography (*2.2.28*) as prescribed in the monograph *Bitter-orange-flower oil (1175)*.

Test solution. The substance to be examined.

Content: minimum 99.0 per cent, calculated by the normalisation procedure.

β-Pinene 6,6-Dimethyl-2-methylenebicyclo[3.1.1]heptane; $C_{10}H_{16}$ = 136.2 (*127-91-3*)

Colourless, oily liquid, odour reminiscent of turpentine, practically insoluble in water, miscible with ethanol (96 per cent).

β-Pinene used in gas chromatography complies with the following additional test.

Assay. Gas chromatography (*2.2.28*) as prescribed in the monograph *Bitter-orange-flower oil (1175)*.

Test solution. The substance to be examined.

Content: minimum 95.0 per cent.

1,4-Piperazinediethanesulfonic Acid Piperazine-1,4-bis(2-ethanesulfonic acid); 2,2′-(Piperazine-1,4-diyl) bis(ethanesulfonic acid); Piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); PIPES; $C_8H_{18}N_2O_6S_2$ = 302.4 (*5625-37-6*)

Content: minimum 99 per cent.

White, crystalline powder.

Piperazine Dipicrate Solution

Dissolve 0.2 g of *piperazine hydrate* in 3.5 mL of 0.5M *sulfuric acid* and 10 mL of *water*. Add 100 mL of *picric acid solution R1*, heat on a water bath for 15 minutes, cool and filter. Wash the precipitate with *water* until the washings are free from sulfate. Shake the precipitate with *water* to produce a saturated solution and filter.

Piperazine Hydrate (*142-63-2*)

See *Piperazine hydrate (0425)*.

Piperidine Hexahydropyridine; $C_5H_{11}N$ = 85.2 (*110-89-4*)

Colourless to slightly yellow, alkaline liquid, miscible with water, with ethanol (96 per cent) and with light petroleum.

bp: about 106 °C.

Piperine (2*E*,4*E*)-1-(Piperidin-1-yl)-5-(1,3-benzodioxol-5-yl)penta-2,4-dien-1-one; 1-Piperoyl-piperidine; 1-[(2*E*,4*E*)-5-(3,4-Methylenedioxyphenyl)-1-oxo-2,4-pentadienyl] piperidine; $C_{17}H_{19}NO_3$ = 285.3 (*94-62-2*)

Piperitone 6-Isopropyl-3-methyl-cyclohex-2-en-1-one; $C_{10}H_{16}O$ = 152.2 (*89-81-6*)

Pirimiphos-ethyl $C_{13}H_{24}N_3O_3PS$ = 333.4 (*23505-41-1*)

mp: 15 °C to 18 °C.

A suitable certified reference solution (10 ng/μL in cyclohexane) may be used.

Plasma, Citrated Rabbit

Collect blood by intracardiac puncture from a rabbit that has been fasted for 12 hours prior to the collection, using a plastic syringe with a No. 1 needle containing a suitable volume of a 3.8% w/v solution of *sodium citrate* so that the final volume ratio of citrate solution to blood is 1:9. Separate the plasma by centrifugation at 1500 to 1800 *g* at 15° to 20° for 30 minutes.

Store at 0° to 6° and use within 4 hours of collection.

Plasma, Platelet-poor

Withdraw 45 mL of human blood into a 50 mL plastic syringe containing 5 mL of a sterile 38 g/L solution of *sodium citrate R*. Without delay, centrifuge at 1500 *g* at 4 °C for 30 min. Remove the upper two-thirds of the supernatant plasma using a plastic syringe and without delay centrifuge at 3500 *g* at 4 °C for 30 min. Remove the upper two-thirds of the liquid and freeze it rapidly in suitable amounts in plastic tubes at or below −40 °C. Use plastic or silicone-treated equipment.

Plasma Substrate Substrate plasma

Separate the plasma from human or bovine blood collected into one-ninth its volume of a 38 g/L solution of *sodium*

citrate R, or into two-sevenths its volume of a solution containing 20 g/L of *disodium hydrogen citrate R* and 25 g/L of *glucose R*. With the former, prepare the substrate on the day of collection of the blood. With the latter, prepare within two days of collection of the blood.

Storage: at −20 °C.

Plasma Substrate Deficient in Factor V

Use preferably a plasma which is congenitally deficient, or prepare it as follows: separate the plasma from human blood collected into one tenth of its volume of a 13.4 g/L solution of *sodium oxalate R*. Incubate at 37 °C for 24 h to 36 h. The coagulation time determined by the method prescribed for *coagulation factor V solution R* should be 70 s to 100 s. If the coagulation time is less than 70 s, incubate again for 12 h to 24 h.

Storage: in small quantities at a temperature of −20 °C or lower.

Factor VII-deficient Plasma

Plasma that is deficient in factor VII.

Plasma Substrate R1 Substrate plasma R1

Use water-repellent equipment (made from materials such as suitable plastics or suitably silicone-treated glass) for taking and handling blood.

Collect a suitable volume of blood from each of at least five sheep; a 285 mL volume of blood collected into 15 mL of anticoagulant solution is suitable but smaller volumes may be collected, taking the blood, either from a live animal or at the time of slaughter, using a needle attached to a suitable cannula which is long enough to reach the bottom of the collecting vessel. Discarding the first few millilitres and collecting only free-flowing blood, collect the blood in a sufficient quantity of an anticoagulant solution containing 8.7 g of *sodium citrate R* and 4 mg of *aprotinin R* per 100 mL of *water R* to give a final ratio of blood to anticoagulant solution of 19 to 1. During and immediately after collection, swirl the flask gently to ensure mixing but do not allow frothing to occur. When collection is complete, close the flask and cool to 10-15 °C. When cold, pool the contents of all the flasks with the exception of any that show obvious haemolysis or clots and keep the pooled blood at 10-15 °C.

As soon as possible and within 4 h of collection, centrifuge the pooled blood at 1000-2000 *g* at 10-15 °C for 30 min. Separate the supernatant and centrifuge it at 5000 *g* for 30 min. (Faster centrifugation, for example 20 000 *g* for 30 min, may be used if necessary to clarify the plasma, but filtration procedures should not be used.) Separate the supernatant and, without delay, mix thoroughly and distribute the plasma substrate into small stoppered containers in portions sufficient for a complete heparin assay (for example 10 mL to 30 mL). Without delay, rapidly cool to a temperature below −70 °C (for example by immersing the containers into liquid nitrogen) and store at a temperature below −30 °C.

The plasma is suitable for use as plasma substrate in the assay for heparin if, under the conditions of the assay, it gives a clotting time appropriate to the method of detection used and if it provides reproducible, steep log dose-response curves.

When required for use, thaw a portion of the plasma substrate in a water-bath at 37 °C, gently swirling until thawing is complete; once thawed it should be kept at 10-20 °C and used without delay. The thawed plasma substrate may be lightly centrifuged if necessary; filtration procedures should not be used.

Plasma Substrate R2

Prepare from human blood containing less than 1 per cent of the normal amount of factor IX. Collect the blood into one-ninth its volume of a 38 g/L solution of *sodium citrate R*.

Storage: in small amounts in plastic tubes at a temperature of −30 °C or lower.

Plasma Substrate R3

Prepare from human blood containing less than 1 per cent of the normal amount of factor XI. Collect the blood into one-ninth its volume of a 38 g/L solution of *sodium citrate R*.

Storage: in small amounts in plastic tubes at a temperature of −30 °C or lower.

Plasminogen, Human (*9001-91-6*)

A substance present in blood that may be activated to plasmin, an enzyme that lyses fibrin in blood clots.

Platelet Substitute

To 0.5 to 1 g of *phospholipid* add 20 mL of *acetone* and allow to stand for 2 hours with frequent shaking. Centrifuge for 2 minutes and discard the supernatant liquid. Dry the residue using a water pump, mix with 20 mL of *chloroform* and shake for 2 hours. Filter under vacuum and suspend the residue obtained in 5 to 10 mL of *saline solution*.

Prepare a dilution in *saline solution* so that it will give clotting time differences between consecutive dilutions of the reference preparation used in the Assay of factor IX fraction of about 10 seconds.

Store the dilute suspensions at −30° and use within 6 weeks.

Plutonium-242 Spiking Solution

Contains 50 Bq/L ^{242}Pu and a 134 mg/L solution of *lanthanum chloride heptahydrate R* in a 284 g/L solution of *nitric acid R*.

Poloxamer 124

General reagent grade of commerce.

Poloxamer 188 See *Poloxamers (1464)*.

Polyamine Grafted Poly(vinyl alcohol) Copolymer

Copolymer beads of poly(vinyl alcohol) to which polyamine is covalently bonded; The size range of the beads is specified after the name of the reagent in the tests where it is used;

Polydatin 3-Hydroxy-5-[2-(4-hydroxyphenyl)eth-1-en-1-yl] phenyl β-D-glucopyranoside; Resveratrol-3-β-mono-D-glucoside; $C_{20}H_{22}O_8$ = 390.4 (*65914-17-2*)

Polyether Hydroxylated Gel for Chromatography

Gel with a small particle size having a hydrophilic surface with hydroxyl groups. It has an exclusion limit for dextran of relative molecular mass 2×10^5 to 2.5×10^6.

Polyethylene Glycol 200 Macrogol 200; (*25322-68-3*)

Clear, colourless or almost colourless viscous liquid, very soluble in acetone and in anhydrous ethanol, practically insoluble in fatty oils.

d_{20}^{20}: about 1.127.

n_D^{20}: about 1.450.

Polyethylene Glycol 200 R1 Macrogol 200 R1

Introduce 500 mL of *macrogol 200 R* into a 1000 mL round bottom flask. Using a rotation evaporator remove any volatile components applying for 6 h a temperature of 60 °C and a vacuum with a pressure of 1.5-2.5 kPa.

Polyethylene Glycol 300 Macrogol 300; (*25322-68-3*)

See *Macrogols (1444)*.

Polyethylene Glycol 400 Macrogol 400; (*25322-68-3*)

See *Macrogols (1444)*.

Polyethylene Glycol 1000 Macrogol 1000; (*25322-68-3*)

See *Macrogols (1444)*.

Polyethylene Glycol 1500 Macrogol 1500; (*25322-68-3*)

See *Macrogols (1444)*.

Polyethylene Glycol 20,000 Macrogol 20 000

See *Macrogols (1444)*.

Polyethylene Glycol Adipate Macrogol adipate; Polyethyleneglycol adipate; $(C_8H_{12}O_4)_n$ = M_r $(172.2)_n$

White or almost white, wax-like mass, practically insoluble in water.

mp: about 43 °C.

Polyethylene Glycol Succinate Macrogol succinate; Polyethyleneglycol succinate; $(C_6H_8O_4)_n$ = M_r $(144.1)_n$

White or almost white, crystalline powder, practically insoluble in water.

mp: about 102 °C.

Polymethacrylate Gel

A methacrylate-based size-exclusion stationary phase for water-soluble samples.

Butylated Polymethacrylate Gel

Gel based on butylated methacrylic acid polymer.

Polymethacrylate Gel, Hydroxylated

Stationary phase for size-exclusion chromatography.

Gel based on hydroxylated methacrylic acid polymer.

Polyorganosiloxane for Oxygen-Containing Compounds

Combination of suitable polyorganosiloxanes with high affinity for oxygen-containing compounds.

Polyoxyethylated Castor Oil

Light yellow liquid. It becomes clear above 26 °C.

Polyoxyethylene 10 Lauryl Ether Decaethylene glycol monododecyl ether; $C_{32}H_{66}O_{11}$ = 626.9 (*9002-92-0*)

bp: about 100°

mp: about 24°

General reagent grade of commerce.

Polyoxyethylene 23 Lauryl Ether Brij 35; $C_{58}H_{120}O_{24}$ = 1199.6 (*9002-92-0*)

bp: about 100°.

mp: about 43°.

General reagent grade of commerce.

Polysorbate 20 (*9005-64-5*)

See *Polysorbate 20 (0426)*.

Polysorbate 65 (*9005-71-4*)

Polysorbate 80 (*9005-65-6*)

See *Polysorbate 80 (0428)*.

Polystyrene 900–1000 (*9003-53-6*)

Organic standard used for calibration in gas chromatography.

M_w: about 950.

M_w/M_n: 1.10.

Potassium Acetate (*127-08-2*)

See *Potassium acetate (1139)*.

Potassium Antimonate(v) Potassium pyroantimonate; $KSb(OH)_6$ = 262.9 (*12208-13-8*)

White or almost white, crystals or crystalline powder, sparingly soluble in water.

Potassium Antimonate(v) Solution Potassium pyroantimonate solution

Dissolve 2 g of *potassium pyroantimonate R* in 95 mL of hot *water R*. Cool quickly and add a solution containing 2.5 g of *potassium hydroxide R* in 50 mL of *water R* and 1 mL of *dilute sodium hydroxide solution R*. Allow to stand for 24 h, filter and dilute to 150 mL with *water R*.

Potassium Bicarbonate (*298-14-6*)

See *Potassium hydrogen carbonate R*.

Potassium Bicarbonate Solution, Saturated Methanolic See *potassium hydrogen carbonate solution, saturated methanolic R*.

Potassium Borohydride Potassium tetrahydroborate; KBH_4 = 53.94 (*13762-51-1*)

General reagent grade of commerce.

Potassium Bromate $KBrO_3$ = 167.0 (*7758-01-2*)

White or almost white granular powder or crystals, soluble in water, slightly soluble in ethanol (96 per cent).

Potassium Bromide (*7758-02-3*)

See *Potassium bromide (0184)*.

Potassium bromide used for infrared absorption spectrophotometry (2.2.24) also complies with the following additional test.

A disc 2 mm thick prepared from the substance previously dried at 250 °C for 1 h, has a substantially flat baseline over the range 4000 cm^{-1} to 620 cm^{-1}. It exhibits no maxima with absorbance greater than 0.02 above the baseline, except maxima for water at 3440 cm^{-1} and 1630 cm^{-1}.

Potassium Carbonate Anhydrous potassium carbonate; K_2CO_3 = 138.2 (*584-08-7*)

White or almost white, granular powder, hygroscopic, very soluble in water, practically insoluble in anhydrous ethanol.

Storage: in an airtight container.

Potassium Carbonate Sesquihydrate $K_2CO_3,1½H_2O$ = 165.2 (*6381-79-9*)

General reagent grade of commerce.

Potassium Chlorate $KClO_3$ = 122.6 (*3811-04-9*)

A white or almost white powder, granules or crystals, soluble in water.

Potassium Chloride (*7447-40-7*)

See *Potassium chloride (0185)*.

Potassium chloride used for infrared absorption spectrophotometry (2.2.24) also complies with the following additional test.

A disc 2 mm thick, prepared from the substance previously dried at 250 °C for 1 h, has a substantially flat baseline over the range 4000 cm^{-1} to 620 cm^{-1}. It exhibits no maxima with absorbance greater than 0.02 above the baseline, except maxima for water at 3440 cm^{-1} and 1630 cm^{-1}.

Potassium Chloride, 0.1M

A solution of *potassium chloride R* containing the equivalent of 7.45 g of KCl in 1000.0 mL.

Potassium Chromate Dipotassium chromate; K_2CrO_4 = 194.2 (*7789-00-6*)

Yellow crystals, freely soluble in water.

Potassium Chromate Solution

A 50 g/L solution of *potassium chromate R*.

Potassium Citrate (*6100-05-6*)

See *Potassium citrate (0400)*.

Potassium Cyanide KCN = 65.1 (*151-50-8*)

White or almost white, crystalline powder or white or almost white mass or granules, freely soluble in water, slightly soluble in ethanol (96 per cent).

Potassium Cyanide Solution

A 100 g/L solution of *potassium cyanide R*.

Potassium Cyanide Solution, Lead-free

Dissolve 10 g of *potassium cyanide R* in 90 mL of *water R*, add 2 mL of *strong hydrogen peroxide solution R* diluted 1 to 5. Allow to stand for 24 h, dilute to 100 mL with *water R* and filter.

The solution complies with the following test: take 10 mL of the solution, add 10 mL of *water R* and 10 mL of *hydrogen sulfide solution R*. No colour is evolved even after addition of 5 mL of *dilute hydrochloric acid R*.

Potassium Cyanide Solution PbT

Dissolve 10 g of *potassium cyanide* in 90 mL of *water*, add 2 mL of *hydrogen peroxide solution (20 vol)*, allow to stand for 24 hours, dilute to 100 mL with *water* and filter.

Potassium Dichromate Dipotassium dichromate; $K_2Cr_2O_7$ = 294.2 (*7778-50-9*)

Potassium dichromate used for the calibration of spectrophotometers (*2.2.25*) contains not less than 99.9 per cent of $K_2Cr_2O_7$, calculated with reference to the substance dried at 130 °C.

Orange-red crystals, soluble in water, practically insoluble in ethanol (96 per cent).

Assay. Dissolve 1.000 g in *water R* and dilute to 250.0 mL with the same solvent. To 50.0 mL of this solution add a freshly prepared solution of 4 g of *potassium iodide R*, 2 g of *sodium hydrogen carbonate R* and 6 mL of *hydrochloric acid R* in 100 mL of *water R* in a 500 mL flask. Stopper the flask and allow to stand protected from light for 5 min. Titrate with *0.1 M sodium thiosulfate*, using 1 mL of *iodide-free starch solution R* as indicator.

1 mL of *0.1 M sodium thiosulfate* is equivalent to 4.903 mg of $K_2Cr_2O_7$.

Potassium Dichromate Solution

A 106 g/L solution of *potassium dichromate R*.

Potassium Dichromate Solution, Dilute

A 7.0% w/v solution of *potassium dichromate*.

Potassium Dichromate Solution R1

A 5 g/L solution of *potassium dichromate R*.

Potassium Dihydrogen Citrate $C_6H_7KO_7$ = 230.2

General reagent grade of commerce.

Potassium Dihydrogen Orthophosphate Potassium dihydrogen phosphate; (*7778-77-0*)

See *Potassium dihydrogen phosphate (0920)*.

Potassium Dihydrogen Phosphate, 0.2M

A solution of *potassium dihydrogen phosphate R* containing the equivalent of 27.22 g of KH_2PO_4 in 1000.0 mL.

Potassium Ferriperiodate Solution

Dissolve 1 g of *potassium periodate R* in 5 mL of a freshly prepared 120 g/L solution of *potassium hydroxide R*. Add 20 mL of *water R* and 1.5 mL of *ferric chloride solution R1*. Dilute to 50 mL with a freshly prepared 120 g/L solution of *potassium hydroxide R*.

Potassium Fluoride KF = 58.1 (*7789-23-3*)

Colourless crystals or white or almost white crystalline powder, deliquescent, soluble in water, practically insoluble in ethanol (96 per cent).

Potassium Hexacyanoferrate(II) Potassium ferrocyanide; $K_4[Fe(CN)_6],3H_2O$ = 422.4 (*14459-95-1*)

Transparent yellow crystals, freely soluble in water, practically insoluble in ethanol (96 per cent).

Potassium Hexacyanoferrate(III) Potassium ferricyanide; $K_3[Fe(CN)_6]$ = 329.3 (*13746-66-2*)

Red crystals, freely soluble in water.

Potassium Hexacyanoferrate(II) Solution Potassium ferrocyanide solution

A 53 g/L solution of *potassium ferrocyanide R*.

Potassium Hexacyanoferrate(III) Solution Potassium ferricyanide solution

Wash 5 g of *potassium ferricyanide R* with a little *water R*, dissolve and dilute to 100 mL with *water R*. Prepare immediately before use.

Potassium Hexacyanoferrate(III) Solution, Dilute

Wash about 1 g of *potassium hexacyanoferrate(III)* crystals with a little *water* and dissolve the washed crystals in 100 mL of *water*.

Produces a blue colour with solutions of iron(II) salts.

Prepare immediately before use.

Potassium Hyaluronate (*31799-91-4*)

General reagent grade of commerce obtained from human umbilical cords and freeze dried.

Protein, not more than 2%; chondroitin sulfate, not more than 3%.

Potassium Hyaluronate Stock Solution

A 0.05% w/v solution of *potassium hyaluronate*.

Store below 0° and use within 30 days.

Potassium Hydrogen Carbonate Potassium bicarbonate; $KHCO_3$ = 100.1 (*298-14-6*)

Transparent, colourless crystals, freely soluble in water, practically insoluble in ethanol (96 per cent).

Potassium Hydrogen Carbonate Solution, Saturated Methanolic

Dissolve 0.1 g of *potassium hydrogen carbonate R* in 0.4 mL of *water R*, heating on water-bath. Add 25 mL of *methanol R* and swirl, keeping the solution on the water-bath until dissolution is complete. Use a freshly prepared solution.

Potassium Hydrogen Phthalate Potassium hydrogen benzene-1,2-dicarboxylate; $C_8H_5KO_4$ = 204.2 (*877-24-7*)

White or almost white crystals, soluble in water, slightly soluble in ethanol (96 per cent).

Potassium Hydrogen Phthalate, 0.2M

A solution of *potassium hydrogen phthalate R* containing the equivalent of 40.84 g of $C_8H_5KO_4$ in 1000.0 mL.

Potassium Hydrogen Sulfate Potassium bisulfate; Potassium bisulphate; Potassium hydrogen sulphate; $KHSO_4$ = 136.2 (*7646-93-7*)

Colourless, transparent, hygroscopic crystals, freely soluble in water giving a strongly acid solution.

Storage: in an airtight container.

Potassium Hydrogen (+)-Tartrate Potassium hydrogen tartrate; $C_4H_5KO_6$ = 188.2 (*868-14-4*)

White or almost white, crystalline powder or colourless, slightly opaque crystals, slightly soluble in water, soluble in boiling water, practically insoluble in ethanol (96 per cent).

Potassium Hydroxide (*1310-58-3*)

See *Potassium hydroxide (0840)*.

Potassium Hydroxide, 2M Alcoholic

Dissolve 12 g of *potassium hydroxide R* in 10 mL of *water R* and dilute to 100 mL with *ethanol (96 per cent) R*.

Potassium Hydroxide, Ethanolic

Solutions of the requisite molarity may be obtained by dissolving the appropriate amount of *potassium hydroxide* in sufficient *ethanol (96%)* to produce 1000 mL.

Potassium Hydroxide in Alcohol (10% v/v), 0.5M

Dissolve 28 g of *potassium hydroxide R* in 100 mL of *ethanol (96 per cent) R* and dilute to 1000 mL with *water R*.

Potassium Hydroxide, Methanolic

Solutions of the requisite molarity may be obtained by dissolving the appropriate amount of *potassium hydroxide* in sufficient *methanol* to produce 1000 mL.

Potassium Hydroxide Solution, Alcoholic

Dissolve 3 g of *potassium hydroxide R* in 5 mL of *water R* and dilute to 100 mL with *aldehyde-free alcohol R*. Decant the clear solution. The solution should be almost colourless.

Potassium Hydroxide Solution R1, Alcoholic

Dissolve 6.6 g of *potassium hydroxide R* in 50 mL of *water R* and dilute to 1000 mL with *anhydrous ethanol R*.

Potassium Iodate KIO_3 = 214.0 *(7758-05-6)*

White or almost white, crystalline powder, soluble in water.

Potassium Iodide *(7681-11-0)*

See *Potassium iodide (0186)*.

Potassium Iodide and Starch Solution

Dissolve 0.75 g of *potassium iodide R* in 100 mL of *water R*. Heat to boiling and add whilst stirring a solution of 0.5 g of *soluble starch R* in 35 mL of *water R*. Boil for 2 min and allow to cool.

Test for sensitivity. A mixture of 15 mL of the potassium iodide and starch solution, 0.05 mL of *glacial acetic acid R* and 0.3 mL of *iodine solution R2* is blue.

Potassium Iodide Solution

A 166 g/L solution of *potassium iodide R*.

Potassium Iodide Solution, Dilute

A 10% w/v solution of *potassium iodide*.

Potassium Iodide Solution, Iodinated

Dissolve 2 g of *iodine R* and 4 g of *potassium iodide R* in 10 mL of *water R*. When solution is complete dilute to 100 mL with *water R*.

Potassium Iodide Solution, Iodinated R1

Dissolve 500 mg of *iodine R* and 1.5 g of *potassium iodide R* in *water R* and dilute to 25 mL with the same solvent.

Potassium Iodide Solution, Saturated

A saturated solution of *potassium iodide R* in *carbon dioxide-free water R*. Make sure the solution remains saturated as indicated by the presence of undissolved crystals.

Test by adding to 0.5 mL of the saturated potassium iodide solution 30 mL of a mixture of 2 volumes of *chloroform R* and 3 volumes of *glacial acetic acid R*, as well as 0.1 mL of *starch solution R*. Any blue colour formed should be discharged by the addition of 0.05 mL of *0.1 M sodium thiosulfate*.

Storage: protected from light.

Potassium Iodobismuthate Solution

To 0.85 g of *bismuth subnitrate R* add 40 mL of *water R*, 10 mL of *glacial acetic acid R* and 20 mL of a 400 g/L solution of *potassium iodide R*.

Potassium Iodobismuthate Solution, Acid

Dissolve 1.7 g of *bismuth oxynitrate* in a mixture of 80 mL of *water* and 20 mL of *glacial acetic acid*, warming if necessary, cool, add 100 mL of a 50% w/v solution of *potassium iodide* and mix. Dilute 10 mL to 100 mL with *water*, add 10 mL of *glacial acetic acid*, mix, add 0.12 g of *iodine* and shake until the iodine has completely dissolved.

Store at a temperature of 2° to 8° and use within 2 weeks.

Potassium Iodobismuthate Solution, Dilute

Dissolve 100 g of *tartaric acid R* in 500 mL of *water R* and add 50 mL of *potassium iodobismuthate solution R1*.

Storage: protected from light.

Potassium Iodobismuthate Solution R1

Dissolve 100 g of *tartaric acid R* in 400 mL of *water R* and add 8.5 g of *bismuth subnitrate R*. Shake for 1 h, add 200 mL of a 400 g/L solution of *potassium iodide R* and shake well. Allow to stand for 24 h and filter.

Storage: protected from light.

Potassium Iodobismuthate Solution R2

Stock solution. Suspend 1.7 g of *bismuth subnitrate R* and 20 g of *tartaric acid R* in 40 mL of *water R*. To the suspension add 40 mL of a 400 g/L solution of *potassium iodide R* and stir for 1 h. Filter. The solution may be kept for several days in brown bottles.

Spray solution. Mix immediately before use 5 mL of the stock solution with 15 mL of *water R*.

Potassium Iodobismuthate Solution R3

Dissolve 0.17 g of *bismuth subnitrate R* in a mixture of 2 mL of *glacial acetic acid R* and 18 mL of *water R*. Add 4 g of *potassium iodide R*, 1 g of *iodine R* and dilute to 100 mL with *dilute sulfuric acid R*.

Potassium Iodobismuthate Solution R4

Dissolve 1.7 g of *bismuth subnitrate R* in 20 mL of *glacial acetic acid R*. Add 80 mL of *distilled water R*, 100 mL of a 400 g/L solution of *potassium iodide R*, 200 mL of *glacial acetic acid R* and dilute to 1000 mL with *distilled water R*. Mix 2 volumes of this solution with 1 volume of a 200 g/L solution of *barium chloride R*.

Potassium Iodobismuthate Solution R5

To 0.85 g of *bismuth subnitrate R* add 10 mL of *glacial acetic acid R* and gently heat until completely dissolved. Add 40 mL of *water R* and allow to cool. To 5 mL of this solution, add 5 mL of a 400 g/L solution of *potassium iodide R*, 20 mL of *glacial acetic acid R* and 70 mL of *water R*.

Potassium Iodoplatinate Solution

Add 5 mL of a 5% w/v solution of *chloroplatinic(IV) acid* to 45 mL of *dilute potassium iodide solution* and dilute to 100 mL with *water*.

Store in an amber glass container.

Potassium Mercuri-iodide Solution, Alkaline

To 3.5 g of *potassium iodide* add 1.25 g of *mercury(II) chloride* dissolved in 80 mL of *water* and a cold, saturated solution of *mercury(II) chloride* in *water*, stirring continuously, until a slight red precipitate remains. Dissolve 12 g of *sodium hydroxide* in the resulting solution and add a little more of the saturated solution of mercury(II) chloride and sufficient *water* to produce 100 mL. Allow to stand and decant the clear, supernatant liquid.

Potassium Nitrate KNO_3 = 101.1 *(7757-79-1)*

Colourless crystals, very soluble in water.

Potassium Periodate Potassium metaperiodate; KIO_4 = 230.0 (*7790-21-8*)

White or almost white, crystalline powder or colourless crystals, soluble in water.

Potassium Permanganate (*7722-64-7*)

See *Potassium permanganate (0121)*.

Potassium Permanganate and Phosphoric Acid Solution

Dissolve 3 g of *potassium permanganate R* in a mixture of 15 mL of *phosphoric acid R* and 70 mL of *water R*. Dilute to 100 mL with *water R*.

Potassium Permanganate Solution

A 30 g/L solution of *potassium permanganate R*.

Potassium Permanganate Solution, Dilute

A 1.0% w/v solution of *potassium permanganate*.

Potassium Perrhenate $KReO_4$ = 289.3 (*10466-65-6*)

White or almost white, crystalline powder, soluble in water, slightly soluble in ethanol (96 per cent), in methanol and in propylene glycol.

Potassium Persulfate Dipotassium peroxodisulphate; Potassium persulphate; $K_2S_2O_8$ = 270.3 (*7727-21-1*)

Colourless crystals or white or almost white, crystalline powder, sparingly soluble in water, practically insoluble in ethanol (96 per cent). Aqueous solutions decompose at room temperature and more rapidly on warming.

Potassium Plumbite Solution

Dissolve 1.7 g of *lead acetate R*, 3.4 g of *potassium citrate R* and 50 g of *potassium hydroxide R* in *water R* and dilute to 100 mL with the same solvent.

Potassium Pyroantimonate Solution R1

Dissolve 2.0 g of *potassium pyroantimonate R* in 100 mL of hot *water R*. Boil for about 5 min, cool quickly and add 10 mL of a 150 g/L solution of *potassium hydroxide R*. Allow to stand for 24 h and filter.

Potassium Sodium (+)-Tartrate Sodium potassium (+)-tartrate; Sodium potassium tartrate; $C_4H_4KNaO_6,4H_2O$ = 282.2 (*6381-59-5*)

Colourless, prismatic crystals, very soluble in water.

Potassium Sorbate Sorbic acid potassium salt; $C_6H_7KO_2$ = 150.2 (*110-44-1*)

General reagent grade of commerce.

Potassium 4-sulfobenzoate 4-Sulfobenzoic acid potassium salt; Potassium 4-carboxybenzenesulfonate; $C_7H_5KO_5S$ = 240.3 (*5399-63-3*)

White, crystalline powder.

Potassium Tetraiodomercurate Solution

Dissolve 1.35 g of *mercuric chloride R* in 50 mL of *water R*. Add 5 g of *potassium iodide R* and dilute to 100 mL with *water R*.

Potassium Tetraiodomercurate Solution, Alkaline

Dissolve 11 g of *potassium iodide R* and 15 g of *mercuric iodide R* in *water R* and dilute to 100 mL with the same solvent. Immediately before use, mix 1 volume of this solution with an equal volume of a 250 g/L solution of *sodium hydroxide R*.

Potassium Tetroxalate Potassium trihydrogen dioxalate; $C_4H_3KO_8,2H_2O$ = 254.2 (*6100-20-5*)

White or almost white, crystalline powder, sparingly soluble in water, soluble in boiling water, slightly soluble in ethanol (96 per cent).

Potassium Thiocyanate KSCN = 97.2 (*333-20-0*)

Colourless crystals, deliquescent, very soluble in water and in ethanol (96 per cent).

Storage: in an airtight container.

Potassium Thiocyanate Solution

A 97 g/L solution of *potassium thiocyanate R*.

Povidone (*9003-39-8*)

See *Povidone (0685)*.

Prednisolone $C_{21}H_{28}O_5$ = 360.5 (*50-24-8*)

$[\alpha]_D^{20}$: about +97 (1% w/v in 1,4-dioxan).

mp: about 230°, with decomposition.

General reagent grade of commerce.

Hygroscopic crystalline powder.

Prednisolone 21-Acetate $C_{23}H_{30}O_6$ = 402.5 (*52-21-1*)

General reagent grade of commerce.

Procaine Hydrochloride See *Procaine hydrochloride (0050)*.

Proline (*147-85-3*)

See *Proline (0785)*.

D-Prolyl-L-phenylalanyl-L-arginine 4-Nitroanilide Hydrochloride D-Prolyl-L-phenylalanyl-L-arginine 4-nitroanilide dihydrochloride; $C_{26}H_{36}Cl_2N_8O_5$ = 612

Propane C_3H_8 = 44.10 (*74-98-6*)

Content: minimum 99.0 per cent *V/V*.

Propane-1,2-diol Propylene glycol; (*57-55-6*)

See *Propylene glycol (0430)*.

Propane-1,3-diol 1,3-Dihydroxypropane; $C_3H_8O_2$ = 76.1 (*504-63-2*)

Colourless, viscous liquid.

bp: about 214 °C.

mp: about −27 °C.

Bis-tris Propane 2,2′-(Propane-1,3-diyldiimino)bis [2-(hydroxymethyl)-1,3-propanediol; $C_{11}H_{26}N_2O_6$ = 282.3 (*64431-96-5*)

Content: minimum 99.0 per cent.

Propanol R1 (*71-23-8*)

See *Propanol (2036)*.

2-Propanol R1

Complies with the requirements prescribed for *2-propanol R* with the following additional requirements.

n_D^{20}: about 1.378.

Water (*2.5.12*): maximum 0.05 per cent, determined on 10 g.

Absorbance (*2.2.25*): maximum 0.60 at 210 nm, 0.26 at 220 nm, 0.13 at 230 nm, 0.02 at 250 nm, 0.01 at 260 nm, determined using *water R* as compensation liquid.

2-Propanol R2 (*67-63-0*)

See *Isopropyl alcohol (0970)*.

Propan-1-ol *N*-Propyl alcohol; Propanol; C_3H_8O = 60.1 (*71-23-8*)

Clear colourless liquid, miscible with water and with ethanol (96 per cent).

d_{20}^{20}: about 0.802 to 0.806.

bp: about 97.2 °C.

Distillation range (*2.2.11*). Not less than 95 per cent distils between 96 °C and 99 °C.

Propan-2-ol Isopropyl alcohol; 2-Propanol; C_3H_8O = 60.1 (*67-63-0*)

Clear, colourless, flammable liquid, miscible with water and with ethanol (96 per cent).

d_{20}^{20}: about 0.785.

bp: 81 °C to 83 °C.

Propan-2-ol MB C_3H_8O = 60.10 (*67-63-0*)

Analytical grade for molecular biology.

Propan-2-ol R1

n_D^{20}, about 1.378.

Complies with the requirements prescribed for *2-propanol* with the following additional requirements.

Water (2.5.12) Maximum 0.05%, determined on 10 g.

Absorbance (2.2.25) Maximum 0.60 at 210 nm, 0.26 at 220 nm, 0.13 at 230 nm, 0.02 at 250 nm, 0.01 at 260 nm, determined using *water* as compensation liquid.

Propanolamine 3-Amino-1-propanol; 3-Aminopropan-1-ol; 3-Aminopropanol; C_3H_9NO = 75.1 (*156-87-6*)

Clear, colourless, viscous liquid.

d_{20}^{20}: about 0.99.

n_D^{20}: about 1.461.

mp: about 11 °C.

Propetamphos $C_{10}H_{20}NO_4PS$ = 281.3 (*31218-83-4*)

A suitable certified reference solution (10 ng/µL in cyclohexane) may be used.

Propidium Iodide 3,8-Diamino-5-[3(diethylmethylammonio)propyl]-6-phenylphenanthridinium diiodide; $C_{27}H_{34}I_2N_4$ = 668.4 (*25535-16-4*)

Dark red solid.

Propionaldehyde Propanal; C_3H_6O = 58.1 (*123-38-6*)

Liquid freely soluble in water, miscible with ethanol (96 per cent).

d_{20}^{20}: about 0.81.

n_D^{20}: about 1.365.

bp: about 49 °C.

mp: about −81 °C.

Propionic Acid $C_3H_6O_2$ = 74.1 (*79-09-4*)

Oily liquid, soluble in ethanol (96 per cent), miscible with water.

d_{20}^{20}: about 0.993.

n_D^{20}: about 1.387.

bp: about 141 °C.

mp: about −21 °C.

Propionic Anhydride $C_6H_{10}O_3$ = 130.1 (*123-62-6*)

Clear, colourless liquid, soluble in ethanol (96 per cent).

d_{20}^{20}: about 1.01.

bp: about 167 °C.

Propionic Anhydride Reagent

Dissolve 1 g of *toluenesulfonic acid R* in 30 mL of *glacial acetic acid R*, add 5 mL of *propionic anhydride R* and allow to stand for at least 15 min before use.

Storage: use within 24 h.

Propranolol Hydrochloride $C_{16}H_{21}NO_2$,HCl = 295.8 (*318-98-9*)

mp: about 164°.

General reagent grade of commerce.

Propyl Acetate $C_5H_{10}O_2$ = 102.1 (*109-60-4*)

d_{20}^{20}: about 0.888.

bp: about 102 °C.

mp: about −95 °C.

Propyl 4-Hydroxybenzoate Propyl parahydroxybenzoate; (*94-13-3*)

See *Propyl parahydroxybenzoate (0431)*.

Propylene Oxide C_3H_6O = 58.1 (*75-56-9*)

Colourless liquid, miscible with ethanol (96 per cent).

Protamine Sulfate (*53597-25-4 (salmine) 9007-31-2 (clupeine)*)

See *Protamine sulfate (0569)*.

Protopine Hydrochloride $C_{20}H_{20}ClNO_5$ = 389.8 (*6164-47-2*)

5-Methyl-4,6,7,14-tetrahydrobis[1,3]benzodioxolo[4,5-*c*:5′,6′-*g*]azecin-13(5*H*)-one hydrochloride.

PSMA-11 (3*S*,7*S*)-22-[3-[[[2-[[[5-(2-Carboxyethyl)-2-hydroxyphenyl]methyl](carboxymethyl)amino]ethyl](carboxymethyl)amino]methyl]-4-hydroxyphenyl]-5,13,20-trioxo-4,6,12,19-tetraazadocosane-1,3,7-tricarboxylic acid supplied as trifluoroacetate salt; $C_{44}H_{62}N_6O_{17}$ = 947 (*1366302-52-4*)

White or almost white powder, freely soluble in water.

Content: minimum 96.0 per cent (anhydrous and trifluoroacetic acid-free substance).

PSMA-1007 (3*S*,10*S*,14*S*)-1-[4-[[(2*S*)-4-Carboxy-2-[(2*S*)-4-carboxy-2-(6-fluoropyridin-3-amido)butanamido]butanamido]methyl]phenyl]-3-[(naphthalen-2-yl)methyl]-1,4,12-trioxo-2,5,11,13-tetraazahexadecane-10,14,16-tricarboxylic acid; $C_{49}H_{55}FN_8O_{16}$ = 1031

White or almost white powder.

Pteroic Acid 4-[[(2-Amino-4-oxo-1,4-dihydropteridin-6-yl)methyl]amino]benzoic acid; $C_{14}H_{12}N_6O_3$ = 312.3 (*119-24-4*)

Crystals, soluble in solutions of alkali hydroxides.

Puerarin 7,4′-Dihydroxy-8-*C*-glucosyliso-haloprone; 8-β-D-Glucopyranosyl-7-hydroxy-3-(4-hydroxyphenyl)-4*H*-1-benzopyran-4-one; $C_{21}H_{20}O_9$ = 416.4 (*3681-99-0*)

Pulegone (*R*)-2-Isopropylidene-5-methylcyclohexanone; (+)-*p*-Menth-4-en-3-one; $C_{10}H_{16}O$ = 152.2 (*89-82-7*)

Oily, colourless liquid, practically insoluble in water, miscible with ethanol (96 per cent).

d_{15}^{20}: about 0.936.

n_D^{20}: 1.485 to 1.489.

bp: 222 °C to 224 °C.

Pulegone used in gas chromatography complies with the following additional test.

Assay. Gas chromatography (*2.2.28*) as prescribed in the monograph *Peppermint oil (0405)*.

Test solution. The substance to be examined.

Content: minimum 98.0 per cent, calculated by the normalisation procedure.

Pullulanase Pullulan-6-glucanohydrolase obtained from *Klebsiella pneumoniae*; (*9075-68-7*)

Content: minimum 30 units/mg of protein.

One unit represents the enzymatic activity required to produce 1.0 µmol of maltotriose from pullulan per minute at pH 5.0 at 30 °C.

DETERMINATION OF PULLULANASE ACTIVITY

Substrate. Dissolve 0.250 g of pullulan in 20.0 mL of *water R*, adding pullulan to the water.

Buffer solution A. 21 g/L solution of *citric acid monohydrate R* adjusted to pH 5.0 with a 27 g/L solution of *disodium hydrogen phosphate dodecahydrate R*.

Buffer solution B. Prepare a 136 g/L solution of *sodium acetate R* adjusted to pH 6.0 with *dilute acetic acid R*. Dilute 1 mL of this solution to 100 mL with *water R*.

Somogyi reagent. To 28 g of *anhydrous disodium hydrogen phosphate R* and 40 g of *sodium potassium tartrate R* add about 700 mL of *water R*. Add 100 mL of a 42 g/L solution of *sodium hydroxide R* and mix. Add 80 mL of a 100 g/L solution of *copper sulfate pentahydrate R*. Heat until complete dissolution. Add 180 g of *anhydrous sodium sulfate R* and adjust the volume to 1 L with *water R*. Allow to stand at room temperature for 1 or 2 days to let insoluble matter precipitate. Filter the solution and keep the filtrate in a brown-glass bottle with a ground-glass stopper.

Nelson reagent. Dissolve 50 g of *ammonium molybdate R* in 900 mL of *water R*. Add 42 g of *sulfuric acid R* and mix. Dissolve 6 g of *disodium arsenate R* in 50 mL of *water R*. Add the latter solution to the 1st solution, and allow to stand in a brown-glass bottle with a ground-glass stopper at 37 °C for 1 or 2 days.

Glucose standard solution. Dry *glucose R* at a pressure less than 6 kPa at 60 °C for 5 h, and calculate the water content. Transfer 10.00 g of dried glucose to a volumetric flask, dissolve with *water R*, dilute to 1.0 L with the same solvent, and mix. Transfer 10.0 mL of this solution to a volumetric flask and dilute to 1.0 L with *water R*. Each millilitre contains 100 µg of glucose.

Pullulanase diluent. Dilute *pullulanase R* with buffer solution B to prepare a solution with an enzyme activity of about 0.2 units/mL. The measurement range is between 0.1 and 0.4 units/mL. Record the dilution factor (D). This diluent is used as a diluted enzyme solution.

Procedure. Transfer 4.0 mL of substrate to a test tube and add 0.5 mL of buffer solution A, mix, and incubate at 30 °C. Add 0.5 mL of pullulanase diluent and mix thoroughly. After 30 s, transfer 1.0 mL of this solution to a test tube labelled "pullulan test solution 1", add 2.0 mL of Somogyi reagent, and mix. After 30.5 min, transfer 1.0 mL of the mixture of substrate and pullulanase diluent to a second test tube labelled "pullulan test solution 2", add 2.0 mL of Somogyi reagent, and mix. In a third test tube labelled "standard blank", mix 2.0 mL of Somogyi reagent and 1.0 mL of *water R*. In a fourth test tube labelled "glucose standard solution", mix 2.0 mL of Somogyi reagent and 1.0 mL of glucose standard solution, and add 1.0 mL of *water R*. Incubate the fourth test tube in a water-bath for exactly 10 min. Remove the tube and allow it to cool under running water. Add 2.0 mL of Nelson reagent, mix well, and allow the solution to stand for at least 15 min. Add 5.0 mL of *water R* to each of the 4 test tubes and mix thoroughly. Determine the absorbance at 520 nm of the standard blank (A_{blank}), the glucose standard solution (A_{Std}), pullulan test solution 1 (A_0) and pullulan test solution 2 (A_{30}), using *water R* as the blank. One unit is defined as the enzymatic activity required to produce 1 µmol of maltotriose (measured as glucose) from pullulan per minute. Calculate the pullulanase activity, P, in units/mL, using the following expression:

$$[(A_{30} - A_0)/(A_{Std} - A_{blank})] \times 0.185 \times D$$

MEASUREMENT OF PROTEIN CONTENT (MEASURED AS ALBUMINOID CONTENT) FOR THE CALCULATION OF SPECIFIC ACTIVITY

Reagent A. Prepare a solution having known concentrations of about 4 g/L of *sodium hydroxide R* and about 21 g/L of *anhydrous sodium carbonate R*.

Reagent B. Transfer 0.5 g of *copper sulfate pentahydrate R* and 1.0 g of *sodium citrate R* to a volumetric flask, dissolve in and dilute with *water R* to 100.0 mL, and mix.

Lowry solution. Mix 50 volumes of reagent A and 1 volume of reagent B.

Diluted Folin-Ciocalteu's phenol reagent (for albuminoid quantification). Prepare a two-fold dilution of the commercially available 2 N Folin-Ciocalteu's phenol reagent or prepare a solution by making an appropriate dilution of *phosphomolybdotungstic reagent R*.

Bovine albumin standard stock solution. Transfer 50.0 mg of *bovine albumin R* to a volumetric flask, dissolve in and dilute with *water R* to 500.0 mL, and mix. It contains 100 µg/mL of bovine albumin.

Standard solutions. Using appropriate dilutions of bovine albumin standard stock solution in *water R*, prepare 5 standard solutions having concentrations equally spaced between 5 µg/mL and 100 µg/mL of bovine albumin.

Test solution. Dilute *pullulanase R* with buffer solution B in order to obtain a solution having a concentration of 60-70 µg/mL of albuminoid. Water may be used as diluent. Record the dilution factor, D_f.

Procedure. Introduce into separate tubes 0.3 mL of each of the standard solutions, the test solution and *water R*. Add 3.0 mL of Lowry solution to each tube and mix. Incubate at room temperature for 10 min. Add 0.3 mL of diluted Folin-Ciocalteu's phenol reagent to each tube, mix immediately, and allow to stand at room temperature for 60 min. Determine the absorbances of the standard solutions and the test solution at the wavelength of maximum absorbance, about 750 nm, using *water R* as the blank.

Calculation. The relationship of absorbance to protein concentration is non-linear; however, if the standard curve concentration range is sufficiently small, it will approach linearity. Using linear regression method, plot the absorbances of the standard solutions versus the protein (bovine albumin) concentrations, in µg/mL. Using the plot, determine the concentration of protein (albuminoid content), $C_{albuminoid}$, in µg/mL, in the test solution. Calculate the albuminoid concentration, in mg/mL, in *pullulanase R* using the following expression:

$$C_{protein} = (C_{albuminoid} \times D_f)/1000$$

Calculate the specific activity, in units/mg, of pullulanase using the formula:

$$P/C_{protein}$$

P = pullulanase activity in units/mL.

Pumice Powder

Pumice of commerce, powdered and sifted, which passes through a 710 µm sieve, but is retained on a 250 µm sieve.

Putrescine 1,4-Butanediamine; Tetramethylenediamine; $C_4H_{12}N_2$ = 88.15 (*110-60-1*)

Colourless oily liquid, very soluble in water. Strong piperidine-like odour.

bp: about 159 °C.

mp: about 23 °C.

Pyrazine-2-carbonitrile 2-Cyanopyrazine; Clear, pale yellow liquid; $C_5H_3N_3$ = 105.1 (*19847-12-2*)

Content: minimum 99 per cent.

Pyridine C_5H_5N = 79.1 (*110-86-1*)

Clear, colourless liquid, hygroscopic, miscible with water and with ethanol (96 per cent).

bp: about 115 °C.

Storage: in an airtight container.

Pyridine, Anhydrous

Dry *pyridine R* over *anhydrous sodium carbonate R*. Filter and distil.

Water (*2.5.12*): maximum 0.01 per cent *m/m*.

Pyridine Bromide Solution

Dissolve 8 g of *pyridine* and 5.4 mL of *sulfuric acid* in 20 mL of *glacial acetic acid*, keeping the mixture cool. Add 2.6 mL of *bromine* dissolved in 20 mL of *glacial acetic acid* and dilute to 1000 mL with *glacial acetic acid*.

Prepare immediately before use.

Pyridine-4-carbonitrile 4-Cyanopyridine; $C_6H_4N_2$ = 104.1 (*100-48-1*)

White or almost white crystalline powder.

bp: 194 °C to 196 °C.

mp: 76 °C to 79 °C.

Pyridine-3-carboxaldehyde Nicotinaldehyde; C_6H_5NO = 107.1 (*500-22-1*)

General reagent grade of commerce.

Weight per mL, 1.14 g.

Pyridinium Hydrobromide Perbromide Pyridinium tribromide(1-); $C_5H_6Br_3N$ = 319.8 (*39416-48-3*)

Red crystals.

2-Pyridylamine Pyrid-2-ylamine; 2-Aminopyridine; Pyridin-2-amine; $C_5H_6N_2$ = 94.1 (*504-29-0*)

Large crystals soluble in water and in ethanol (96 per cent).

bp: about 210 °C.

mp: about 58 °C.

Pyridylazonaphthol PAN; $C_{15}H_{11}N_3O$ = 249.3 (*85-85-8*)

Brick-red powder, practically insoluble in water, soluble in ethanol (96 per cent), in methanol and in hot dilute alkali solutions.

mp: about 138 °C.

Pyridylazonaphthol Solution

A 1 g/L solution of *pyridylazonaphthol R* in *anhydrous ethanol R*.

Test for sensitivity. To 50 mL of *water R* add 10 mL of *acetate buffer solution pH 4.4 R*, 0.10 mL of *0.02 M sodium edetate* and 0.25 mL of the pyridylazonaphthol solution. After addition of 0.15 mL of a 5 g/L solution of *copper sulfate pentahydrate R*, the colour changes from light yellow to violet.

4-(2-Pyridylazo)resorcinol Monosodium Salt $C_{11}H_8N_3NaO_2$, H_2O = 255.2 (*16593-81-0*)

Orange crystalline powder.

Pyrogallol Benzene-1,2,3-triol; $C_6H_6O_3$ = 126.1 (*87-66-1*)

White or almost white crystals, becoming brownish on exposure to air and light, very soluble in water and in ethanol (96 per cent), slightly soluble in carbon disulfide.
On exposure to air, aqueous solutions, and more rapidly alkaline solutions, become brown owing to the absorption of oxygen.

mp: about 131 °C.

Storage: protected from light.

Pyrogallol Solution, Alkaline

Dissolve 0.5 g of *pyrogallol R* in 2 mL of *carbon dioxide-free water R*. Dissolve 12 g of *potassium hydroxide R* in 8 mL of *carbon dioxide-free water R*. Mix the two solutions immediately before use.

Pyrrolidine C_4H_9N = 71.1 (*123-75-1*)

Content: minimum 99 per cent.

bp: 87 °C to 88 °C.

2-Pyrrolidone Pyrrolidin-2-one; C_4H_7NO = 85.1 (*616-45-5*)

Liquid above 25 °C, miscible with water, with anhydrous ethanol and with ethyl acetate.

d_4^{25}: 1.116.

Water (*2.5.12*): maximum 0.2 per cent determined on 2.00 g.

Assay. Gas chromatography (*2.2.28*): use the normalisation procedure.

Test solution. Dissolve 1.0 g in *methanol R* and dilute to 10.0 mL with the same solvent.

Column:
— *material*: glass;
— *size*: *l* = 30 m; Ø = 0.53 mm;
— *stationary phase*: *macrogol 20 000 R* (1.0 µm).

Carrier gas: *helium for chromatography R*.

Flow rate: adjusted so that the retention time of 2-pyrrolidone is about 10 min.

Split ratio: 1:20.

Temperature:

	Time (min)	Temperature (°C)
Column	0 - 1	80
	1 - 12	80 → 190
	12 - 32	190
Injection port		200

Detection: flame ionisation.

Injection: 1 µL of the test solution.

Content: minimum 98.0 per cent.

Pyruvic Acid 2-Oxopropanoic acid; $C_3H_4O_3$ = 88.1 (*127-17-3*)

Yellowish liquid, miscible with water and with anhydrous ethanol.

d_{20}^{20}: about 1.267.

n_D^{20}: about 1.43.

bp: about 165 °C.

Quercetin Dihydrate 2-(3,4-Dihydroxyphenyl)-3,5,7-trihydroxy-4*H*-1-benzopyran-4-one; $C_{15}H_{10}O_7$,$2H_2O$ = 338.2

Yellow crystals or yellowish powder, practically insoluble in water, soluble in acetone and in methanol.

Water (*2.5.12*): maximum 12.0 per cent, determined on 0.100 g.

Assay. Liquid chromatography (*2.2.29*) as prescribed in the monograph *Ginkgo leaf (1828)*.

Content:minimum 90 per cent (anhydrous substance) calculated by the normalisation procedure.

Storage: protected from light.

Quercitrin Quercetin 3-L-rhamnopyranoside; 3-[(6-Deoxy-α-L-mannopyranosyl)oxy]-2-(3,4-dihydroxyphenyl)-5,7-dihydroxy-4*H*-1-benzopyran-4-one; Quercitroside; $C_{21}H_{20}O_{11}$ = 448.4 (*522-12-3*)

Yellow crystals, practically insoluble in cold water, soluble in ethanol (96 per cent).

mp: 176 °C to 179 °C.

Chromatography. Thin-layer chromatography (*2.2.27*) as prescribed in the monograph *Goldenrod (1892)*: apply 20 µL of the solution; after spraying, the chromatogram shows a yellowish-brown fluorescent zone with an R_F of about 0.6.

Storage: at a temperature of 2 °C to 8 °C.

Quillaia Saponins, Purified

A mixture of related saponins obtained from the bark of *Quillaja saponaria* Molina *s.l.*

Chromatography. Thin-layer chromatography (*2.2.27*) as prescribed in the monograph *Quillaia bark (1843)*: apply 5 µL of the solution; after treating with a 10 per cent *V/V* solution of *sulfuric acid R* in *methanol R*, heat at 120 °C for 5 min and examine in daylight; the chromatogram shows 3 principal zones in the upper part of the middle third.

Quinaldine Red 2-(4-dimethylaminostyryl)quinoline ethiodide; $C_{21}H_{23}IN_2$ = 430.3 (*117-92-0*)

Dark bluish-black powder, sparingly soluble in water, freely soluble in ethanol (96 per cent).

Quinaldine Red Solution

Dissolve 0.1 g of *quinaldine red R* in *methanol R* and dilute to 100 mL with the same solvent.

Colour change: pH 1.4 (colourless) to pH 3.2 (red).

Quinhydrone Equimolecular compound of 1,4-benzoquinone and hydroquinone; $C_{12}H_{10}O_4$ = 218.2 (*106-34-3*)

Dark green, lustrous crystals or a crystalline powder, slightly soluble in water, sparingly soluble in hot water, soluble in ethanol (96 per cent) and in concentrated ammonia.

mp: about 170 °C.

Quinidine (*S*)-(6-Methoxyquinol-4-yl)[(2*R*,4*S*,5*R*)-5-vinylquinuclidin-2-yl]methanol; $C_{20}H_{24}N_2O_2$ = 324.4 (*56-54-2*)

White or almost white crystals, very slightly soluble in water, sparingly soluble in ethanol (96 per cent), slightly soluble in methanol.

$[\alpha]_D^{20}$: about + 260, determined on a 10 g/L solution in *anhydrous ethanol R*.

mp: about 172 °C.

Storage: protected from light.

Quinidine Sulfate Quinidine sulphate; (*6591-63-5*)

See *Quinidine sulfate (0017)*.

Quinine (*R*)-(6-Methoxyquinol-4-yl)[(2*S*,4*S*,5*R*)-5-vinylquinuclidin-2-yl]methanol; $C_{20}H_{24}N_2O_2$ = 324.4 (*130-95-0*)

White or almost white, microcrystalline powder, very slightly soluble in water, slightly soluble in boiling water, very soluble in anhydrous ethanol.

$[\alpha]_D^{20}$: about −167, determined on a 10 g/L solution in *anhydrous ethanol R*.

mp: about 175 °C.

Storage: protected from light.

Quinine Hydrochloride (*6119-47-7*)

See *Quinine hydrochloride (0018)*.

Quinine Sulfate Quinine sulphate; (*6119-70-6*)

See *Quinine sulfate (0019)*.

Quinoline C_9H_7N = 129.2 (*91-22-5*)

n_D^{20}: 1.624 to 1.627.

A pale yellow liquid.

General reagent grade of commerce, suitable for phosphate assay.

Complies with the following requirement.

Tarry impurities Dissolve 1.0 g in a mixture of 2.5 mL of *water* and 2.5 mL of *hydrochloric acid*. Add 3 mL of *water* and 1 mL of *potassium chromate solution* and heat to boiling. The solution shows no darkening and no black deposit.

Store protected from light.

Quinoline Solution

Dissolve 50 mL of *quinoline* in a mixture of 60 mL of *hydrochloric acid* and 300 mL of *water* previously heated to 70°, cool and filter.

3-Quinuclidinol (3*R*)-1-Azabicyclo[2.2.2]octan-3-ol; $C_7H_{13}NO$ = 127.2 (*1619-34-7*)

Content: minimum 99 per cent.

Light yellow powder.

Rabbit Erythrocyte Suspension

Prepare a 1.6 per cent *V/V* suspension of rabbit erythrocytes as follows: defibrinate 15 mL of freshly drawn rabbit blood by shaking with glass beads, centrifuge at 2000 *g* for 10 min and wash the erythrocytes with three quantities, each of 30 mL, of a 9 g/L solution of *sodium chloride R*. Dilute 1.6 mL of the suspension of erythrocytes to 100 mL with a mixture of 1 volume of *phosphate buffer solution pH 7.2 R* and 9 volumes of a 9 g/L solution of *sodium chloride R*.

Rabies Antiserum, Fluorescein-conjugated

Immunoglobulin fraction with a high rabies antibody titre, prepared from the sera of suitable animals that have been immunised with inactivated rabies virus; the immunoglobulin is conjugated with fluorescein isothiocyanate.

Raclopride Tartrate Raclopride L-tartrate; $C_{19}H_{26}Cl_2N_2O_9$ = 497.3 (*98185-20-7*)

White or almost white solid, sensitive to light, soluble in water.

$[\alpha]_D^{25}$: + 0.3, determined on a 3 g/L solution.

mp: about 141 °C.

Raffinose β-D-Fructofuranosyl α-D-galactopyranosyl-(1→6)-α-D-glucopyranoside; $C_{18}H_{32}O_{16}$ = 504.4 (*512-69-6*)

Raffinose Pentahydrate β-D-Fructofuranosyl α-D-galactopyranosyl-(1→6)-α-D-glucopyranoside pentahydrate; $C_{18}H_{32}O_{16}$,$5H_2O$ = 594.5 (*17629-30-0*)

Content: minimum 98.0 per cent.

Crystalline powder.

mp: about 80 °C.

Raltegravir Potassium $C_{20}H_{20}FKN_6O_5$ (*871038-72-1*)

See *Raltegravir potassium (2887)*.

Rapeseed Oil See *Rapeseed oil, refined (1369)*.

Reducing Mixture Hydrazine reducing mixture

Grind the substances added in the following order to obtain a homogeneous mixture: 20 mg of *potassium bromide R*, 0.5 g of *hydrazine sulfate R* and 5 g of *sodium chloride R*.

Reichstein's Substance S $C_{21}H_{30}O_4$ = 346.5 (*152-58-9*)

Content: minimum 95.0 per cent.

mp: about 208 °C.

Reserpine Methyl 11,17α-dimethoxy-18β-[(3,4,5-trimethoxybenzoyl)oxy]-3β,20α-yohimban-16β-carboxylate; $C_{33}H_{40}N_2O_9$ = 609 (*50-55-5*)

General reagent grade of commerce.

Resin for Hydrophobic Interaction Chromatography

Non-porous resin consisting of spherical polymethacrylate particles bonded with butyl groups.

pH limits of use: 2 to 12.

Resin for Reversed-phase Ion Chromatography

A neutral, macroporous, high specific surface area with a non-polar character resin consisting of polymer lattice of polystyrene cross-linked with divinylbenzene.

Resin, Weak Cationic See *weak cationic resin R*.

Resorcinol Benzene-1,3-diol; (*108-46-3*)

See *Resorcinol (0290)*.

Resorcinol Reagent

To 80 mL of *hydrochloric acid R1* add 10 mL of a 20 g/L solution of *resorcinol R* and 0.25 mL of a 25 g/L solution of *copper sulfate pentahydrate R* and dilute to 100.0 mL with *water R*. Prepare the solution at least 4 h before use.

Storage: at 2 °C to 8 °C for 1 week.

Resveratrol 3,4′,5-Stilbenetriol; 5-[(*E*)-2-(4-Hydroxyphenyl)ethenyl]benzene-1,3-diol; $C_{14}H_{12}O_3$ = 228.2 (*501-36-0*)

L-Rhamnose (2R,3R,4R,5R,6S)-6-Methyltetrahydro-2H-pyran-2,3,4,5-tetrol monohydrate; 6-Deoxy-α-L-mannopyranose monohydrate; α-L-Rhamnopyranose monohydrate; L-(+)-Rhamnose monohydrate; Rhamnose; $C_6H_{12}O_5,H_2O$ = 182.2 (*6155-35-7*)

White or almost white, crystalline powder, freely soluble in water.

$[\alpha]_D^{20}$: + 7.8 to + 8.3, determined on a 50 g/L solution in *water R* containing about 0.05 per cent of NH_3.

Rhaponticin Rhapontin; 4′-methoxystilbene-3,3′,5-triol 3-glucoside; $C_{21}H_{24}O_9$ = 420.4 (*155-58-8*)

Yellowish-grey, crystalline powder, soluble in ethanol (96 per cent) and in methanol.

Chromatography. Thin-layer chromatography (*2.2.27*) as prescribed in the monograph *Rhubarb (0291)*; the chromatogram shows only one principal spot.

Rhein 4,5-Dihydroxy-9,10-dioxo-9,10-dihydroanthracene-2-carboxylic acid; 1,8-Dihydroxy-3-carboxyanthraquinone; $C_{15}H_8O_6$ = 284.2 (*478-43-3*)

Rhodamine 6 G 9-[2-(Ethoxycarbonyl)phenyl]-3,6-bis(ethylamino)-2,7-dimethylxanthenylium chloride; $C_{28}H_{31}ClN_2O_3$ = 479.0 (*989-38-8*)

Colour Index No. 45160

Brownish-red powder.

Rhodamine B Basic violet 10; $C_{28}H_{31}ClN_2O_3$ = 479.0 (*81-88-9*)

Schultz No. 864

Colour Index No. 45170

Green crystals or reddish-violet powder, very soluble in water and in ethanol (96 per cent).

Rhynchophylline Methyl (16*E*)-17-methoxy-2-oxo-16,17-didehydro-7β,20α-corynoxan-16-carboxylate; Methyl (16*E*)-16-(methoxymethylidene)-2-oxo-7β,20α-corynoxan-17-oate; $C_{22}H_{28}N_2O_4$ = 384.5 (*76-66-4*)

Ribose D-Ribose; $C_5H_{10}O_5$ = 150.1 (*50-69-1*)

Soluble in water, slightly soluble in ethanol (96 per cent).

mp: 88 °C to 92 °C.

Ricinoleic Acid (9*Z*,12*R*)-12-Hydroxyoctadec-9-enoic acid; 12-Hydroxyoleic acid; $C_{18}H_{34}O_3$ = 298.5 (*141-22-0*)

Yellow or yellowish-brown viscous liquid, consisting of a mixture of fatty acids obtained by the hydrolysis of castor oil, practically insoluble in water, very soluble in anhydrous ethanol.

d_{20}^{20}: about 0.942.

n_D^{20}: about 1.472.

mp: about 285 °C, with decomposition.

Rosmarinic Acid $C_{18}H_{16}O_8$ = 360.3 (*20283-92-5*)

mp: 170 °C to 174 °C.

Rosuvastatin Ethyl Ester Ethyl (3*R*,5*S*,6*E*)-7-[4-(4-fluorophenyl)-2-(*N*-methylmethanesulfonamido)-6-(propan-2-yl)pyrimidin-5-yl]-3,5-dihydroxyhept-6-enoate; $C_{24}H_{32}FN_3O_6S$ = 509.6 (*851443-04-4*)

Content: minimum 98 per cent.

White or pale yellow powder.

Ruscogenins

Mixture of neoruscogenin ($C_{27}H_{40}O_4$ = 428.6) and ruscogenin ($C_{27}H_{42}O_4$ = 430.6)

General reagent grade of commerce.

Ruscogenins used in liquid chromatography complies with the following test.

Assay Carry out the method described in the monograph for Butcher's Broom. The content is not less than 90% of ruscogenins of which at least 60% consists of neoruscogenin, calculated by the *normalisation* procedure.

The reagent from Vinyals laboratory has been found suitable.

Rutecarpine 8,13-Dihydroindolo[2′,3′:3,4]pyrido[2,1-*b*]quinazolin-5(7*H*)-one; $C_{18}H_{13}N_3O$ = 287.3 (*84-26-4*)

Ruthenium Red Ammoniated ruthenium oxychloride; $[(NH_3)_5RuORu(NH_3)_4ORu(NH_3)_5]Cl_6,4H_2O$ = 858 (*11103-72-3*)

Brownish-red powder, soluble in water.

Ruthenium Red Solution

A 0.8 g/L solution of *ruthenium red R* in *lead acetate solution R*.

Rutin (*250249-75-3*)

See *Rutoside trihydrate R*.

Rutoside Trihydrate (*250249-75-3*)

See *Rutoside trihydrate (1795)*.

Sabinene Thuj-4(10)-ene; 4-Methylene-1-isopropylbicyclo[3.1.0]hexane; $C_{10}H_{16}$ = 136.2 (*3387-41-5*)

A colourless, oily liquid.

Sabinene used in gas chromatography complies with the following additional test.

Assay. Gas chromatography (*2.2.28*) as prescribed in the monograph *Bitter-orange-flower oil (1175)*.

Test solution. The substance to be examined.

Content: minimum 95.0 per cent, calculated by the normalisation procedure.

Saccharin $C_7H_5NO_3S$ = 183.2 (*81-07-2*)

mp: about 230°.

General reagent grade of commerce.

Saccharin Sodium (*128-44-9*)

See *Saccharin sodium (0787)*.

Safrole 5-(Prop-2-enyl)-1,3-benzodioxole; 4-Allyl-1,2-(methylenedioxy)benzene; $C_{10}H_{10}O_2$ = 162.2 (*94-59-7*)

Colourless or slightly yellow, oily liquid, with the odour of sassafras, insoluble in water, very soluble in ethanol (96 per cent), miscible with hexane.

d_{20}^{20}: 1.095 to 1.096.

n_D^{20}: 1.537 to 1.538.

bp: 232 °C to 234 °C.

Freezing point: about 11 °C.

Safrole used in gas chromatography complies with the following additional test.

Assay. Gas chromatography (*2.2.28*) as prescribed in the monograph *Cinnamon bark oil, Ceylon (1501)*.

Content: minimum 96.0 per cent, calculated by the normalisation procedure.

Saikosaponin A 13,28-Epoxy-16β,23-dihydroxy-4α-olean-11-en-3β-yl 6-deoxy-3-*O*-β-D-glucopyranosyl-β-D-galactopyranoside; $C_{42}H_{68}O_{13}$ = 781 (*20736-09-8*)

Saikosaponin D 13,28-Epoxy-16α,23-dihydroxy-4α-olean-11-en-3β-yl 6-deoxy-3-*O*-β-D-glucopyranosyl-β-D-galactopyranoside; $C_{42}H_{68}O_{13}$ = 781 (*20874-52-6*)

Salicin 2-(Hydroxymethyl)phenyl-β-D-glucopyranoside; Salicoside; $C_{13}H_{18}O_7$ = 286.3 (*138-52-3*)

$[\alpha]_D^{20}$: −62.5 ± 2.

mp: 199 °C to 201 °C.

Assay. Liquid chromatography (*2.2.29*) as prescribed in the monograph *Willow bark (1583)* at the concentration of the reference solution.

Content: minimum 99.0 per cent, calculated by the normalisation procedure.

Salicylaldehyde 2-Hydroxybenzaldehyde; $C_7H_6O_2$ = 122.1 (*90-02-8*)

Clear, colourless, oily liquid.

d_{20}^{20}: about 1.167.

n_D^{20}: about 1.574.

bp: about 196 °C.

mp: about −7 °C.

Salicylaldehyde Azine 2,2′-Azinodimethyldiphenol; $C_{14}H_{12}N_2O_2$ = 240.3 (*959-36-4*)

Dissolve 0.30 g of *hydrazine sulfate R* in 5 mL of *water R*, add 1 mL of *glacial acetic acid R* and 2 mL of a freshly prepared 20 per cent *V/V* solution of *salicylaldehyde R* in *2-propanol R*. Mix, allow to stand until a yellow precipate is formed. Shake with two quantities, each of 15 mL, of *methylene chloride R*. Combine the organic layers and dry over *anhydrous sodium sulfate R*. Decant or filter the solution and evaporate to dryness. Recrystallise from a mixture of 40 volumes of *methanol R* and 60 volumes of *toluene R* with cooling. Dry the crystals *in vacuo*.

mp: about 213 °C.

Chromatography. Thin-layer chromatography (*2.2.27*) as prescribed in the test for hydrazine in the monograph *Povidone (0685)*; the chromatogram shows only one principal spot.

Salicylic Acid (*69-72-7*)

See *Salicylic acid (0366)*.

Saline Solution

A 0.9% w/v solution of *sodium chloride* in freshly prepared *water*, sterilised by *heating in an autoclave*.

Salvianolic Acid B (2*R*)-2-[[(2*E*)-3-[(2*S*,3*S*)-3-[[(1*R*)-1-Carboxy-2-(3,4-dihydroxyphenyl)ethoxy]carbonyl]-2-(3,4-dihydroxyphenyl)-7-hydroxy-2,3-dihydrobenzofuran-4-yl]prop-2-enoyl]oxy]-3-(3,4-dihydroxyphenyl)propanoic acid; $C_{36}H_{30}O_{16}$ = 719 (*121521-90-2*)

Sand

White or slightly greyish grains of silica with a particle size between 150 µm and 300 µm.

Santonin $C_{15}H_{15}O_3$ = 246.3 (*481-06-1*)

$[\alpha]_D^{18}$: 173 in ethanol.

mp: 174° to 176°.

General reagent grade of commerce.

Chromatography Carry out Identification test C in the monograph for Arnica Flower using a 0.025% w/v solution of the reagent being examined in *methanol*. The chromatogram obtained with 10 µL of the solution shows a quenching zone with an Rf value of about 0.5. Spray the plate with *anisaldehyde solution* and examine while heating at 105° for 5 to 10 minutes. In daylight the quenching zone is at first a yellow zone that quickly changes to a violet-red zone.

Sarafloxacin Hydrochloride 6-Fluoro-1-(4-fluorophenyl)-4-oxo-7-piperazin-1-yl-1,4-dihydroquinoline-3-carboxylic acid hydrochloride; $C_{20}H_{18}ClF_2N_3O_3$ = 421.8 (*91296-87-6*)

Schisandrin Schisandrol A; Wuweizichun A; (6*S*,7*S*,12a*R*$_a$)-5,6,7,8-Tetrahydro-1,2,3,10,11,12-hexamethoxy-6,7-dimethyldibenzo[*a*,*c*]cyclooctan-6-ol; $C_{24}H_{32}O_7$ = 432.5 (*7432-28-2*)

White or almost white, crystalline powder.

Schisandrin used in the assay in the monograph *Schisandra fruit (2428)* complies with the following additional test.

Assay. Liquid chromatography (*2.2.29*) as prescribed in the monograph *Schisandra fruit (2428)*.

Content: minimum 95 per cent, calculated by the normalisation procedure.

Storage: in an airtight container, at −20 °C or below.

γ-Schisandrin Schisandrin B; Wuweizisu B; *rac*-(6*R*,7*S*,13a*R*$_a$)-1,2,3,13-Tetramethoxy-6,7-dimethyl-5,6,7,8-tetrahydrobenzo[3,4]cycloocta[1,2-*f*][1,3]benzodioxole; $C_{23}H_{28}O_6$ = 400.5 (*61281-37-6*)

White or almost white, crystalline powder.

Storage: in an airtight container, at −20 °C or below.

Sclareol (1*R*,2*R*,4a*S*,8a*S*)-1-[(3*R*)-3-Hydroxy-3-methylpent-4-enyl]-2,5,5,8a-tetramethyldecahydronaphthalen-2-ol; $C_{20}H_{36}O_2$ = 308.5 (*515-03-7*)

Odourless crystals.

$[\alpha]_D^{20}$: 6.7, determined with a solution in anhydrous ethanol.

$bp_{19\ mm}$: 218 °C to 220 °C.

mp: 96 °C to 98 °C.

Sclareol used in the chromatographic profile test in the monograph Clary sage oil (1850) complies with the following additional test.

Assay. Gas chromatography (*2.2.28*) as prescribed in the monograph *Clary sage oil (1850)*.

Content: minimum 97 per cent, calculated by the normalisation procedure.

Scopoletin 7-Hydroxy-6-methoxy-2*H*-1-benzopyran-2-one; 7-Hydroxy-6-methoxycoumarin; $C_{10}H_8O_4$ = 192.2 (*92-61-5*)

Faintly beige, fine crystals.

mp: 202 °C to 208 °C.

SDS-PAGE Running Buffer

Dissolve 151.4 g of *tris(hydroxymethyl)aminomethane R*, 721.0 g of *glycine R* and 50.0 g of *sodium laurilsulfate R* in *water R* and dilute to 5000 mL with the same solvent. Immediately before use, dilute to 10 times its volume with

water R and mix. Measure the pH (*2.2.3*) of the diluted solution. The pH is between 8.1 and 8.8.

SDS-PAGE Sample Buffer (Concentrated)

Dissolve 1.89 g of *tris(hydroxymethyl)aminomethane R*, 5.0 g of *sodium laurilsulfate R* and 50 mg of *bromophenol blue R* in *water R*. Add 25.0 mL of *glycerol R* and dilute to 100 mL with *water R*. Adjust the pH to 6.8 with *hydrochloric acid R*, and dilute to 125 mL with *water R*.

SDS-PAGE Sample Buffer for Reducing Conditions (Concentrated)

Dissolve 3.78 g of *tris(hydroxymethyl)aminomethane R*, 10.0 g of *sodium dodecyl sulfate R* and 100 mg of *bromophenol blue R* in *water R*. Add 50.0 mL of *glycerol R* and dilute to 200 mL with *water R*. Add 25.0 mL of *2-mercaptoethanol R*. Adjust to pH 6.8 with *hydrochloric acid R*, and dilute to 250.0 mL with *water R*.

Alternatively, dithiothreitol may be used as reducing agent instead of 2-mercaptoethanol. In this case prepare the sample buffer as follows: dissolve 3.78 g of *tris(hydroxymethyl) aminomethane R*, 10.0 g of *sodium dodecyl sulfate R* and 100 mg of *bromophenol blue R* in *water R*. Add 50.0 mL of *glycerol R* and dilute to 200 mL with *water R*. Adjust to pH 6.8 with *hydrochloric acid R*, and dilute to 250.0 mL with *water R*. Immediately before use, add *dithiothreitol R* to a final concentration of 100 mM.

Selenious Acid Selenous acid; Selenic(IV) acid; H_2SeO_3 = 129.0 (*7783-00-8*)

Deliquescent crystals, freely soluble in water.

Storage: in an airtight container.

Selenium Se = 79.0 (*7782-49-2*)

Brown-red or black powder or granules, practically insoluble in water and in ethanol (96 per cent), soluble in nitric acid.

mp: about 220 °C.

Selenium Dioxide SeO_2 = 111.0 (*7446-08-4*)

General reagent grade of commerce.

Semicarbazide Acetate Solution Triturate 2.5 g of *semicarbazide hydrochloride* with 3.3 g of *sodium acetate*, add 10 mL of *methanol*, mix, transfer to a flask with the aid of 20 mL of *methanol*and allow to stand at a temperature of about 4° for 30 minutes

Triturate 2.5 g of *semicarbazide hydrochloride* with 3.3 g of *sodium acetate*, add 10 mL of *methanol*, mix, transfer to a flask with the aid of 20 mL of *methanol* and allow to stand at a temperature of about 4° for 30 minutes; filter and add sufficient *methanol* to produce 100 mL.

Semicarbazide Hydrochloride CH_5N_3O,HCl = 111.5 (*563-41-7*)

mp: about 175°, with decomposition.

Analytical reagent grade of commerce.

A white, crystalline powder.

Sennoside A (9*R*,9′*R*)-5,5′-Bis(β-D-glucopyranosyloxy)-4,4′-dihydroxy-10,10′-dioxo-9,9′,10,10′-tetrahydro[9,9′-bianthracene]-2,2′-dicarboxylic acid; $C_{42}H_{38}O_{20}$ = 863 (*81-27-6*)

Sennoside B (9*R*,9′*S*)-5,5′-Bis(β-D-glucopyranosyloxy)-4,4′-dihydroxy-10,10′-dioxo-9,9′,10,10′-tetrahydro-9,9′-bianthracene-2,2′-dicarboxylic acid; $C_{42}H_{38}O_{20}$ = 863 (*128-57-4*)

Pale yellow crystals, practically insoluble in water, very slightly soluble in ethanol (96 per cent), soluble in dilute solutions of alkali hydroxides.

mp: 180 °C to 186 °C.

L-Serine Serine; (*56-45-1*)

See *Serine (0788)*.

Sesame Oil

General reagent grade of commerce.

Sialic Acid (*131-48-6*)

See *N-acetylneuraminic acid R*.

Silibinin Silybin; (2*R*,3*R*)-3,5,7-Trihydroxy-2-[(2*R*,3*R*)-3-(4-hydroxy-3-methoxyphenyl)-2-(hydroxymethyl)-2,3-dihydro-1,4-benzodioxin-6-yl]-2,3-dihydro-4*H*-1-benzopyran-4-one; $C_{25}H_{22}O_{10}$ = 482.4 (*22888-70-6*)

White or yellowish powder, practically insoluble in water, soluble in acetone and in methanol.

Silibinin used in the assay of Milk thistle fruit (1860) complies with the following additional test.

Assay. Liquid chromatography (*2.2.29*) as prescribed in the monograph *Milk thistle fruit (1860)*.

Test solution. Dissolve 5.0 mg of silibinin, dried *in vacuo*, in *methanol R* and dilute to 50.0 mL with the same solvent.

Silibinin A and silibinin B content: minimum 95.0 per cent, calculated by the normalisation procedure.

Silica for Chromatography, Porous

Porous silica with porous layer open tubular (PLOT) design.

Silica Gel

A fine, white, homogeneous powder of an average particle size of about 15 µm containing a suitable binding agent.

Silica Gel π-Acceptor/π-Donor for Chiral Separations

A very finely divided silica gel for chromatography consisting of spherical particles to which 1-(3,5-dinitrobenzamido)-1,2,3,4-tetrahydrophenantrene has been covalently bound, showing both π-electron acceptor and π-electron donor characteristics.

Silica Gel AGP for Chiral Chromatography See *α1-Acid-glycoprotein silica gel for chiral separation R*.

Silica Gel, Anhydrous (*112926-00-8*)

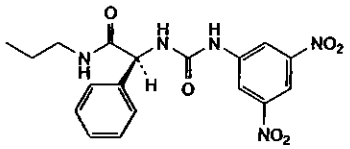
Partly dehydrated polymerised, amorphous silicic acid, absorbing at 20 °C about 30 per cent of its mass of water. Practically insoluble in water, partly soluble in solutions of sodium hydroxide. It contains a suitable indicator for detection of the humidity status, for which the colour change from the hydrated to anhydrous form is given on the label.

Silica Gel BC for Chiral Chromatography

A very finely divided silica gel for chromatography (5 µm) coated with β-cyclodextrin. Higher selectivity may be obtained when cyclodextrin has been derivatized with propylene oxide.

Silica Gel for Chiral Chromatography, Urea Type

A very finely divided silica gel (5 µm) coated with the following derivative:



Silica Gel for Chiral Separation, Amylose Derivative of

Substituted amylose coated on a very finely divided silica gel for chromatography.

Silica Gel for Chiral Separation, Cellulose Derivative of Silica Gel OD for Chiral Separations

Substituted cellulose coated on a very finely divided silica gel for chromatography.

Silica Gel for Chiral Separation, Protein Derivative of

A very finely divided silica gel for chromatography consisting of spherical particles coated with a protein derivative.

Silica Gel for Chiral Separation, Vancomycin-bonded

High-purity silica gel chemically modified by the bonding of vancomycin through multiple covalent linkages.

Silica Gel for Chromatography

A very finely divided silica gel.

Silica Gel for Chromatography, Alkyl-bonded for use with Highly Aqueous Mobile Phases

A very finely divided silica gel with bonded alkyl groups suitable for use with highly aqueous mobile phases.

Silica Gel for Chromatography, Alkyl-bonded for use with Highly Aqueous Mobile Phases, End-capped

A very finely divided silica gel with bonded alkyl groups suitable for use with highly aqueous mobile phases.
To minimise any interaction with basic compounds it is carefully end-capped to cover most of the remaining silanol groups.

Silica Gel for Chromatography, Alkylsilyl, Solid Core, End-capped

Silica gel with spherical silica particles containing a non-porous solid silica core surrounded by a thin outer porous silica coating with alkylsilyl groups. To minimise any interaction with basic compounds, it is carefully end-capped to cover most of the remaining silanol groups.

Silica Gel for Chromatography, Amido-alkylsilyl Silica gel for chromatography, amidoalkylsilyl

A very finely divided silica gel, chemically modified at the surface by the bonding of amidoalkylsilyl groups.

Silica Gel for Chromatography, Amidohexadecylsilyl

A very finely divided silica gel with a fine particle size, chemically modified at the surface by the bonding of amidohexadecylsilyl groups.

Silica Gel for Chromatography, Amidohexadecylsilyl, End-capped

A very finely divided silica gel, chemically modified at the surface by the bonding of amidohexadecylsilyl groups.
To minimise any interaction with basic compounds, it is carefully end-capped to cover most of the remaining silanol groups.

Silica Gel for Chromatography, Aminopropylmethylsilyl

Silica gel with a fine particle size, chemically modified by bonding aminopropylmethylsilyl groups on the surface.

Silica Gel for Chromatography, Aminopropylsilyl

Silica gel with a fine particle size, chemically modified by bonding aminopropylsilyl groups on the surface.

Silica Gel for Chromatography R1, Aminopropylsilyl

Silica gel with a particle size of about 55 µm, chemically modified by bonding aminopropylsilyl groups on the surface.

Silica Gel for Chromatography, Amylose-derivative of

A very finely divided (10 µm) silica gel, chemically modified at the surface by the bonding of an amylose derivative.

Silica Gel for Chromatography, Butylsilyl

A very finely divided silica gel, chemically modified at the surface by the bonding of butylsilyl groups.

Silica Gel for Chromatography, Butylsilyl, End-capped

A very finely divided silica, chemically modified at the surface by the bonding of butylsilyl groups. To minimise any interaction with basic compounds, it is carefully end-capped to cover most of the remaining silanol groups.

Silica Gel for Chromatography, Carbamoylsilyl

A very finely divided silica gel, chemically modified at the surface by the bonding of carbamoylsilyl groups.

Silica Gel for Chromatography Compatible with Highly Aqueous Mobile Phases, Octadecylsilyl Diol, End-capped

A very finely divided silica gel, chemically modified at the surface by the bonding of octadecylsilyl groups and end-capping. Free diol groups are also present. For use with highly aqueous mobile phases.

Silica Gel for Chromatography, Crown-ether

Stationary phase for liquid chromatography.

Crown ether coated on silica gel.

Silica Gel for Chromatography, Cyanopropylsilyl, End-capped, Base-deactivated

A very finely divided silica gel, pre-treated by various techniques before the bonding of cyanopropylsilyl groups.
To minimise any interaction with basic compounds, it is carefully end-capped to cover most of the remaining silanol groups.

Silica Gel for Chromatography, Cyanosilyl

A very finely divided silica gel chemically modified at the surface by the bonding of cyanosilyl groups.

Silica Gel for Chromatography, Cyanosilyl, End-capped

A very finely divided silica gel chemically modified at the surface by the bonding of cyanosilyl groups. To minimise any interaction with basic compounds it is carefully end-capped to cover most of the remaining silanol groups.

Silica Gel for Chromatography, Cyanosilyl, End-capped, Base-deactivated

A very finely divided silica gel pre-treated before the bonding of cyanosilyl groups by washing and hydrolysing most of the superficial siloxane bridges, chemically modified at the surface by bonding of cyano groups. To further minimise any interaction with basic compounds it is carefully end-capped to cover most of the remaining silanol groups.

Silica Gel for Chromatography, Di-isobutyloctadecylsilyl

A very finely divided silica gel chemically modified at the surface by the bonding of di-isobutyloctadecylsilyl groups.

Silica Gel for Chromatography, Diisopropylcyanosilyl Silica Gel for Chromatography, Diisopropylcyanopropylsilyl

A very finely divided silica gel chemically modified at the surface by the bonding of diisopropylcyanosilyl groups.

Silica Gel for Chromatography, 4-dimethylaminobenzylcarbamidesilyl

A very finely divided silica gel, chemically modified at the surface by the bonding of 4-dimethylaminobenzylcarbamide groups.

Silica Gel for Chromatography, Dimethyloctadecylsilyl

A very finely divided silica gel, chemically modified at the surface by the bonding of dimethyloctadecylsilyl groups.

Specific surface area: 300 m^2/g.

Silica Gel for Chromatography, Diol

Spherical silica particles to which dihydroxypropyl groups are bonded. Pore size 10 nm.

Silica Gel for Chromatography, Dodecylsilyl, End-capped

A very finely divided silica gel, chemically modified at the surface by the introduction of dodecylsilyl groups.
To minimise any interaction with basic compounds, it is carefully end-capped to cover most of the remaining silanol groups.

Silica Gel for Chromatography, Hexadecylamidylsilyl

A very finely divided (5 µm) silica gel, chemically modified at the surface by the introduction of hexadecylcarboxamidopropyldimethylsilyl groups.

Silica Gel for Chromatography, Hexadecylamidylsilyl, End-capped

A very finely divided (5 µm) silica gel, chemically modified at the surface by the introduction of hexadecylcarboxamidopropyldimethylsilyl groups.
To minimise any interaction with basic compounds it is carefully end-capped to cover most of the remaining silanol groups.

Silica Gel for Chromatography, Hexylsilyl

A very finely divided silica gel, chemically modified at the surface by the bonding of hexylsilyl groups.

Silica Gel for Chromatography, Hexylsilyl, End-capped

A very finely divided silica gel, chemically modified at the surface by the bonding of hexylsilyl groups. To minimise any interaction with basic compounds it is carefully end-capped to cover most of the remaining silanol groups.

Silica Gel for Chromatography (Hybrid Material), Octadecylsilyl, Ethylene-bridged, Charged Surface, End-capped

Synthetic, spherical ethylene-bridged hybrid particles with a charged surface, containing both inorganic (silica) and organic (organosiloxanes) components, chemically modified at the surface by the bonding of octadecylsilyl groups.
To minimise any interaction with basic compounds they are carefully end-capped to cover most of the remaining silanol groups.

Silica Gel for Chromatography (hybrid material), Octylsilyl, Ethylene-bridged, End-capped

Synthetic, spherical ethylene-bridged hybrid particles, containing both inorganic (silica) and organic (organosiloxanes) components, chemically modified at the surface by the bonding of octylsilyl groups. To minimise any interaction with basic compounds they are carefully end-capped to cover most of the remaining silanol groups.

Silica Gel for Chromatography (Hybrid Material), Phenylhexylsilyl, Ethylene-bridged, Charged Surface, End-capped

Synthetic, spherical ethylene-bridged hybrid particles with a charged surface, containing both inorganic (silica) and organic (organosiloxanes) components, chemically modified at the surface by the bonding of phenylhexylsilyl groups.
To minimise any interaction with basic compounds they are carefully end-capped to cover most of the remaining silanol groups.

Silica Gel for Chromatography (Hybrid Material), Phenylsilyl, Ethylene-bridged, End-capped

Synthetic, spherical, ethylene-bridged hybrid particles, containing both inorganic (silica) and organic (organosiloxanes) components, chemically modified at the surface by the bonding of phenylsilyl groups. To minimise any interaction with basic compounds, they are carefully end-capped to cover most of the remaining silanol groups.

Silica Gel for Chromatography (Hybrid Material), Polar-embedded, Octadecylsilyl, Ethylene-bridged, End-capped

Synthetic, spherical, ethylene-bridged hybrid particles containing both inorganic (silica) and organic (organosiloxanes) components, chemically modified at the surface by the bonding of polar-embedded octadecylsilyl groups. To minimise any interaction with basic compounds, they are carefully end-capped to cover most of the remaining silanol groups.

Silica Gel for Chromatography, Hydrophilic

A very finely divided silica gel whose surface has been modified to provide hydrophilic characteristics.

Silica Gel for Chromatography, Hydroxypropylsilyl

A very finely divided silica gel, chemically modified at the surface by the bonding of hydroxypropylsilyl groups.

Silica Gel for Chromatography, Methylsilyl

Liquid chromatographic reagent grade of commerce.

A very finely divided silica gel (3 to 10 µm) chemically modified at the surface by the introduction of methylsilyl groups. The particle size is specified after the name of the reagent in tests where it is used.

Silica Gel for Chromatography, Nitrile

A very finely divided silica gel, chemically modified at the surface by the bonding of cyanopropylsilyl groups.

Silica Gel for Chromatography, 4-Nitrophenylcarbamidesilyl

A very finely divided silica gel, chemically modified at the surface by bonding of 4-nitrophenylcarbamide groups.

Silica Gel for Chromatography, Octadecanoylaminopropylsilyl

A very finely divided silica gel, chemically modified at the surface by the bonding of aminopropylsilyl groups which are acylated with octadecanoyl groups.

Silica Gel for Chromatography, Octadecylphenylsilyl, End-capped

A very finely divided silica gel, chemically modified at the surface by bonding of octadecylphenylsilyl groups.
To minimise any interaction with basic compounds it is carefully end-capped to cover most of the remaining silanol groups.

Silica Gel for Chromatography, Octadecylsilyl

A very finely divided silica gel, chemically modified at the surface by the bonding of octadecylsilyl groups.

Silica Gel for Chromatography, Octadecylsilyl, Base-deactivated

A very finely divided silica gel, pretreated by various techniques before the bonding of octadecylsilyl groups to minimise the interaction with basic components.

Silica Gel for Chromatography, Octadecylsilyl, Cross-linked, End-capped

A very finely divided silica gel, chemically modified at the surface by the cross-linking and bonding of octadecylsilyl groups. To minimise any interaction with basic compounds it is carefully end-capped to cover most of the remaining silanol groups.

Silica Gel for Chromatography, Octadecylsilyl, End-capped

A very finely divided silica gel, chemically modified at the surface by the bonding of octadecylsilyl groups. To minimise any interaction with basic compounds it is carefully end-capped to cover most of the remaining silanol groups.

Silica Gel for Chromatography, Octadecylsilyl, End-capped, Base-deactivated

A very finely divided silica gel, pretreated by various techniques before the bonding of octadecylsilyl groups. To further minimise any interaction with basic compounds, it is carefully end-capped to cover most of the remaining silanol groups.

Silica Gel for Chromatography, Octadecylsilyl, Ethylene-Bridged (Hybrid Material)

Synthetic, spherical, ethylene-bridged hybrid particles, containing both inorganic (silica) and organic (organosiloxanes) components, chemically modified at the surface by the bonding of octadecylsilyl groups. To minimise any interaction with basic compounds, they are carefully end-capped to cover most of the remaining silanol groups.

Silica Gel for Chromatography, Octadecylsilyl, Extra-dense Bonded, End-capped

A very finely divided silica gel, chemically modified at the surface by the extra-dense bonding of octadecylsilyl groups. To minimise any interaction with basic compounds it is carefully end-capped to cover most of the remaining silanol groups.

Silica Gel for Chromatography, Octadecylsilyl, for Separation of Polycyclic Aromatic Hydrocarbons

A very finely divided ultrapure silica gel, chemically modified at the surface by the bonding of octadecylsilyl groups, optimised for the analysis of polycyclic aromatic hydrocarbons.

Silica gel for Chromatography, Octadecylsilyl, Monolithic

Monolithic rods of highly porous (greater than 80 per cent) metal-free silica with a bimodal pore structure, modified at the surface by the bonding of octadecylsilyl groups.

Silica Gel for Chromatography, Octadecylsilyl, Monolithic, End-capped

Monolithic rods of highly porous (greater than 80 per cent) metal-free silica with a bimodal pore structure, modified at the surface by the bonding of octadecylsilyl groups. To minimise any interaction with basic compounds, they are carefully end-capped to cover most of the remaining silanol groups.

Silica Gel for Chromatography, Octadecylsilyl, Polar-embedded, Encapsulated

Silica gel chemically modified at the surface by the bonding of polar-embedded octadecylsilyl groups. To minimise any interaction with basic compounds, it is carefully encapsulated to cover most of the remaining silanol groups.

Silica Gel for Chromatography, Octadecylsilyl, Polar End-capped

A very finely divided silica gel, chemically modified at the surface by the bonding of octadecylsilyl groups. To minimise any interaction with basic compounds it is carefully polar end-capped to cover most of the remaining silanol groups.

Silica Gel for Chromatography, Octadecylsilyl, Solid Core Silica gel with spherical silica particles containing a non-porous solid silica core surrounded by a thin outer porous silica coating with octadecylsilyl groups; **Silica Gel for Chromatography, Octadecylsilyl, Solid Core, End-capped**

Silica gel with spherical silica particles containing a non-porous solid silica core surrounded by a thin outer porous silica coating with octadecylsilyl groups. To minimise any interaction with basic compounds it is carefully end-capped to cover most of the remaining silanol groups.

Silica Gel for Chromatography, Octadecylsilyl, with Embedded Polar Groups, End-capped

A very finely divided silica gel, chemically modified at the surface by the bonding of polar-embedded octadecylsilyl groups. To minimise any interaction with basic compounds, it is carefully end-capped to cover most of the remaining silanol groups.

Silica Gel for Chromatography, Octadecylsilyl, with Extended pH Range, End-Capped

A very finely divided silica gel, chemically modified at the surface by the bonding of octadecylsilyl groups resistant to bases up to pH 11. To minimise any interaction with basic compounds it is carefully end-capped to cover most of the remaining silanol groups.

Silica Gel for Chromatography, Octylsilyl

A very finely divided silica gel, chemically modified at the surface by the bonding of octylsilyl groups.

Silica Gel for Chromatography, Octylsilyl and Octadecylsilyl Multi-Alkyl

High purity base deactivated silica (10 µm particle sizes) chemically modified at the surface with binding of octylsilyl and octadecylsilyl groups.

Silica Gel for Chromatography, Octylsilyl, Base-deactivated

A very finely divided silica gel, pretreated by various techniques before the bonding of octylsilyl groups to minimise the interaction with basic components.

Silica Gel for Chromatography, Octylsilyl, End-capped

A very finely divided silica gel, chemically modified at the surface by the bonding of octylsilyl groups. To minimise any interaction with basic compounds, it is carefully end-capped to cover most of the remaining silanol groups.

Silica Gel for Chromatography, Octylsilyl, End-capped, Base-deactivated

A very finely divided silica gel, pre-treated by various techniques before the bonding of octylsilyl groups. To further minimise any interaction with basic compounds it is carefully end-capped to cover most of the remaining silanol groups.

Silica Gel for Chromatography, Octylsilyl, Extra-dense Bonded, End-capped

A very finely divided silica gel, chemically modified at the surface by the extra-dense bonding of octylsilyl groups. To minimise any interaction with basic compounds, it is carefully end-capped to cover most of the remaining silanol groups.

Silica Gel for Chromatography, Octylsilyl, Solid Core

Silica gel with spherical particles containing a non-porous solid silica core surrounded by a thin outer porous silica coating with octylsilyl groups.

Silica Gel for Chromatography, Octylsilyl, Solid Core, End-capped

Silica gel with spherical silica particles containing a non-porous solid silica core surrounded by a thin outer porous silica coating with octylsilyl groups. To minimise any interaction with basic compounds it is carefully end-capped to cover most of the remaining silanol groups.

Silica Gel for Chromatography, Octylsilyl, with Embedded Polar Groups, End-capped

A very finely divided silica gel, chemically modified at the surface by the bonding of polar-embedded octylsilyl groups. To minimise any interaction with basic compounds, it is carefully end-capped to cover most of the remaining silanol groups.

Silica Gel for Chromatography, Oxypropionitrilsilyl

A very finely divided silica gel chemically modified at the surface by the bonding of oxypropionitrilsilyl groups.

Silica Gel for Chromatography, Palmitamidopropylsilyl, End-capped

A very finely divided silica gel, chemically modified at the surface by the bonding of palmitamidopropyl groups and end-capped with acetamidopropyl groups.

Silica Gel for Chromatography, Pentafluorophenylpropylsilyl, Solid Core, End-capped

Silica gel with spherical silica particles containing a non-porous solid silica core surrounded by a thin outer porous silica coating with pentafluorophenylpropylsilyl groups. To minimise any interaction with basic compounds it is carefully end-capped to cover most of the remaining silanol groups.

Silica Gel for Chromatography, Phenyl

Liquid chromatographic reagent grade of commerce.

A very finely divided silica gel, chemically modified at the surface by the bonding of phenyl groups. The particle size is indicated after the name of the reagent in the tests where it is used.

Silica Gel for Chromatography, Phenylethyl, End-capped

Liquid chromatographic reagent grade of commerce.

A very finely divided silica gel (2.5 to 10 µm) chemically modified at the surface by the introduction of ether-linked phenyl groups. To minimise any interaction with basic compounds it is carefully end-capped to cover most of the remaining silanol groups. The particle size is specified after the name of the reagent in tests where it is used.

Silica Gel for Chromatography, Phenylhexylsilyl

A very finely divided silica gel, chemically modified at the surface by the bonding of phenylhexyl groups.

Silica Gel for Chromatography, Phenylhexylsilyl, End-capped

A very finely divided silica gel, chemically modified at the surface by the bonding of phenylhexylsilyl groups. To minimise any interaction with basic compounds it is carefully end-capped to cover most of the remaining silanol groups.

Silica Gel for Chromatography, Phenylhexylsilyl, Solid Core, End-capped

Silica gel with spherical silica particles containing a non-porous solid silica core surrounded by a thin outer porous silica coating with phenylhexylsilyl groups. To minimise any interaction with basic compounds it is carefully end-capped to cover most of the remaining silanol groups.

Silica Gel for Chromatography, Phenylsilyl

A very finely divided silica gel, chemically modified at the surface by the bonding of phenyl groups.

Silica Gel for Chromatography, Phenylsilyl, End-capped

A very finely divided silica gel, chemically modified at the surface by the bonding of phenyl groups. To minimise any interaction with basic compounds it is carefully end-capped to cover most of the remaining silanol groups.

Silica Gel for Chromatography, Phenylsilyl, End-Capped, Base-Deactivated

A very finely divided silica gel, pre-treated by various techniques before the bonding of phenylsilyl groups. To further minimise any interaction with basic compounds it is carefully end-capped to cover most of the remaining silanol groups.

Silica Gel for Chromatography, Phenylsilyl, Extra-dense Bonded, End-capped

A very finely divided silica gel, chemically modified at the surface by the extra-dense bonding of phenylsilyl groups. To minimise any interaction with basic compounds, it is carefully end-capped to cover most of the remaining silanol groups.

Silica Gel for Chromatography, Propoxybenzene, End-capped

A very finely divided silica gel, chemically modified at the surface by the bonding of propoxybenzene groups.

Silica Gel for Chromatography, Propylsilyl

A very finely divided silica gel, chemically modified at the surface by the bonding of propylsilyl groups.

Silica Gel for Chromatography Compatible with 100% Aqueous Mobile Phases, Octadecylsilyl, End-capped

A very finely divided silica gel with bonded octadecylsilyl groups suitable for use with highly aqueous mobile phases including 100 per cent aqueous phases. To minimise any interaction with basic compounds it is carefully end-capped to cover most of the remaining silanol groups.

Silica Gel for Chromatography compatible with 100 per cent Aqueous Mobile Phases, Octadecylsilyl

A very finely divided silica gel with bonded octadecylsilyl groups suitable for use with highly aqueous mobile phases including 100 per cent aqueous phases.

Silica Gel for Chromatography R1, Nitrile

A very finely divided silica gel consisting of porous, spherical particles with chemically bonded nitrile groups.

Silica Gel for Chromatography R1, Octadecylsilyl

A very finely divided ultrapure silica gel, chemically modified at the surface by the bonding of octadecylsilyl groups.

Silica Gel for Chromatography R2, Octadecylsilyl

A very finely divided (15 nm pore size) ultrapure silica gel, chemically modified at the surface by the bonding of octadecylsilyl groups (20 per cent carbon load), optimised for the analysis of polycyclic aromatic hydrocarbons.

Silica Gel for Chromatography R1, Octadecylsilyl, End-capped

A very finely divided ultrapure silica gel, chemically modified at the surface by the bonding of octadecylsilyl groups. To minimise any interaction with basic compounds it is carefully end-capped to cover most of the remaining silanol groups.

Silica Gel for Chromatography R1, Octadecylsilyl, End-capped, Base-deactivated

A very finely divided silica gel pre-treated before the bonding of octadecylsilyl groups by washing and hydrolysing most of the superficial siloxane bridges. To further minimise any interaction with basic compounds it is carefully end-capped to cover most of the remaining silanol groups.

Silica Gel for Chromatography R1, Octylsilyl

A very finely divided silica gel, chemically modified at the surface by the bonding of octylsilyl and methyl groups (double bonded phase).

Silica Gel for Chromatography R2, Octylsilyl

Ultrapure very finely divided (10 nm pore size) silica gel, chemically modified at the surface by the bonding of octylsilyl groups (19 per cent carbon load). Less than 20 ppm of metals.

Silica Gel for Chromatography R3, Octylsilyl

A very finely divided ultrapure silica gel, chemically modified at the surface by the bonding of octylsilyl groups and sterically protected with branched hydrocarbons at the silanes.

Silica Gel for Chromatography, Strong-anion-exchange

A very finely divided silica gel, chemically modified at the surface by the bonding of quaternary ammonium groups.

pH limit of use: 2 to 8.

Silica Gel for Chromatography, Strong Cation-exchange

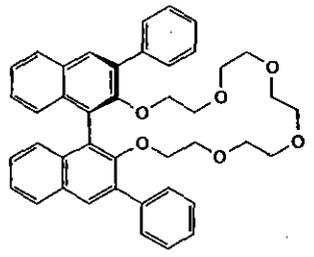
A very finely divided silica gel, chemically modified at the surface by the bonding of sulfonic acid groups.

Silica Gel for Chromatography, Trimethylsilyl

A very finely divided silica gel, chemically modified at the surface by the bonding of trimethylsilyl groups.

Crown-ether Silica Gel for Chiral Separation

A very finely divided silica gel for chromatography coated with the following chiral crown ether:



(R_a)-6,23-Diphenyl-8,9,11,12,14,15,17,18,20,21-decahydrodinaphtho[2,1-*q*:1′,2′-*s*][1,4,7,10,13,16]hexaoxacycloicosine.

Silica Gel for HPTLC, Octadecylsilyl

A finely divided silica gel, chemically modified at the surface by the bonding of octadecylsilyl groups.

Fine, white or almost white, homogeneous powder, practically insoluble in water and in ethanol (96%).

Silica Gel for Size-exclusion Chromatography

A very finely divided silica gel (10 µm) with a very hydrophilic surface. The average diameter of the pores is about 30 nm. It is compatible with aqueous solutions between pH 2 and 8 and with organic solvents. It is suitable for the separation of proteins with relative molecular masses of 1×10^3 to 3×10^5.

Silica Gel F_{254}

A fine, white, homogeneous powder of an average particle size of about 15 µm containing a suitable binding agent and about 1.5% of a florescent indicator having a maximum intensity at 254 nm. It complies with the following requirement.

Fluorescence Carry out the method for thin-layer chromatography using a mixture of 10 volumes of *anhydrous formic* acid and 90 volumes of *propan-2-ol* as the mobile phase, but allowing the solvent front to ascend 10 cm above the line of application. Apply separately to the plate 10 quantities from 1 to 10 uL of a 0.1% w/v solution of *benzoic acid* in the mobile phase. After removal of the plate, dry it in a current of warm air and examine under ultraviolet light (254 nm). The benzoic acid appears as dark spots on a fluorescent background in the upper third of the chromatogram at levels of 2 µg and greater.

Silica Gel G (*112926-00-8*)

Contains about 13 per cent of calcium sulfate hemihydrate. The particle size is about 15 µm.

Calcium sulfate content. Place 0.25 g in a ground-glass stoppered flask, add 3 mL of *dilute hydrochloric acid R* and 100 mL of *water R* and shake vigorously for 30 min. Filter through a sintered-glass filter (*2.1.2*) and wash the residue. Carry out on the combined filtrate and washings the complexometric assay of calcium (*2.5.11*).

1 mL of *0.1 M sodium edetate* is equivalent to 14.51 mg of $CaSO_4,{}^1/_2H_2O$.

pH (*2.2.3*). Shake 1 g for 5 min with 10 mL of *carbon dioxide-free water R*. The pH of the suspension is about 7.

Silica Gel GF_{254} (*112926-00-8*)

Contains about 13 per cent of calcium sulfate hemihydrate and about 1.5 per cent of a fluorescent indicator having an optimal intensity at 254 nm. The particle size is about 15 µm.

Calcium sulfate content. Determine by the method prescribed for *silica gel G R*.

pH. Complies with the test prescribed for *silica gel G R*.

Fluorescence. Thin-layer chromatography (*2.2.27*) using *silica gel GF_{254} R* as the coating substance. Apply separately to the plate at ten points increasing volumes from 1 µL to 10 µL of a 1 g/L solution of *benzoic acid R* in a mixture of 10 volumes of *anhydrous formic acid R* and 90 volumes of *2-propanol R*. Develop over a path of 10 cm with the same mixture of solvents. After evaporating the solvents examine the chromatogram in ultraviolet light at 254 nm. The benzoic acid appears as dark spots on a fluorescent background in the upper third of the chromatogram for quantities of 2 µg and greater.

Silica Gel H (*112926-00-8*)

The particle size is of about 15 µm.

pH (2.2.3). Complies with the test prescribed for *silica gel G R*.

Silica Gel H, Silanised

Preparation of a thin layer. See *silanised silica gel HF_{254} R*

Chromatographic separation. Complies with the test prescribed for *silanised silica gel HF_{254} R.*

Silica Gel HF_{254}

Contains about 1.5 per cent of a fluorescent indicator having an optimal intensity at 254 nm. The particle size is about 15 µm.

pH. Complies with the test prescribed for *silica gel G R.*

Fluorescence. Complies with the test prescribed for *silica gel GF_{254} R.*

Silica Gel HF_{254}, Silanised

Contains about 1.5 per cent of a fluorescent indicator having an optimal intensity at 254 nm.

Preparation of a thin layer. Vigorously shake 30 g for 2 min with 60 mL of a mixture of 1 volume of *methanol R* and 2 volumes of *water R.* Coat carefully cleaned plates with a layer 0.25 mm thick using a spreading device. Allow the coated plates to dry in air and then heat in an oven at 100 °C to 105 °C for 30 min.

Chromatographic separation. Introduce 0.1 g each of *methyl laurate R, methyl myristate R, methyl palmitate R* and *methyl stearate R* into a 250 mL conical flask. Add 40 mL of *alcoholic potassium hydroxide solution R* and heat under a reflux condenser on a water-bath for 1 h. Allow to cool, transfer the solution to a separating funnel by means of 100 mL of *water R,* acidify (pH 2 to 3) with *dilute hydrochloric acid R* and shake with three quantities, each of 10 mL of *chloroform R.* Dry the combined chloroform extracts over *anhydrous sodium sulfate R,* filter and evaporate to dryness on a water-bath. Dissolve the residue in 50 mL of *chloroform R.* Examine by thin-layer chromatography (*2.2.27*), using silanised silica gel HF_{254} as the coating substance. Apply to the plate at each of three separate points 10 µL of the chloroformic solution. Develop over a path of 14 cm with a mixture of 10 volumes of *glacial acetic acid R,* 25 volumes of *water R* and 65 volumes of *dioxan R.* Dry the plate at 120 °C for 30 min. Allow to cool, spray with a 35 g/L solution of *phosphomolybdic acid R* in *2-propanol R* and heat at 150 °C until the spots become visible. Treat the plate with ammonia vapour until the background is white. The chromatograms show four clearly separated, well-defined spots.

Silica Gel OD for Chiral Separations

See *Cellulose derivative of silica gel for chiral separation R*

Silicotungstic Acid Dodecatungstosilicic acid; $H_4SiW_{12}O_{40},xH_2O$ (*11130-20-4*)

White or yellowish-white crystals, deliquescent, very soluble in water and in ethanol (96 per cent).

Storage: in an airtight container.

Silicristin (2*R*,3*R*)-3,5,7-Trihydroxy-2-[(2*R*,3*S*)-7-hydroxy-2-(4-hydroxy-3-methoxyphenyl)-3-hydroxymethyl-2,3-dihydro-1-benzofuran-5-yl]chroman-4-one; $C_{25}H_{22}O_{10}$ = 482.4 (*33889-69-9*)

White or yellowish powder, practically insoluble in water, soluble in acetone and in methanol.

Silidianin (3*R*,3a*R*,6*R*,7a*R*,8*R*)-7a-Hydroxy-8-(4-hydroxy-3-methoxyphenyl)-4-[(2*R*, 3*R*)-3,5,7-trihydroxy-4-oxochroman-2-yl]-2,3,3a,7a-tetrahydro-3,6-methano-1-benzofuran-7(6a*H*)-one; $C_{25}H_{22}O_{10}$ = 482.4 (*29782-68-1*)

White or yellowish powder, practically insoluble in water, soluble in acetone and in methanol.

Silver Diethyldithiocarbamate Silver diethylcarbamodithioate; $C_5H_{10}AgNS_2$ = 256.1 (*1470-61-7*)

Pale-yellow or greyish-yellow powder, practically insoluble in water, soluble in pyridine.

Storage below 8 °C is recommended.

It may be prepared as follows. Dissolve 1.7 g of *silver nitrate R* in 100 mL of *water R.* Separately dissolve 2.3 g of *sodium diethyldithiocarbamate R* in 100 mL of *water R.* Cool both solutions to 10 °C, then mix, and while stirring, collect the yellow precipitate on a sintered-glass filter (16) (*2.1.2*) and wash with 200 mL of cold *water R.* Dry the precipitate *in vacuo* for 10 h (*2.2.32*).

Silver Diethyldithiocarbamate Solution

Prepare the solution immediately before use. Dissolve 0.100 g of *silver diethyldithiocarbamate R* in *pyridine R* and dilute to 20.0 mL with the same solvent.

Suitability test. The solution is clear (*2.2.1*). The absorbance (*2.2.25*) of the solution is maximum 0.20 at 450 nm, maximum 0.01 at 510 nm and maximum 0.010 at 538 nm.

Silver Manganese Paper

Immerse strips of slow filter paper into a solution containing 8.5 g/L of *manganese sulfate R* and 8.5 g/L of *silver nitrate R.* Maintain for a few minutes and allow to dry over an appropriate desiccant, protected from acid and alkaline vapours.

Silver Nitrate (*7761-88-8*)

See *Silver nitrate (0009).*

Silver Nitrate Reagent

Prepare immediately before use. To a mixture of 3 mL of *concentrated ammonia R* and 40 mL of *1 M sodium hydroxide,* add 8 mL of a 200 g/L solution of *silver nitrate R,* dropwise, with stirring. Dilute to 200 mL with *water R.*

Silver Nitrate Solution

A freshly prepared 5.0% w/v solution of *silver nitrate.*

Store protected from light.

Silver Nitrate Solution, Ammoniacal

Dissolve 2.5 g of *silver nitrate R* in 80 mL of *water R* and add *dilute ammonia R1* dropwise until the precipitate has dissolved. Dilute to 100 mL with *water R.* Prepare immediately before use.

Silver Nitrate Solution in Pyridine

An 85 g/L solution of *silver nitrate R* in *pyridine R.*

Storage: protected from light.

Silver Nitrate Solution R1

A 42.5 g/L solution of *silver nitrate R.*

Storage: protected from light.

Silver Nitrate Solution R2

A 17 g/L solution of *silver nitrate R.*

Storage: protected from light.

Silver Oxide Disilver oxide; Ag_2O = 231.7 (*20667-12-3*)

Brownish-black powder, practically insoluble in water and in ethanol (96 per cent), freely soluble in dilute nitric acid and in ammonia.

Storage: protected from light.

Silver Sulfate Ag_2SO_4 = 311.8 (*10294-26-5*)

Content: minimum 99.0 per cent.

White or light grey powder, slightly soluble in water.

mp: about 652 °C.

Storage: protected from light.

Sinensetin 3′,4′,5,6,7-Pentamethoxyflavone; $C_{20}H_{20}O_7$ = 372.4 (*2306-27-6*)

White or almost white, crystalline powder, practically insoluble in water, soluble in ethanol (96 per cent).

mp: about 177 °C.

Absorbance (*2.2.25*). A solution in *methanol R* shows 3 absorption maxima, at 243 nm, 268 nm and 330 nm.

Assay. Liquid chromatography (*2.2.29*) as prescribed in the monograph *Java tea (1229)*.

Content: minimum 95 per cent, calculated by the normalisation procedure.

Sinomenine 7,8-Didehydro-4-hydroxy-3,7-dimethoxy-17-methyl-9α,13α,14α-morphinan-6-one; Cucoline; $C_{19}H_{23}NO_4$ = 329.4 (*115-53-7*)

Sirolimus Rapamycin; $C_{51}H_{79}NO_{13}$ = 914 (*53123-88-9*)

mp: 183 °C to 185 °C.

Sitostanol Dihydro-β-sitosterol; $C_{29}H_{52}O$ = 416.7 (*19466-47-8*)

Content: minimum 95.0 per cent.

β-Sitosterol Stigmast-5-en-3β-ol; 22,23-Dihydrostigmasterol; $C_{29}H_{50}O$ = 414.7 (*83-46-5*)

White or almost white powder, practically insoluble in water, sparingly soluble in tetrahydrofuran.

Content: minimum 75.0 per cent *m/m* (dried substance).

Assay. Gas chromatography (*2.2.28*), as prescribed in the monograph *Phytosterol (1911)*.

Test solution. Dissolve 0.100 g of the substance to be examined in *tetrahydrofuran R* and dilute to 10.0 mL with the same solvent. Introduce 100 µL of this solution into a suitable 3 mL flask and evaporate to dryness under *nitrogen R*. To the residue add 100 µL of a freshly prepared mixture of 50 µL of *1-methylimidazole R* and 1.0 mL of *heptafluoro-N-methyl-N-(trimethylsilyl)butanamide R*. Close the flask tightly and heat at 100 °C for 15 min. Allow to cool.

Injection: 1 µL of the test solution.

Sodium Na = 22.99 (*7440-23-5*)

A metal whose freshly cut surface is bright silver-grey. It rapidly tarnishes in contact with air and is oxidised completely to sodium hydroxide and converted to sodium carbonate. It reacts violently with water, yielding hydrogen and a solution of sodium hydroxide; soluble in anhydrous methanol, yielding hydrogen and a solution of sodium methoxide; practically insoluble in light petroleum.

Storage: under light petroleum or liquid paraffin.

Sodium Acetate (*6131-90-4*)

See *Sodium acetate trihydrate (0411)*.

Sodium Acetate, Anhydrous $C_2H_3NaO_2$ = 82.0 (*127-09-3*)

Colourless crystals or granules, very soluble in water, sparingly soluble in ethanol (96 per cent).

Loss on drying (*2.2.32*). Not more than 2.0 per cent, determined by drying in an oven at 105 °C.

Sodium Arsenite Sodium metaarsenite; $NaAsO_2$ = 129.9 (*7784-46-5*)

Sodium Arsenite Solution

Dissolve 5.0 g of *sodium arsenite R* in 30 mL of *1 M sodium hydroxide*. Cool to 0 °C and add, while stirring, 65 mL of *dilute hydrochloric acid R*.

Sodium Ascorbate Solution (*134-03-2*)

Dissolve 3.5 g of *ascorbic acid R* in 20 mL of *1 M sodium hydroxide*. Prepare immediately before use.

Sodium Azide NaN_3 = 65.0 (*26628-22-8*)

White or almost white, crystalline powder or crystals, freely soluble in water, slightly soluble in ethanol (96 per cent).

Sodium Benzenesulfonate $C_6H_5SO_3Na$ = 180.16 (*515-42-4*)

White crystalline powder, soluble in water.

Sodium Bicarbonate (*144-55-8*)

See *sodium hydrogen carbonate R*.

Sodium Bismuthate $NaBiO_3$ = 280.0 (*12232-99-4*)

Content: minimum 85.0 per cent.

Yellow or yellowish-brown powder, slowly decomposing when moist or at a high temperature, practically insoluble in cold water.

Assay. Suspend 0.200 g in 10 mL of a 200 g/L solution of *potassium iodide R* and add 20 mL of *dilute sulfuric acid R*. Using 1 mL of *starch solution R* as indicator, titrate with *0.1 M sodium thiosulfate* until an orange colour is obtained.

1 mL of *0.1 M sodium thiosulfate* is equivalent to 14.00 mg of $NaBiO_3$.

Sodium Bromide (*7647-15-6*)

See *Sodium bromide (0190)*.

Sodium Butanesulfonate 1-Butanesulfonic acid sodium salt; 1-Butanesulfonic acid sodium salt; Sodium butanesulfonate; $C_4H_9NaO_3S$ = 160.2 (*2386-54-1*)

White or almost white, crystalline powder, soluble in water.

mp: greater than 300 °C.

Sodium Calcium Edetate (*62-33-9*)

See *sodium calcium edetate (0231)*.

Sodium Carbonate (*6132-02-1*)

See *Sodium carbonate decahydrate (0191)*.

Sodium Carbonate, Anhydrous Disodium carbonate; Na_2CO_3 = 106.0 (*497-19-8*)

White or almost white powder, hygroscopic, freely soluble in water.

When heated to about 300 °C it loses not more than 1 per cent of its mass.

Storage: in an airtight container.

Sodium Carbonate Monohydrate (*5968-11-6*)

See *Sodium carbonate monohydrate (0192)*.

Sodium Carbonate Solution

A 106 g/L solution of *anhydrous sodium carbonate R*.

Sodium Carbonate Solution, Dilute

A 10% w/v solution of *sodium carbonate*.

Sodium Carbonate Solution R1

A 20 g/L solution of *anhydrous sodium carbonate R* in *0.1 M sodium hydroxide*.

Sodium Carbonate Solution R2

A 40 g/L solution of *anhydrous sodium carbonate R* in *0.2 M sodium hydroxide*.

Sodium Cetostearyl Sulfate See *Sodium cetostearyl sulfate (0847)*.

Sodium Chloride (*7647-14-5*)

See *Sodium chloride (0193)*.

Sodium Chloride Solution

A 20 per cent *m/m* solution of *sodium chloride R*.

Sodium Chloride Solution, Saturated

Mix 1 part of *sodium chloride R* with 2 parts of *water R*, shake from time to time and allow to stand. Before use, decant the

solution from any undissolved substance and filter, if necessary.

Sodium Cholate Cholic acid sodium salt hydrate; $C_{24}H_{39}NaO_5$ = 430.5 (*73163-53-8*)

$[\alpha]_D^{20}$: +31.3 (0.5% w/v in water).

General reagent grade of commerce.

Sodium Citrate Trisodium citrate; (*6132-04-3*)

See *Sodium citrate (0412)*.

Sodium Cobaltinitrite Trisodium hexanitrocobaltate(III); $Na_3[Co(NO_2)_6]$ = 403.9 (*13600-98-1*)

Orange-yellow powder, freely soluble in water, slightly soluble in ethanol (96 per cent).

Sodium Cobaltinitrite Solution Sodium hexanitritocobaltate(III)

A 100 g/L solution of *sodium cobaltinitrite R*. Prepare immediately before use.

Sodium Decanesulfonate Sodium decanesulfonate; $C_{10}H_{21}NaO_3S$ = 244.3 (*13419-61-9*)

Crystalline powder or flakes, white or almost white, freely soluble in water, soluble in methanol.

Sodium Decyl Sulfate Sodium decyl sulfate; $C_{10}H_{21}NaO_4S$ = 260.3 (*142-87-0*)

Content: minimum 95.0 per cent.

White or almost white powder, freely soluble in water.

Sodium Deoxycholate Deoxycholic acid, sodium salt; $C_{24}H_{39}NaO_4$ = 414.6 (*302-95-4*)

Sodium Deoxyribonucleate About 85 per cent has a relative molecular mass of 2×10^7 or greater (*73049-39-5*)

White or almost white, fibrous preparation obtained from calf thymus.

Test for suitability. Dissolve 10 mg in *imidazole buffer solution pH 6.5 R* and dilute to 10.0 mL with the same buffer solution (solution A). Dilute 2.0 mL of solution A to 50.0 mL with *imidazole buffer solution pH 6.5 R*. The absorbance (*2.2.25*) of the solution, measured at 260 nm, is 0.4 to 0.8.

To 0.5 mL of solution A add 0.5 mL of *imidazole buffer solution pH 6.5 R* and 3 mL of perchloric acid (25 g/L $HClO_4$). A precipitate is formed. Centrifuge. The absorbance of the supernatant, measured at 260 nm using a mixture of 1 mL of *imidazole buffer solution pH 6.5 R* and 3 mL of perchloric acid (25 g/L $HClO_4$) as compensation liquid, is not greater than 0.3.

In each of two tubes, place 0.5 mL of solution A and 0.5 mL of a solution of a reference preparation of streptodornase containing 10 IU/mL in *imidazole buffer solution pH 6.5 R*. To one tube add immediately 3 mL of perchloric acid (25 g/L $HClO_4$). A precipitate is formed. Centrifuge and collect supernatant A. Heat the other tube at 37 °C for 15 min and add 3 mL of perchloric acid (25 g/L $HClO_4$). Centrifuge and collect supernatant B. The absorbance of supernatant B, measured at 260 nm with reference to supernatant A is not less than 0.15.

Sodium Diethyldithiocarbamate $C_5H_{10}NNaS_2,3H_2O$ = 225.3 (*20624-25-3*)

White or almost white or colourless crystals, freely soluble in water, soluble in ethanol (96 per cent). The aqueous solution is colourless.

Sodium Diethyldithiocarbamate Solution

A 0.1% w/v solution of *sodium diethyldithiocarbamate* in *water*.

Prepare immediately before use.

Sodium Dihydrogen Orthophosphate Sodium dihydrogen phosphate dihydrate; Sodium Dihydrogen Phosphate; (*13472-35-0*)

See *Sodium dihydrogen phosphate dihydrate (0194)*.

Sodium Dihydrogen Orthophosphate, Anhydrous Sodium dihydrogen phosphate, anhydrous; NaH_2PO_4 = 120.0 (*7558-80-7*)

White or almost white powder, hygroscopic.

Storage: in an airtight container.

Sodium Dihydrogen Orthophosphate Monohydrate Sodium dihydrogen phosphate monohydrate; NaH_2PO4, H_2O = 138.0 (*10049-21-5*)

White or almost white, slightly deliquescent crystals or granules, freely soluble in water, practically insoluble in ethanol (96 per cent).

Storage: in an airtight container.

Sodium Dithionite $Na_2S_2O_4$ = 174.1 (*7775-14-6*)

White or greyish-white, crystalline powder, oxidises in air, very soluble in water, slightly soluble in ethanol (96 per cent).

Storage: in an airtight container.

Sodium Dodecyl Sulfate Sodium dodecyl sulphate; Sodium lauryl sulfate; Sodium lauryl sulphate; Sodium laurilsulfate; (*151-21-3*)

See *Sodium laurilsulfate (0098)*.

Content: minimum 99.0 per cent.

Sodium Fluoride (*7681-49-4*)

See *Sodium fluoride (0514)*.

When used in the Assay of preparations containing Sodium Fluoride, use a grade containing not less than 99.9% of NaF.

Sodium Formate Sodium methanoate; $CHNaO_2$ = 68.0 (*141-53-7*)

White or almost white, crystalline powder or deliquescent granules, soluble in water and in glycerol, slightly soluble in ethanol (96 per cent).

mp: about 253 °C.

Sodium Glucuronate D-Glucuronic acid, sodium salt; $C_6H_9NaO_7,H_2O$ = 234.1

$[\alpha]_D^{20}$: about + 21.5, determined on a 20 g/L solution.

Sodium Glycocholate Sodium [(3,7,12-trihydroxy-5-cholan-24-oyl)amino]acetate dihydrate; *N*-[(3,5,7,12)-3,7,12-Trihydroxy-24-oxocholan-24-yl]glycine monosodium salt dihydrate; $C_{26}H_{42}NNaO_6,2H_2O$ = 523.6 (*207300-80-9*)

Content: minimum 97 per cent of $C_{26}H_{42}NNaO_6,2H_2O$.

Sodium Heptanesulfonate 1-Heptanesulfonic acid sodium salt; 1-Heptanesulphonic acid sodium salt; Sodium heptanesulphonate; $C_7H_{15}NaO_3S$ = 202.3 (*22767-50-6*)

White or almost white, crystalline mass, freely soluble in water, soluble in methanol.

Sodium Heptanesulfonate Monohydrate 1-Heptanesulfonic acid sodium salt monohydrate; 1-Heptanesulphonic acid sodium salt monohydrate; Sodium heptanesulphonate monohydrate; $C_7H_{15}NaO_3S,H_2O$ = 220.3

Content: minimum 96 per cent (anhydrous substance).

White or almost white, crystalline powder, soluble in water, very slightly soluble in anhydrous ethanol.

Water (2.5.12): maximum 8 per cent, determined on 0.300 g.

Assay. Dissolve 0.150 g in 50 mL of *anhydrous acetic acid R*. Titrate with *0.1 M perchloric acid*, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M perchloric acid* is equivalent to 20.22 mg of $C_7H_{15}NaO_3S$.

Sodium Hexanesulfonate Hexanesulfonic acid sodium salt; Hexanesulphonic acid sodium salt; Sodium hexanesulphonate; $C_6H_{13}NaO_3S$ = 188.2 (*2832-45-3*)

White or almost white powder, freely soluble in water.

Sodium Hexanesulfonate Monohydrate Hexanesulfonic acid sodium salt monohydrate; Sodium hexanesulfonate monohydrate; $C_6H_{13}NaO_3S,H_2O$ = 206.2 (*207300-91-2*)

White or almost white powder, soluble in water.

Sodium Hexanesulfonate Monohydrate for Ion-pair Chromatography $C_6H_{13}NaO_3S,H_2O$ = 206.2 (*207300-91-2*)

Content: minimum 99.0 per cent.

Sodium Hydrogen Carbonate Sodium bicarbonate; (*144-55-8*)

See *Sodium hydrogen carbonate (0195)*.

Sodium Hydrogen Carbonate Solution

A 42 g/L solution of *sodium hydrogen carbonate R*.

Sodium Hydrogen Sulfate Sodium bisulphate; Sodium hydrogen sulphate; $NaHSO_4$ = 120.1 (*7681-38-1*)

Freely soluble in water, very soluble in boiling water. It decomposes in ethanol (96 per cent) into sodium sulfate and free sulfuric acid.

mp: about 315 °C.

Sodium Hydrogensulfite Sodium hydrogensulphite; $NaHO_3S$ = 104.1 (*7631-90-5*)

White or almost white, crystalline powder, freely soluble in water, sparingly soluble in ethanol (96 per cent).

On exposure to air, some sulfur dioxide is lost and the substance is gradually oxidated to sulfate.

Sodium Hydroxide (*1310-73-2*)

See *Sodium hydroxide (0677)*.

2M Sodium Hydroxide

Dissolve 84 g of *sodium hydroxide R* in *carbon dioxide-free water R* and dilute to 1000.0 mL with the same solvent.

4M Sodium Hydroxide

Dissolve 168 g of *sodium hydroxide R* in *carbon dioxide-free water R* and dilute to 1.0 L with the same solvent.

Sodium Hydroxide, Ethanolic

Solutions of the requisite molarity may be obtained by dissolving the appropriate amount of *sodium hydroxide* in sufficient *ethanol (96%)* to produce 1000 mL.

Sodium Hydroxide, Methanolic

Solutions of the requisite molarity may be obtained by dissolving the appropriate amount of *sodium hydroxide* in sufficient *methanol* to produce 1000 mL.

Sodium Hydroxide Solution

Dissolve 20.0 g of *sodium hydroxide R* in *water R* and dilute to 100.0 mL with the same solvent. Verify the concentration by titration with *1 M hydrochloric acid*, using *methyl orange solution R* as indicator, and adjust if necessary to 200 g/L.

Sodium Hydroxide Solution, Carbonate-free

Dissolve *sodium hydroxide R* in *carbon dioxide-free water R* to give a concentration of 500 g/L and allow to stand. Decant the clear supernatant, taking precautions to avoid the introduction of carbon dioxide.

Sodium Hydroxide Solution, Dilute

Dissolve 8.5 g of *sodium hydroxide R* in *water R* and dilute to 100 mL with the same solvent.

Sodium Hydroxide Solution, Methanolic

Dissolve 40 mg of *sodium hydroxide R* in 50 mL of *water R*. Cool and add 50 mL of *methanol R*.

Sodium Hydroxide Solution R1, Methanolic

Dissolve 200 mg of *sodium hydroxide R* in 50 mL of *water R*. Cool and add 50 mL of *methanol R*.

Sodium Hydroxide Solution, Strong

Dissolve 42 g of *sodium hydroxide R* in *water R* and dilute to 100 mL with the same solvent.

Sodium 2-Hydroxybutyrate Sodium (2*RS*)-2-hydroxybutanoate; $C_4H_7NaO_3$ = 126.1 (*19054-57-0*)

Sodium Hypobromite Solution

In a bath of iced water mix 20 mL of *strong sodium hydroxide solution R* and 500 mL of *water R*, add 5 mL of *bromine solution R* and stir gently until solution is complete. Prepare immediately before use.

Sodium Hypochlorite Solution

General reagent grade of commerce containing 10 to 14% w/v of available chlorine.

Sodium Hypochlorite Solution (3% Cl) Sodium hypochlorite solution, strong

Content: 25 g/L to 30 g/L of active chlorine.

Yellowish liquid with an alkaline reaction.

Assay. Introduce into a flask, successively, 50 mL of *water R*, 1 g of *potassium iodide R* and 12.5 mL of *dilute acetic acid R*. Dilute 10.0 mL of the substance to be examined to 100.0 mL with *water R*. Introduce 10.0 mL of this solution into the flask and titrate with *0.1 M sodium thiosulfate*, using 1 mL of *starch solution R* as indicator.

1 mL of *0.1 M sodium thiosulfate* is equivalent to 3.546 mg of active chlorine.

Storage: protected from light.

see Strong Sodium Hypochlorite Solution

Sodium Hypochlorite Solution, Dilute

Dilute 35 mL of *sodium hypochlorite solution* to 100 mL with *water* immediately before use.

The solution contains approximately 3.5% w/v of available chlorine.

Sodium Hypophosphite Sodium phosphinate monohydrate; NaH_2PO_2,H_2O = 106.0 (*10039-56-2*)

White or almost white, crystalline powder or colourless crystals, hygroscopic, freely soluble in water, soluble in ethanol (96 per cent).

Storage: in an airtight container.

Sodium Iodide (*7681-82-5*)

See *Sodium iodide (0196)*.

Sodium Iodobismuthate Solution

Boil for a few minutes a mixture of 2.6 g of *bismuth oxycarbonate*, 7.0 g of *sodium iodide* and 25 mL of *glacial acetic acid*. Allow to stand for 12 hours and filter, if necessary, through sintered glass. To 20 mL of the filtrate add 80 mL of *ethyl acetate* (solution A). Immediately before use, mix 2 mL of solution A, 20 mL of *glacial acetic acid* and 40 mL of *ethyl acetate*.

For use in connection with thin-layer chromatography the sensitivity may be increased by spraying first with this solution and then with *sulfuric acid (0.2%)*.

Solution A should be kept in a well-closed container.

Sodium Laurilsulfate Sodium dodecyl sulphate; (*151-21-3*)

See *Sodium laurilsulfate (0098)*.

Sodium Laurilsulfate R1 (*151-21-3*)

Content: minimum 99.0 per cent.

Sodium Lauryl Sulfate (*151-21-3*)

See *Sodium laurilsulfate R*.

Sodium Laurylsulfonate for Chromatography Sodium laurylsulphonate for chromatography; $C_{12}H_{25}NaO_3S$ = 272.4 (*2386-53-0*)

White or almost white powder or crystals, freely soluble in water.

Absorbance $A_{1\ cm}^{5\%}$ (*2.2.25*), determined in *water R*: about 0.05 at 210 nm; about 0.03 at 220 nm; about 0.02 at 230 nm; about 0.02 at 500 nm.

Sodium Metabisulfite Sodium metabisulphite; Sodium pyrosulfite; Sodium pyrosulphite; (*7681-57-4*)

See *Sodium metabisulfite (0849)*.

Sodium Methanesulfonate Methanesulfonic acid, sodium salt; Methanesulphonic acid, sodium salt; Sodium methanesulphonate; CH_3SO_3Na = 118.1 (*2386-57-4*)

White or almost white, crystalline powder, hygroscopic.

Storage: in an airtight container.

Sodium 2-methyl-2-thiazoline-4-carboxylate $C_5H_6NNaO_2S$ = 167.2 (*15058-19-2*)

Sodium 2-methyl-4,5-dihydro-1,3-thiazole-4-carboxylate.

White solid.

Content: minimum 95 per cent.

Sodium Molybdate Disodium molybdate dihydrate; $Na_2MoO_4,2H_2O$ = 242.0 (*10102-40-6*)

White or almost white, crystalline powder or colourless crystals, freely soluble in water.

Sodium 1,2-Naphthoquinone-4-sulfonate Sodium naphthoquinonesulfonate; $C_{10}H_5NaO_5S$ = 260.2 (*521-24-4*)

Yellow or orange-yellow, crystalline powder, freely soluble in water, practically insoluble in ethanol (96 per cent).

Sodium Nitrate $NaNO_3$ = 85.0 (*7631-99-4*)

White or almost white powder or granules or colourless, transparent crystals, deliquescent in moist air, freely soluble in water, slightly soluble in ethanol (96 per cent).

Storage: in an airtight container.

Sodium Nitrite $NaNO_2$ = 69.0 (*7632-00-0*)

Content: minimum 97.0 per cent.

White or almost white, granular powder or a slightly yellow, crystalline powder, freely soluble in water.

Assay. Dissolve 0.100 g in 50 mL of *water R*. Add 50.0 mL of *0.02 M potassium permanganate* and 15 mL of *dilute sulfuric acid R*. Add 3 g of *potassium iodide R*. Titrate with *0.1 M sodium thiosulfate*, using 1.0 mL of *starch solution R* added towards the end of the titration as indicator.

1 mL of *0.02 M potassium permanganate* is equivalent to 3.450 mg of $NaNO_2$.

Sodium Nitrite Solution

A 100 g/L solution of *sodium nitrite R*. Prepare immediately before use.

Sodium Nitroprusside Sodium pentacyanonitrosylferrate(III) dihydrate; $Na_2[Fe(CN)_5(NO)],2H_2O$ = 298.0 (*13755-38-9*)

Reddish-brown powder or crystals, freely soluble in water, slightly soluble in ethanol (96 per cent).

Sodium Nitroprusside-Carbonate Solution

Dissolve 1 g of *sodium nitroprusside* and 1 g of *anhydrous sodium carbonate* in sufficient *water* to produce 100 mL.

Sodium Octanesulfonate Octanesulfonic acid sodium salt; Octanesulphonic acid sodium salt; Sodium octanesulphonate; $C_8H_{17}NaO_3S$ = 216.3 (*5324-84-5*)

Content: minimum 98.0 per cent.

White or almost white, crystalline powder or flakes, freely soluble in water, soluble in methanol.

Absorbance (*2.2.25*): maximum 0.10, determined at 200 nm and maximum 0.01, determined at 250 nm using a 54 g/L solution.

Sodium Octanesulfonate Monohydrate Sodium octanesulphonate monohydrate; $C_8H_{17}NaO_3S,H_2O$ = 234.3 (*207596-29-0*)

White or almost white powder.

Sodium Octyl Sulfate Octyl sulfate sodium salt; 4-Octyl sulphate sodium salt; Sodium octyl sulphate; $C_8H_{17}NaO_4S$ = 232.3 (*142-31-4*)

White or almost white, crystalline powder or flakes, freely soluble in water, soluble in methanol.

Sodium Oxalate $C_2Na_2O_4$ = 134.0 (*62-76-0*)

White or almost white, crystalline powder, soluble in water, practically insoluble in ethanol (96 per cent).

Sodium Oxidronate Sodium hydroxymethylenediphosphonate; $CH_4Na_2O_7P_2$ = 236.0 (*14255-61-9*)

White or almost white powder or colourless crystals, very soluble in water, very slightly soluble in ethanol (96 per cent), practically insoluble in methylene chloride.

Sodium Pentanesulfonate 1-Pentanesulfonic acid sodium salt; 1-Pentanesulphonic acid sodium salt; Sodium pentanesulphonate; $C_5H_{11}NaO_3S$ = 174.2 (*22767-49-3*)

White or almost white, crystalline solid, soluble in water.

Sodium Pentanesulfonate Monohydrate 1-Pentanesulfonic acid sodium salt monohydrate; 1-Pentanesulphonic acid sodium salt monohydrate; Sodium pentanesulphonate monohydrate; $C_5H_{11}NaO_3S,H_2O$ = 192.2 (*207605-40-1*)

White or almost white crystalline solid, soluble in water.

Sodium Pentanesulfonate Monohydrate R1 Sodium pentanesulphonate monohydrate R1; $C_5H_{11}NaO_3S$, H_2O = 192.2 (*207605-40-1*)

Content: minimum 99 per cent of $C_5H_{11}NaO_3S,H_2O$.

Sodium Perchlorate $NaClO_4,H_2O$ = 140.5 (*7791-07-3*)

Content: minimum 99.0 per cent of $NaClO_4,H_2O$.

White or almost white, deliquescent crystals, very soluble in water.

Storage: in a well-closed container.

Sodium Periodate Sodium metaperiodate; $NaIO_4$ = 213.9 (*7790-28-5*)

Content: minimum 99.0 per cent.

White or almost white, crystalline powder or crystals, soluble in water and in mineral acids.

Sodium Periodate Solution

Dissolve 1.07 g of *sodium periodate R* in *water R*, add 5 mL of *dilute sulfuric acid R* and dilute to 100.0 mL with *water R*. Use a freshly prepared solution.

Sodium Phosphite Sodium phosphite pentahydrate; $Na_2HPO_3,5H_2O$ = 216.0 (*13517-23-2*)

White or almost white, crystalline powder, hygroscopic, freely soluble in water.

Storage: in an airtight container.

Sodium Picrate Solution, Alkaline

Mix 20 mL of *picric acid solution R* and 10 mL of a 50 g/L solution of *sodium hydroxide R* and dilute to 100 mL with *water R*.

Storage: use within 2 days.

Sodium 1-Propanesulfonate Sodium propane-1-sulfonate monohydrate; $C_3H_9SO_4Na$ = 164.2 (*304672-01-3*)

mp: about 250 °C.

Sodium Pyrophosphate Tetrasodium diphosphate decahydrate; $Na_4P_2O_7,10H_2O$ = 446.1 (*13472-36-1*)

Colourless, slightly efflorescent crystals, freely soluble in water.

Sodium Pyruvate 2-Oxopropanoic acid sodium salt; $C_3H_3NaO_3$ = 110.0 (*113-24-6*)

White or faint yellow powder, soluble in water (100 mg/mL).

mp: greater than 300 °C.

Sodium Rhodizonate Rhodizonic acid disodium salt; $C_6Na_2O_6$ = 214.0 (*523-21-7*)

Violet crystals, soluble in water with an orange-yellow colour.

Solutions are unstable and must be prepared on the day of use.

Sodium Salicylate (*54-21-7*)

See *Sodium salicylate (0413)*.

Sodium Stearyl Fumarate $C_{22}H_{39}NaO_4$ (*4070-80-8*)

See *Sodium stearyl fumarate (1567)*.

Sodium Sulfate Sodium sulfate decahydrate; $Na_2SO_4,10H_2O$ = 322.2 (*7727-73-3*)

See *Sodium sulfate decahydrate (0100)*.

Sodium Sulfate, Anhydrous Sodium sulphate, anhydrous; (*7757-82-6*)

Ignite at 600 °C to 700 °C anhydrous sodium sulfate complying with the requirements prescribed in the monograph on *Anhydrous sodium sulfate (0099)*.

Loss on drying (*2.2.32*): maximum 0.5 per cent, determined by drying in an oven at 130 °C.

Sodium Sulfate, Anhydrous R1

Complies with the requirements prescribed for *anhydrous sodium sulfate R* with the following maximum contents.

Cl: 20 ppm.

Pb: 10 ppm.

As: 3 ppm.

Ca: 50 ppm.

Fe: 10 ppm.

Mg: 10 ppm.

Sodium Sulfide Sodium sulphide; $Na_2S,9H_2O$ = 240.2 (*1313-84-4*)

Colourless, rapidly yellowing crystals, deliquescent, very soluble in water.

Storage: in an airtight container.

Sodium Sulfide Solution Sodium sulphide solution

Dissolve 12 g of *sodium sulfide R* with heating in 45 mL of a mixture of 10 volumes of *water R* and 29 volumes of *glycerol (85 per cent) R*, allow to cool and dilute to 100 mL with the same mixture of solvents.

The solution should be colourless.

Sodium Sulfide Solution R1 Sodium sulphide solution R1

Prepare by one of the following methods.

— Dissolve 5 g of *sodium sulfide R* in a mixture of 10 mL of *water R* and 30 mL of *glycerol R*.

— Dissolve 5 g of *sodium hydroxide R* in a mixture of 30 mL of *water R* and 90 mL of *glycerol R*. Divide the solution into 2 equal portions. Saturate 1 portion with *hydrogen sulfide R*, with cooling. Mix the 2 portions.

Storage: in a well-filled container, protected from light; use within 3 months.

Sodium Sulfite Sodium sulphite; Sodium sulfite heptahydrate; (*10102-15-5*)

See *Sodium sulfite heptahydrate (0776)*.

Sodium Sulfite, Anhydrous Sodium sulphite, anhydrous; (*7757-83-7*)

See *Sodium sulfite (0775)*.

Sodium (+)-Tartrate Disodium (2R,3R)-2,3-dihydroxybutanedioate dehydrate; Sodium tartrate; $C_4H_4Na_2O_6,2H_2O$ = 230.1 (*6106-24-7*)

White or almost white crystals or granules, very soluble in water, practically insoluble in ethanol (96 per cent).

Sodium Taurodeoxycholate Sodium 2-[(3,12-dihydroxy-5-cholan-24-oyl)amino]ethanesulfonate monohydrate; 2-[[(3,5,12)-3,12-Dihydroxy-24-oxocholan-24-yl]amino] ethanesulfonic acid monosodium salt monohydrate; $C_{26}H_{44}NNaO_6S,H_2O$ = 539.7 (*110026-03-4*)

Content: minimum 94 per cent of $C_{26}H_{44}NNaO_6S,H_2O$.

Sodium Tetraborate Borax; Disodium tetraborate; (*1303-96-4*)

See *Borax (0013)*.

Sodium Tetrahydroborate Sodium borohydride; $NaBH_4$ = 37.8 (*16940-66-2*)

Colourless, hygroscopic crystals, freely soluble in water, soluble in anhydrous ethanol, decomposing at higher temperature or in the presence of acids or certain metal salts forming borax and hydrogen.

Storage: in an airtight container.

Sodium Tetrahydroborate Reducing Solution

Introduce about 100 mL of *water R* into a 500 mL volumetric flask containing a stirring bar. Add 5.0 g of *sodium hydroxide R* in pellets and 2.5 g of *sodium tetrahydroborate R*. Stir until complete dissolution, dilute to 500.0 mL with *water R* and mix. Prepare immediately before use.

Sodium Tetraphenylborate $NaB(C_6H_5)_4$ = 342.2 (*143-66-8*)

White or slightly yellowish, bulky powder, freely soluble in water and in acetone.

Sodium Tetraphenylborate Solution

Filter before use if necessary.

A 10 g/L solution of *sodium tetraphenylborate R*.

Storage: use within 1 week.

Sodium Thioglycollate Sodium mercaptoacetate; $C_2H_3NaO_2S$ = 114.1 (*367-51-1*)

White or almost white, granular powder or crystals, hygroscopic, freely soluble in water and in methanol, slightly soluble in ethanol (96 per cent).

Storage: in an airtight container.

Sodium Thiosulfate Sodium thiosulphate; (*10102-17-7*)

See *Sodium thiosulfate (0414)*.

Sodium Thiosulfate, Anhydrous Disodium thiosulfate; $Na_2S_2O_3$ = 158.1 (*7772-98-7*)

Content: minimum 98.0 per cent.

Sodium Tungstate Disodium tungstate dihydrate; $Na_2WO_4,2H_2O$ = 329.9 (*10213-10-2*)

White or almost white, crystalline powder or colourless crystals, freely soluble in water forming a clear solution, practically insoluble in ethanol (96 per cent).

Solochrome Dark Blue CI 15705; calcon; mordant black 17; $C_{20}H_{13}N_2NaO_5S$ = 416.4 (*2538-85-4*)

General reagent grade of commerce.

A brownish black powder with a violet sheen. Produces a purplish red colour with calcium ions in alkaline solutions. When metal ions are absent, for example, in the presence of an excess of disodium edetate, the solution is blue.

Solochrome Dark Blue Mixture

A mixture of 1 part of *solochrome dark blue* with 99 parts of freshly ignited *anhydrous sodium sulfate*.

Complies with the following test.

Sensitivity to calcium Dissolve 0.2 g in 5 mL of *water*. To 1 mL of the solution add 50 mL of *water*, 10 mL of 1M *sodium hydroxide* and 1 mL of a 1% w/v solution of *magnesium sulfate*; a blue colour is produced. Add 0.1 mL of a 0.15% w/v solution of *calcium chloride*; a violet colour is produced. Add 0.1 mL of 0.01M *disodium edetate VS*; a pure blue colour is produced.

Sorbic Acid Hexa-2,4-dienoic acid; $C_6H_8O_2$ = 112.1 (*110-44-1*)

mp: about 136°.

General reagent grade of commerce.

D-Sorbitol Sorbitol; (*50-70-4*)

See *Sorbitol (0435)*.

Soya Bean Lecithin (*8030-76-0*)

Soya-bean Oil, Refined See *Soya-bean oil, refined (1473)*.

Sphingomyelin from Egg Yolk (*85187-10-6*)

(2*R*,3*S*,4*E*)-2-(Acylamino)-3-hydroxyoctadec-4-en-1-yl 2-(trimethylazaniumyl)ethyl phosphate.

Squalane (6*E*,10*E*,15*E*,19*E*)-2,6,10,15,19,23-Hexamethyltetracosane; Perhydrosqualene; $C_{30}H_{62}$ = 422.8 (*111-01-3*)

Colourless, oily liquid, freely soluble in fatty oils, slightly soluble in acetone, in ethanol (96 per cent), in glacial acetic acid and in methanol.

d_{20}^{20}: 0.811 to 0.813.

n_D^{20}: 1.451 to 1.453.

Stanolone 17β-Hydroxy-5α-androstan-3-one; $C_{19}H_{30}O_2$ = 290.4 (*521-18-6*)

White or almost white powder.

mp: about 180 °C.

Staphylococcus Aureus Strain V8 Protease Type XVII-B (*66676-43-5*)

Microbial extracellular proteolytic enzyme. A lyophilised powder containing 500 units to 1000 units per milligram of solid.

Starch

Potato starch of commerce.

Starch, Hydrolysed

Electrophoretic grade of commerce.

Starch Iodate Paper

Immerse strips of filter paper in 100 mL of *iodide-free starch solution R* containing 0.1 g of *potassium iodate R*. Drain and allow to dry protected from light.

Starch Iodide Paper

Immerse strips of filter paper in 100 mL of *potassium iodide and starch solution R*. Drain and allow to dry protected from light.

Test for sensitivity. Mix 0.05 mL of *0.1 M sodium nitrite* with 4 mL of *hydrochloric acid R* and dilute to 100 mL with *water R*. Apply one drop of the solution to starch iodide paper; a blue spot appears.

Starch Mucilage

Triturate 0.5 g of *starch* or *soluble starch* with 5 mL of *water* and add, stirring continuously, to sufficient *water* to produce about 100 mL. Boil for a few minutes, cool and filter.

Produces a blue colour with free iodine in the presence of a soluble iodide.

It must be recently prepared.

Starch, Soluble (*9005-84-9*)

White or almost white powder.

Starch Solution

Triturate 1.0 g of *soluble starch R* with 5 mL of *water R* and whilst stirring pour the mixture into 100 mL of boiling *water R* containing 10 mg of *mercuric iodide R*.

NOTE: commercially available reagents may be used; including mercury-free solutions or those containing alternative preservatives.

Carry out the test for sensitivity each time the reagent is used.

Test for sensitivity. To a mixture of 1 mL of the starch solution and 20 mL of *water R*, add about 50 mg of *potassium iodide R* and 0.05 mL of *iodine solution R1*. The solution is blue.

Starch Solution, Iodide-free

Prepare the solution as prescribed for *starch solution R* omitting the mercuric iodide. Prepare immediately before use.

Starch Solution R1

Mix 1 g of *soluble starch R* and a small amount of cold *water R*. Add this mixture, while stirring, to 200 mL of boiling *water R*. Add 0.25 g of *salicylic acid R* and boil for 3 min. Immediately remove from the heat and cool.

Storage: if long storage is required, the solution shall be stored at 4 °C to 10 °C. A fresh starch solution shall be prepared when the end-point of the titration from blue to colourless fails to be sharp. If stored under refrigeration, the starch solution is stable for about 2 to 3 weeks.

Test for sensitivity. A mixture of 2 mL of *starch solution R1*, 20 mL of *water R*, about 50 mg of *potassium iodide R* and 0.05 mL of *iodine solution R1* is blue.

Starch Solution R2

Triturate 1.0 g of *soluble starch R* with 5 mL of *water R* and whilst stirring pour the mixture into 100 mL of boiling *water R*. Use a freshly prepared solution.

Test for sensitivity. To a mixture of 1 mL of the starch solution and 20 mL of *water R*, add about 50 mg of *potassium iodide R* and 0.05 mL of *iodine solution R1*. The solution is blue.

Starch Substrate

Determine the water content of *starch EPBRP* by heating at 120° for 4 hours. Stir a quantity of *starch EPBRP* equivalent to 2.0 g of the dried substance with 10 mL of *water* and add, stirring continuously, to 160 mL of boiling *water*. Rinse the container with several 10-mL quantities of *water*, add the washings to the hot starch solution and heat to boiling, stirring continuously. Cool to 20° and add sufficient *water* to produce 200 mL.

Use on the day of preparation.

Stavudine (*3056-17-5*)

See *Stavudine (2130)*.

Stearic Acid Octadecanoic acid; $C_{18}H_{36}O_2$ = 284.5 (*57-11-4*)

White or almost white powder or flakes, greasy to the touch, practically insoluble in water, soluble in hot ethanol (96 per cent).

mp: about 70 °C.

Stearic acid used in the assay of total fatty acids in Saw palmetto fruit (1848) complies with the following additional test.

Assay. Gas chromatography (*2.2.28*) as prescribed in the monograph *Saw palmetto fruit (1848)*.

Content: minimum 98 per cent, calculated by the normalisation procedure.

Stearic Anhydride $C_{36}H_{70}O_3$ = 551.0 (*638-08-4*)

mp: about 70°.

General reagent grade of commerce.

White, waxy flakes.

Stigmasterol (22*E*)-Stigmasta-5,22-dien-3β-ol; (22*E*)-24-Ethylcholesta-5,22-dien-3β-ol; $C_{29}H_{48}O$ = 412.7 (*83-48-7*)

White or almost white powder, insoluble in water.

mp: about 170 °C.

$[\alpha]_D^{22}$: about – 51, determined with a 20 g/L solution in *chloroform R*.

Streptomycin Sulfate Streptomycin sulphate; (*3810-74-0*)

See *Streptomycin sulfate (0053)*.

Strongly Acidic Ion-exchange Resin See *ion-exchange resin, strongly acidic R*.

Strontium Carbonate $SrCO_3$ = 147.6 (*1633-05-2*)

White or almost white, crystalline powder.

Content: minimum 99.5 per cent.

Strontium Chloride Strontium chloride hexahydrate; $SrCl_2,6H_2O$ = 266.6 (*10025-70-4*)

White or almost white crystals, very soluble in water.

mp: about 115 °C (loss of water) and 872 °C.

Strontium Selective Extraction Resin

Commercially available resin prepared by loading a suspension of 4,4′(5′)-di-*tert*-butylcyclohexano-18-crown-6 (crown ether) in octanol onto an inert chromatographic support. The bed density of this resin is approximately 0.35 g/mL.

Strontium-85 Spiking Solution

Dilute *strontium-85 standard solution R* to a radioactivity concentration of approximately 10 kBq/mL with a 0.27 g/L solution of *strontium chloride hexahydrate R* in a 1.03 g/L solution of *hydrochloric acid R*.

Strontium-85 Standard Solution

A solution of strontium-85 in the form of Sr^{2+} ions in a 51.5 g/L solution of *hydrochloric acid R*.

Strychnine $C_{21}H_{22}N_2O_2$ = 334.4 (*57-24-9*)

(4a*R*,4b*R*,5a*S*,8a*R*,13a*S*,15a*S*)-2,4a,4b,5,5a,7,8,13a,15,15a-Decahydro-4,6-methano-6*H*-indolo[3,2,1-*ij*]oxepino[2,3,4-*de*]pyrrolo[2,3-*h*]quinolin-14-one. Strychnidin-10-one.

White or almost white, crystalline powder, sparingly soluble in water.

mp: about 285 °C.

Styrene Ethenylbenzene; C_8H_8 = 104.2 (*100-42-5*)

bp: about 145 °C.

Colourless, oily liquid, very slightly soluble in water.

Styrene–Divinylbenzene Copolymer

Porous, rigid, cross-linked polymer beads. Several grades are available with different sizes of beads. The size range of the beads is specified after the name of the reagent in the tests where it is used.

Succinic Acid Butanedioic acid; $C_4H_6O_4$ = 118.1 (*110-15-6*)

White or almost white, crystalline powder or colourless crystals, soluble in water and in ethanol (96 per cent).

mp: 184 °C to 187 °C.

Sucrose (*57-50-1*)

See *Sucrose (0204)*.

Sudan Red CI 26100; sudan III; solvent red 23; 1-(4-phenylazophenylazo)-2-naphthol; $C_{22}H_{16}N_4O$ = 352.4 (*85-86-9*)

Technical reagent grade of commerce.

A reddish brown powder.

Sudan Red G Sudan red I; $C_{17}H_{14}N_2O_2$ = 278.3

Schultz No. 149

Colour Index No. 12150

Reddish-brown powder, practically insoluble in water.

Chromatography. Thin-layer chromatography (*2.2.27*) using *silica gel G R* as the coating substance: apply 10 µL of a 0.1 g/L solution in *methylene chloride R* and develop over a path of 10 cm with the same solvent; the chromatogram shows only one principal spot.

Sudan Red Solution

A 0.5% w/v solution of *sudan red* in *anhydrous acetic acid*.

Sudan Yellow 1-(phenylazo)naphthalen-2-ol; Sudan I; Sudan orange; $C_{16}H_{12}N_2O$ = 248.3 (*842-07-9*)

Colour Index No. 12055

Orange-red powder, practically insoluble in water, soluble in methylene chloride.

mp: about 131 °C.

Sudan Yellow Solution

A 0.2% w/v solution of *sudan yellow* in a mixture of 1 volume of *benzene* and 4 volumes of *petroleum spirit (boiling range, 60° to 80°)*.

Sulfanilamide 4-Aminobenzenesulphonamide; Sulphanilamide; $C_6H_8N_2O_2S$ = 172.2 (*63-74-1*)

White or almost white powder, slightly soluble in water, freely soluble in boiling water, in acetone, in dilute acids and in solutions of the alkali hydroxides, sparingly soluble in ethanol (96 per cent) and in light petroleum.

mp: about 165 °C.

Sulfathiazole 4-Amino-*N*-(thiazol-2-yl) benzenesulfonamide; $C_9H_9N_3O_2S_2$ = 255.3 (*72-14-0*)

White or yellowish-white powder or crystals, very slightly soluble in water, soluble in acetone, slightly soluble in

ethanol (96 per cent). It dissolves in dilute mineral acids and in solutions of alkali hydroxides and carbonates.

mp: about 200 °C.

Sulfamic Acid H_3NO_3S = 97.1 (*5329-14-6*)

White or almost white crystalline powder or crystals, freely soluble in water, sparingly soluble in acetone, in ethanol (96 per cent) and in methanol.

mp: about 205 °C, with decomposition.

Sulfan Blue Sulphan blue; $C_{27}H_{31}N_2NaO_6S_2$ = 566.6 (*129-17-9*)

Schultz No. 769

Colour Index No. 42045

Violet powder, soluble in water. Dilute solutions are blue and turn yellow on the addition of concentrated hydrochloric acid.

Sulfanilic Acid Sulphanilic acid; $C_6H_7NO_3S$ = 173.2 (*121-57-3*)

Colourless crystals, sparingly soluble in water, practically insoluble in ethanol (96 per cent).

Sulfanilic Acid Solution Sulphanilic acid solution

Dissolve 0.33 g of *sulfanilic acid R* in 75 mL of *water R* heating gently if necessary and dilute to 100 mL with *glacial acetic acid R*.

Sulfanilic Acid Solution R1 Sulphanilic acid solution R1

Dissolve 0.5 g of *sulfanilic acid R* in a mixture of 75 mL of *dilute acetic acid R* and 75 mL of *water R*.

Sulfanilic Acid Solution, Diazotised Sulphanilic acid solution, diazotised

Dissolve, with warming, 0.9 g of *sulfanilic acid R* in 9 mL of *hydrochloric acid R*, and dilute to 100 mL with *water R*. Cool 10 mL of this solution in iced water and add 10 mL of an ice-cold 45 g/L solution of *sodium nitrite R*. Allow to stand at 0 °C for 15 min (if stored at this temperature, the solution is stable for 3 days) and immediately before use add 20 mL of a 100 g/L solution of *sodium carbonate R*.

Sulfomolybdic Reagent R2 Sulphomolybdic reagent R2

Dissolve about 50 mg of *ammonium molybdate R* in 10 mL of *sulfuric acid R*.

Sulfomolybdic Reagent R3 Sulphomolybdic reagent R3

Dissolve with heating 2.5 g of *ammonium molybdate R* in 20 mL of *water R*. Dilute 28 mL of *sulfuric acid R* in 50 mL of *water R*, then cool. Mix the two solutions and dilute to 100 mL with *water R*.

Storage: in a polyethylene container.

Sulfosalicylic Acid 5-Sulfo-2-hydroxybenzoic acid; 5-Sulpho-2-hydroxybenzoic acid; 3-Sulpho-6-hydroxybenzoic acid; 3-Sulpho-6-hydroxybenzoic acid; Sulfosalicylic acid; $C_7H_6O_6S,2H_2O$ = 254.2 (*5965-83-3*)

White or almost white, crystalline powder or crystals, very soluble in water and in ethanol (96 per cent).

mp: about 109 °C.

Sulfur (*7704-34-9*)

See *Sulfur (0953)*.

Sulfur Dioxide Sulphur dioxide; SO_2 = 64.1 (*7446-09-5*)

A colourless gas. When compressed it is a colourless liquid.

Sulfur Dioxide R1 Sulphur dioxide R1; SO_2 = 64.1 (*7446-09-5*)

Content: minimum 99.9 per cent *V/V*.

Sulfur Dioxide Solution Sulphur dioxide solution, sulfurous acid, sulphurous acid; H_2SO_3 = 82.08

Use a solution of commerce containing about 5% w/v of SO_2; weight per mL, about 1.03 g.

Sulfuric Acid Sulphuric acid; H_2SO_4 = 98.1 (*7664-93-9*)

Content: 95.0 per cent *m/m* to 97.0 per cent *m/m*.

Colourless, caustic liquid with an oily consistency, highly hygroscopic, miscible with water and with ethanol (96 per cent) producing intense heat.

d_{20}^{20}: 1.834 to 1.837.

A 10 g/L solution is strongly acid and gives the reactions of sulfates (*2.3.1*).

Appearance. It is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

Oxidisable substances. Pour 20 g cautiously, with cooling, into 40 mL of *water R*. Add 0.5 mL of *0.002 M potassium permanganate*. The violet colour persists for at least 5 min.

Chlorides: maximum 0.5 ppm.

Pour 10 g, carefully and while cooling, into 10 mL of *water R* and after cooling dilute to 20 mL with the same solvent. Add 0.5 mL of *silver nitrate solution R2*. Allow to stand for 2 min protected from bright light. The solution is not more opalescent than a standard prepared at the same time using a mixture of 1 mL of *chloride standard solution (5 ppm Cl) R*, 19 mL of *water R* and 0.5 mL of *silver nitrate solution R2*.

Nitrates: maximum 0.5 ppm.

Pour 50 g or 27.2 mL, carefully and while cooling, into 15 mL of *water R*. Add 0.2 mL of a freshly prepared 50 g/L solution of *brucine R* in *glacial acetic acid R*. After 5 min any colour is less intense than that of a reference mixture prepared in the same manner and containing 12.5 mL of *water R*, 50 g of *nitrogen-free sulfuric acid R*, 2.5 mL of *nitrate standard solution (10 ppm NO_3) R* and 0.2 mL of a 50 g/L solution of *brucine R* in *glacial acetic acid R*.

Ammonium: maximum 2 ppm.

Pour 2.5 g, carefully and while cooling, into *water R* and dilute to 20 mL with the same solvent. Cool, and add dropwise 10 mL of a 200 g/L solution of *sodium hydroxide R*, followed by 1 mL of *alkaline potassium tetraiodomercurate solution R*. The colour of the solution is less intense than that of a mixture of 5 mL of *ammonium standard solution (1 ppm NH_4) R*, 15 mL of *water R*, 10 mL of a 200 g/L solution of *sodium hydroxide R* and 1 mL of *alkaline potassium tetraiodomercurate solution R*.

Arsenic (*2.4.2, Method A*): maximum 0.02 ppm.

To 50 g add 3 mL of *nitric acid R* and evaporate carefully until the volume is reduced to about 10 mL. Cool, add to the residue 20 mL of *water R* and concentrate to 5 mL. Prepare the standard using 1.0 mL of *arsenic standard solution (1 ppm As) R*.

Iron (*2.4.9*): maximum 1 ppm.

Dissolve the residue on ignition with slight heating in 1 mL of *dilute hydrochloric acid R* and dilute to 50.0 mL with *water R*. Dilute 5 mL of this solution to 10 mL with *water R*.

Heavy metals (*2.4.8*): maximum 2 ppm.

Dilute 10 mL of the solution obtained in the test for iron to 20 mL with *water R*. 12 mL of the solution complies with test A. Prepare the reference solution using *lead standard solution (2 ppm Pb) R*.

Residue on ignition: maximum 0.001 per cent, determined on 100 g by evaporating cautiously in a small crucible over a naked flame and igniting the residue to redness.

Assay. Weigh accurately a ground-glass-stoppered flask containing 30 mL of *water R*, introduce 0.8 mL of the sulfuric acid, cool and weigh again. Titrate with *1 M sodium hydroxide*, using 0.1 mL of *methyl red solution R* as indicator.

1 mL of *1 M sodium hydroxide* is equivalent to 49.04 mg of H_2SO_4.

Storage: in a ground-glass-stoppered container made of glass or other inert material.

Sulfuric Acid, 5M Sulphuric acid, 5M

Dilute 28 mL of *sulfuric acid R* to 100 mL with *water R*.

Sulfuric Acid, Dilute Sulphuric acid, dilute

Contains 98 g/L of H_2SO_4.

Add 5.5 mL of *sulfuric acid R* to 60 mL of *water R*, allow to cool and dilute to 100 mL with the same solvent.

Assay. Into a ground-glass-stoppered flask containing 30 mL of *water R*, introduce 10.0 mL of the dilute sulfuric acid. Titrate with *1 M sodium hydroxide*, using 0.1 mL of *methyl red solution R* as indicator.

1 mL of *1 M sodium hydroxide* is equivalent to 49.04 mg of H_2SO_4.

Sulfuric Acid, Dilute R1

Contains 4.9 g/L of H_2SO_4.

Prepared from *sulfuric acid R*.

Sulfuric Acid, 2.5M Alcoholic Sulphuric acid, 2.5M alcoholic

Carefully and with constant cooling, stir 14 mL of *sulfuric acid R* into 60 mL of *anhydrous ethanol R*. Allow to cool and dilute to 100 mL with *anhydrous ethanol R*. Prepare immediately before use.

Sulfuric Acid, 0.25M Alcoholic Sulphuric acid, 0.25M alcoholic

Dilute 10 mL of *2.5 M alcoholic sulfuric acid R* to 100 mL with *anhydrous ethanol R*. Prepare immediately before use.

Sulfuric Acid, Alcoholic Solution of Sulphuric acid, alcoholic solution of

Carefully and with constant cooling, stir 20 mL of *sulfuric acid R* into 60 mL of *ethanol (96 per cent) R*. Allow to cool and dilute to 100 mL with *ethanol (96 per cent) R*. Prepare immediately before use.

Sulfuric Acid, Ethanolic

Sulphuric acid, ethanolic

Solutions of the requisite molarity may be obtained by mixing *sulfuric acid* with *ethanol (96%)* as directed under *sulfuric acid*.

When '*ethanolic sulfuric acid*' is followed by a percentage figure, an instruction to use *sulfuric acid* diluted with *ethanol (96%)* to produce the specified percentage v/v proportion of sulfuric acid is implied.

Sulfuric Acid–Formaldehyde Reagent Sulphuric acid–formaldehyde reagent

Mix 2 mL of *formaldehyde solution R* with 100 mL of *sulfuric acid R*.

Sulfuric Acid, Heavy Metal-free Sulphuric acid, heavy metal-free

Complies with the requirements prescribed for *sulfuric acid R* with the following maximum contents of heavy metals.

As: 0.005 ppm.

Cd: 0.002 ppm.

Cu: 0.001 ppm.

Fe: 0.05 ppm.

Hg: 0.005 ppm.

Ni: 0.002 ppm.

Pb: 0.001 ppm.

Zn: 0.005 ppm.

Sulfuric Acid, Methanolic

Sulphuric acid, methanolic

Solutions of the requisite molarity may be obtained by mixing *sulfuric acid* with *methanol* as directed under *sulfuric acid*.

When '*methanolic sulfuric acid*' is followed by a percentage figure, an instruction to use *sulfuric acid* diluted with *methanol* to produce the specified percentage v/v proportion of sulfuric acid is implied.

Sulfuric Acid, Nitrogen-free Sulphuric acid, nitrogen-free

Complies with the requirements prescribed for *sulfuric acid R* with the following additional test.

Nitrates. To 5 mL of *water R* add carefully 45 mL of the sulfuric acid, allow to cool to 40 °C and add 8 mg of *diphenylbenzidine R*. The solution is colourless or very pale blue.

Sulfuric Acid, Nitrogen-free R1

Complies with the requirements prescribed for *nitrogen-free sulfuric acid R*.

Content: 95.0 per cent *m/m* to 95.5 per cent *m/m*.

Sulfuric Acid R1 H_2SO_4 = 98.1 (*7664-93-9*)

Content: 75 per cent *V/V*.

Sunflower Oil See *Sunflower oil, refined (1371)*.

Swertiamarin Swertiamaroside; (4*R*,5*R*,6*S*)-5-Ethenyl-6-(β-D-glucopyranosyloxy)-4a-hydroxy-4,4a,5,6-tetrahydro-1*H*,3*H*-pyrano[3,4-*c*]pyran-1-one; $C_{16}H_{22}O_{10}$ = 374.3 (*17388-39-5*)

Tagatose D-*lyxo*-Hexulose; $C_6H_{12}O_6$ = 180.16 (*87-81-0*)

White or almost white powder.

$[\alpha]_D^{20}$: −2.3 determined on a 21.9 g/L solution.

mp: 134 °C to 135 °C.

Talc (*14807-96-6*)

See *Talc (0438)*.

Tannic Acid (*1401-55-4*)

Yellowish or light-brown, glistening scales or amorphous powder, very soluble in water, freely soluble in ethanol (96 per cent), soluble in acetone.

Storage: protected from light.

Tannic Acid Reagent

Dissolve 25 mg of *tannic acid* in 20 mL of *glacial acetic acid* and add 80 mL of *orthophosphoric acid*.

Prepare immediately before use.

Tanshinone IIA $C_{19}H_{18}O_3$ = 294.3 (*568-72-9*)

1,6,6-Trimethyl-6,7,8,9-tetrahydrophenanthro[1,2-*b*]furan-10,11-dione.

(+)-Tartaric Acid Tartaric acid; (*87-69-4*)

See *Tartaric acid (0460)*.

Taurodeoxycholic Acid Sodium Salt

$C_{26}H_{44}NaNO_6S$ = 521.7 (*1180-95-6*)

General reagent grade of commerce.

Taxifolin (2*R*,3*R*)-2-(3,4-Dihydroxyphenyl)-3,5,7-trihydroxy-2,3-dihydro-4*H*-1-benzopyran-4-one; $C_{15}H_{12}O_7$ = 304.3 (*480-18-2*)

White or almost white powder, slightly soluble in anhydrous ethanol.

Absorbance (2.2.25). A solution in *anhydrous ethanol R* shows an absorption maximum at 290 nm.

Tecnazene $C_6HCl_4NO_2$ = 260.9 (*117-18-0*)

bp: about 304 °C.

mp: 99 °C to 100 °C.

A suitable certified reference solution (10 ng/µL in cyclohexane) may be used.

***trans*-Terpin** (1*r*,4*r*)-4-(2-Hydroxypropan-2-yl)-1-methylcyclohexan-1-ol; *p*-Menthane-1,8-diol; $C_{10}H_{20}O_2$ = 172.3 (*565-50-4*)

mp: about 116 °C.

Terpinene 1-Isopropyl-4-methylcyclohexa-1,3-diene; α-Terpinene; $C_{10}H_{16}$ = 136.2 (*99-86-5*)

Clear, almost colourless liquid.

d_4^{20}: about 0.837.

n_D^{20}: about 1.478.

bp: about 174 °C.

α-Terpinene used in gas chromatography complies with the following additional test.

Assay. Gas chromatography (*2.2.28*) as prescribed in the monograph *Tea tree oil (1837)*.

Content: minimum 90 per cent, calculated by the normalisation procedure.

γ-Terpinene 1-Isopropyl-4-methylcyclohexa-1,4-diene; $C_{10}H_{16}$ = 136.2 (*99-85-4*)

Oily liquid.

γ-Terpinene used in gas chromatography complies with the following additional test.

Assay. Gas chromatography (*2.2.28*) as prescribed in the monograph *Peppermint oil (0405)*.

Test solution. The substance to be examined.

Content: minimum 93.0 per cent, calculated by the normalisation procedure.

Terpinen-4-ol 4-Methyl-1-(1-methylethyl)cyclohex-3-en-1-ol; *p*-Menth-1-en-4-ol; $C_{10}H_{18}O$ = 154.2 (*562-74-3*)

Oily, colourless liquid.

Terpinen-4-ol used in gas chromatography complies with the following additional test.

Assay. Gas chromatography (*2.2.28*) as prescribed in the monograph *Lavender oil (1338)*.

Test solution. The substance to be examined.

Content: minimum 90.0 per cent, calculated by the normalisation procedure.

α-Terpineol (*RS*)-2-(4-Methylcyclohex-3-enyl)-2-propanol; $C_{10}H_{18}O$ = 154.2 (*98-55-5*)

Colourless crystals, practically insoluble in water, soluble in ethanol (96 per cent).

d_{20}^{20}: about 0.935.

n_D^{20}: about 1.483.

$[\alpha]_D^{20}$: about 92.5.

mp: about 35 °C.

It may contain 1 to 3 per cent of β-terpineol.

α-Terpineol used in gas chromatography complies with the following test.

Assay. Gas chromatography (*2.2.28*) as prescribed in the monograph *Anise oil (0804)*.

Test solution. A 100 g/L solution in *hexane R*.

Content: minimum 97.0 per cent, calculated by the normalisation procedure.

Terpinolene *p*-Mentha-1,4(8)-diene; 4-Isopropylidene-1-methylcyclohexene; $C_{10}H_{16}$ = 136.2 (*586-62-9*)

Clear, almost colourless liquid.

d_4^{20}: about 0.863.

n_D^{20}: about 1.488.

bp: about 184 °C.

Terpinolene used in gas chromatography complies with the following additional test.

Assay. Gas chromatography (*2.2.28*) as prescribed in the monograph *Tea tree oil (1837)*.

Content: minimum 90 per cent, calculated by the normalisation procedure.

Testosterone (*58-22-0*)

See *Testosterone (1373)*.

Testosterone Propionate (*57-85-2*)

See *Testosterone propionate (0297)*.

1,2,3,4-Tetra-*O*-acetyl-β-D-glucopyranose $C_{14}H_{20}O_{10}$ = 348.3 (*13100-46-4*)

White or almost white powder, soluble in water with gentle heating.

$[\alpha]_D^{20}$: + 11, determined on a 6 g/L solution in *chloroform R*.

mp: 126 °C to 128 °C.

1,3,4,6-Tetra-*O*-acetyl-β-D-mannopyranose $C_{14}H_{20}O_{10}$ = 348.3 (*18968-05-3*)

Colourless or white powder or crystals.

mp: 160 °C to 161 °C.

$[\alpha]_D^{20}$: − 68, determined on a 7 g/L solution in *methylene chloride R*.

Tetrabutylammonium Bromide $C_{16}H_{36}BrN$ = 322.4 (*1643-19-2*)

White or almost white crystals.

mp: 102 °C to 104 °C.

Tetrabutylammonium Dihydrogen Orthophosphate Tetrabutylammonium dihydrogen phosphate; $C_{16}H_{38}NO_4P$ = 339.5 (*5574-97-0*)

White or almost white powder, hygroscopic.

pH (2.2.3): about 7.5 for a 170 g/L solution.

Absorbance (2.2.25): about 0.10 determined at 210 nm using a 170 g/L solution.

Storage: in an airtight container.

Tetrabutylammonium Dihydrogen Phosphate Solution

A 1.0 M solution of *tetrabutylammonium dihydrogen phosphate R*. This solution is commercially available.

Tetrabutylammonium Hydrogen Sulfate Tetrabutylammonium hydrogen sulphate; $C_{16}H_{37}NO_4S$ = 339.5 (*32503-27-8*)

Crystalline powder or colourless crystals, freely soluble in water and in methanol.

mp: 169 °C to 173 °C.

Absorbance (2.2.25): maximum 0.05, determined between 240 nm and 300 nm using a 50 g/L solution.

Tetrabutylammonium Hydrogen Sulfate R1 Tetrabutylammonium hydrogen sulphate R1

Complies with the requirements prescribed for *tetrabutylammonium hydrogen sulfate R* with the following additional requirement.

Absorbance (*2.2.25*): maximum 0.02, determined between 215 nm and 300 nm using a 50 g/L solution.

Tetrabutylammonium Hydroxide
$C_{16}H_{37}NO,30H_2O$ = 800 (*147741-30-8*)

Content: minimum 98.0 per cent of $C_{16}H_{37}NO,30H_2O$.

White or almost white crystals, soluble in water.

Assay. Dissolve 1.000 g in 100 mL of *water R*. Titrate immediately with *0.1 M hydrochloric acid* determining the end-point potentiometrically (*2.2.20*). Carry out a blank titration.

1 mL of *0.1 M hydrochloric acid* is equivalent to 80.0 mg of $C_{16}H_{37}NO,30H_2O$.

Tetrabutylammonium Hydroxide Solution

General reagent grade of commerce containing 40.0% w/v of $C_{16}H_{37}NO$.

Tetrabutylammonium Hydroxide Solution (104 g/L)

A solution containing 104 g/L of $C_{16}H_{37}NO$ (M_r 259.5), prepared by dilution of a suitable reagent grade.

Tetrabutylammonium Hydroxide Solution (400 g/L)

A solution containing 400 g/L of $C_{16}H_{37}NO$ (M_r 259.5) of a suitable grade.

Tetrabutylammonium Hydroxide, 0.4M

Use a grade of commerce suitable for chromatography.

Tetrabutylammonium Iodide $C_{16}H_{36}IN$ = 369.4 (*311-28-4*)

Content: minimum 98.0 per cent.

White or slightly coloured, crystalline powder or crystals, soluble in ethanol (96 per cent).

Sulfated ash (*2.4.14*): maximum 0.02 per cent.

Assay. Dissolve 1.200 g in 30 mL of *water R*. Add 50.0 mL of *0.1 M silver nitrate* and 5 mL of *dilute nitric acid R*. Titrate the excess of silver nitrate with *0.1 M ammonium thiocyanate*, using 2 mL of *ferric ammonium sulfate solution R2* as indicator.

1 mL of *0.1 M silver nitrate* is equivalent to 36.94 mg of $C_{16}H_{36}IN$.

Tetrabutylammonium Perchlorate
$C_{16}H_{36}ClNO_4$ = 341.91 (*1923-70-2*)

General reagent grade of commerce containing not less than 98.0% of $C_{16}H_{36}ClNO_4$.

Tetrachloroethane 1,1,2,2-Tetrachloroethane; $C_2H_2Cl_4$ = 167.9 (*79-34-5*)

Clear, colourless liquid, slightly soluble in water, miscible with ethanol (96 per cent).

d_{20}^{20}: about 1.59.

n_D^{20}: about 1.495.

Distillation range (*2.2.11*). Not less than 95 per cent distils between 145 °C and 147 °C.

Tetrachlorvinphos $C_{10}H_9Cl_4O_4P$ = 366.0 (*22248-79-9*)

mp: about 95 °C.

A suitable certified reference solution (10 ng/µL in iso-octane) may be used.

Tetracos-15-enoic Acid Methyl Ester 15-Tetracosaenoic acid methyl ester; Methyl tetracos-15-enoate; Nervonic acid methyl ester; $C_{25}H_{48}O_2$ = 380.7 (*2733-88-2*)

Content: minimum 99.0 per cent, determined by gas chromatography.

Liquid.

Tetracycline $C_{22}H_{24}N_2O_8$ = 444.4 (*60-54-8*)

mp: about 176°.

General reagent grade of commerce.

Store protected from light.

Tetracycline Hydrochloride

See *Tetracycline hydrochloride (0210)*.

***N*-Tetradecane** Tetradecane; $C_{14}H_{30}$ = 198.4 (*629-59-4*)

Content: minimum 99.5 per cent *m/m*.

A colourless liquid.

d_{20}^{20}: about 0.76.

n_D^{20}: about 1.429.

bp: about 252 °C.

mp: about −5 °C.

Tetradecylammonium Bromide Tetrakis(decyl) ammonium bromide; $C_{40}H_{84}BrN$ = 659 (*14937-42-9*)

White or slightly coloured, crystalline powder or crystals.

mp: 88 °C to 89 °C.

Tetraethylammonium Hydrogen Sulfate
Tetraethylammonium hydrogen sulphate; $C_8H_{21}NO_4S$ = 227.3 (*16873-13-5*)

Hygroscopic powder.

mp: about 245 °C.

Tetraethylammonium Hydroxide Solution
$C_8H_{21}NO$ = 147.3 (*77-98-5*)

A 200 g/L solution.

Colourless liquid, strongly alkaline.

d_{20}^{20}: about 1.01.

n_D^{20}: about 1.372.

HPLC grade.

Tetraethylene Pentamine 3,6,9-Triazaundecan-1,11-diamine; $C_8H_{23}N_5$ = 189.3 (*112-57-2*)

Colourless liquid, soluble in acetone.

n_D^{20}: about1.506.

Storage: protected from humidity and heat.

Tetraheptylammonium Bromide $C_{28}H_{60}BrN$ = 490.7 (*4368-51-8*)

White or slightly coloured, crystalline powder or crystals.

mp: 89 °C to 91 °C.

Tetrahexylammonium Bromide *N,N,N*-Trihexylhexan-1-aminium bromide; $C_{24}H_{52}BrN$ = 434.6 (*4328-13-6*)

White or almost white, crystalline powder, hygroscopic.

mp: about 100 °C.

Tetrahexylammonium Hydrogen Sulfate
Tetrahexylammonium hydrogen sulphate; $C_{24}H_{53}NO_4S$ = 451.8 (*32503-34-7*)

White or almost white crystals.

mp: 100 °C to 102 °C.

Tetrahydrofuran Tetramethylene oxide; C_4H_8O = 72.1 (*109-99-9*)

Clear, colourless, flammable liquid, miscible with water, with ethanol (96 per cent).

d_{20}^{20}: about 0.89.

Do not distil if the tetrahydrofuran does not comply with the test for peroxides.

Peroxides. Place 8 mL of *potassium iodide and starch solution R* in a 12 mL ground-glass-stoppered cylinder about 1.5 cm in diameter. Fill completely with the substance to be examined, shake vigorously and allow to stand protected from light for 30 min. No colour is produced.

Tetrahydrofuran used in spectrophotometry complies with the following additional test.

Absorbance (*2.2.25*): maximum 0.70 at 255 nm, 0.10 at 270 nm, 0.01 at 310 nm, determined using *water R* as compensation liquid.

Tetrahydrofuran for Chromatography

Complies with the requirements prescribed for *tetrahydrofuran R* with the following additional requirements:

d_4^{20} = 0.8892.

bp: about 66 °C.

Content: minimum 99.8 per cent of C_4H_8O.

Tetrahydrofuran, Stabiliser-free

Tetrahydrofuran that is free from stabilisers and inhibitors.

Reagent grade of commerce.

Tetrahydropalmatine (13a*RS*)-5,8,13,13a-Tetrahydro-2,3,9,10-tetramethoxy-6*H*-dibenzo[*a*,*g*]quinolizine; $C_{21}H_{25}NO_4$ = 355.4 (*2934-97-6*)

D-Tetrahydropalmatine Hydrochloride $C_{21}H_{25}NO_4$, HCl = 391.90 (*6024-83-5*)

General reagent grade of commerce.

α-Tetralone 1-Oxotetraline; 3,4-Dihydronaphthalen-1(2*H*)-one; $C_{10}H_{10}O$ = 146.2 (*529-34-0*)

bp: about 115 °C.

mp: about 5 °C.

Tetramethylammonium Bromide *N,N,N*-Trimethylmethanaminium bromide; $C_4H_{12}BrN$ = 154.1 (*64-20-0*)

White or slightly yellow crystals, freely soluble in water.

mp: about 285 °C, with decomposition.

Tetramethylammonium Chloride $C_4H_{12}ClN$ = 109.6 (*75-57-0*)

Colourless crystals, soluble in water and in ethanol (96 per cent).

mp: about 300 °C, with decomposition.

Tetramethylammonium Hydrogen Sulfate Tetramethylammonium hydrogen sulphate; $C_4H_{13}NO_4S$ = 171.2 (*80526-82-5*)

Hygroscopic powder.

mp: about 295 °C.

Tetramethylammonium Hydroxide Pentahydrate Tetramethylammonium hydroxide; $C_4H_{13}NO,5H_2O$ = 181.2 (*10424-65-4*)

Suitable grade for HPLC.

Tetramethylammonium Hydroxide Solution (*75-59-2*)

Content: minimum 10.0 per cent *m/m* of $C_4H_{13}NO$. (M_r 91.2).

Clear, colourless or very pale yellow liquid, miscible with water and with ethanol (96 per cent).

Assay. To 1.000 g add 50 mL of *water R* and titrate with *0.05 M sulfuric acid*, using 0.1 mL of *methyl red solution R* as indicator.

1 mL of *0.05 M sulfuric acid* is equivalent to 9.12 mg of $C_4H_{13}NO$.

Tetramethylammonium Hydroxide Solution, Dilute

Dilute 10 mL of *tetramethylammonium hydroxide solution R* to 100 mL with *aldehyde-free alcohol R*. Prepare immediately before use.

Tetramethylbenzidine 3,3′,5,5′-Tetramethylbiphenyl-4,4′-diamine; $C_{16}H_{20}N_2$ = 240.3 (*54827-17-7*)

Powder, practically insoluble in water, very soluble in methanol.

mp: about 169 °C.

1,1,3,3-Tetramethylbutylamine 2-Amino-2,4,4-trimethylpentane; $C_8H_{19}N$ = 129.3 (*107-45-9*)

Clear, colourless liquid.

d_{20}^{20}: about 0.805.

n_D^{20}: about 1.424.

bp: about 140 °C.

Tetramethylethylenediamine Tetramethylethane-1,2-diamine; $C_6H_{16}N_2$ = 116.2 (*110-18-9*)

Colourless liquid, miscible with water and with ethanol (96 per cent).

d_{20}^{20}: about 0.78.

n_D^{20}: about 1.418.

bp: about 121 °C.

***N,N,N′,N′*-Tetramethyl-p-phenylenediamine Dihydrochloride** $C_{10}H_{16}N_2,2HCl$ = 237.2 (*637-01-4*)

General reagent grade of commerce.

Whitish grey crystals.

Tetramethylsilane TMS; $C_4H_{12}Si$ = 88.2 (*75-76-3*)

Clear, colourless liquid, very slightly soluble in water, soluble in acetone and in ethanol (96 per cent).

d_{20}^{20}: about 0.64.

n_D^{20}: about 1.358.

bp: about 26 °C.

Tetramethylsilane used in nuclear magnetic resonance spectrometry complies with the following additional test.

In the nuclear magnetic resonance spectrum of an approximately 10 per cent *V/V* solution of the tetramethylsilane in *deuterated chloroform R*, the intensity of any foreign signal, excluding those due to spinning side bands and to chloroform, is not greater than the intensity of the C-13 satellite signals located at a distance of 59.1 Hz on each side of the principal signal of tetramethylsilane.

Tetrandrine $C_{38}H_{42}N_2O_6$ = 623 (*518-34-3*)

1,2,3,4-Tetraphenylcyclopenta-1,3-diene $C_{29}H_{22}$ = 370.5 (*15570-45-3*)

mp: about 178°.

General reagent grade of commerce.

1,2,3,4-Tetraphenylcyclopenta-1,3-dienone Tetraphenylcyclopentadienone; $C_{29}H_{20}O$ = 384.5 (*479-33-4*)

mp: about 218°.

General reagent grade of commerce.

Tetraphenylethylene $C_{26}H_{20}$ = 332.5 (*632-51-9*)

mp: about 223°.

General reagent grade of commerce.

Tetrapropylammonium Chloride $C_{12}H_{28}ClN$ = 221.8 (*5810-42-4*)

White or almost white, crystalline powder, sparingly soluble in water.

mp: about 241 °C.

Tetrapropylammonium Hydrogen Sulfate *N,N,N*-Tripropylpropan-1-aminium hydrogen sulfate; $C_{12}H_{29}NO_4S$ = 283.4 (*56211-70-2*)

White or almost white, crystalline, hygroscopic powder.

Tetrazolium Blue Blue tetrazolium salt; $C_{40}H_{32}Cl_2N_8O_2$ = 728 (*1871-22-3*)

Yellow crystals, slightly soluble in water, freely soluble in ethanol (96 per cent) and in methanol, practically insoluble in acetone.

mp: about 245 °C, with decomposition.

Tetrazolium Blue Solution, Alkaline

Immediately before use mix 1 volume of a 0.2% w/v solution of *tetrazolium blue* in *methanol* with 3 volumes of a 12% w/v solution of *sodium hydroxide* in *methanol*.

Tetrazolium Bromide 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; MTT; $C_{18}H_{16}BrN_5S$ = 414.3 (*298-93-1*)

Tetrazolium Salt 5-(3-Carboxymethoxyphenyl)-3-(4,5-dimethylthiazol-2-yl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt; MTS; $C_{20}H_{17}N_5O_6S_2$ = 487.5 (*138169-43-4*)

Thallium(I) Nitrate Thallous nitrate; $TlNO_3$ = 266.4 (*10102-45-1*)

General reagent grade of commerce.

Thallium(I) Sulfate Thallium(I) sulphate; Thallous sulphate; Thallous sulfate; Tl_2SO_4 = 504.8 (*7446-18-6*)

White or almost white, rhomboid prisms, slightly soluble in water, practically insoluble in ethanol (96 per cent).

Thebaine (5*R*,9*R*,13*S*)-4,5-Epoxy-3,6-dimethoxy-9a-methylmorphina-6,8-diene; $C_{19}H_{21}NO_3$ = 311.4 (*115-37-7*)

White or pale yellow, crystalline powder, very slightly soluble in water, soluble in hot anhydrous ethanol and in toluene.

mp: about 193 °C.

Chromatography (*2.2.27*). Thin-layer chromatography (*2.2.27*) as prescribed in identification test B in the monograph *Raw opium (0777)*: apply to the plate as a band (20 mm × 3 mm) 20 µL of a 0.5 g/L solution; the chromatogram shows an orange-red or red principal band with an R_F of about 0.5.

Theobromine $C_7H_8N_4O_2$ = 180.2 (*83-67-0*)

A white or almost white powder, very slightly soluble in water and in anhydrous ethanol, slightly soluble in ammonia. It dissolves in dilute solutions of alkali hydroxides and in mineral acids.

Content: minimum 99.0 per cent.

Theophylline (*58-55-9*)

See *Theophylline (0299)*.

2-(2-Thienyl)acetic Acid 2-Thiopheneacetic acid; $C_6H_6O_2S$ = 142.1 (*1918-77-0*)

Brown powder.

mp: about 65 °C.

Thioacetamide C_2H_5NS = 75.1 (*62-55-5*)

Crystalline powder or colourless crystals, freely soluble in water and in ethanol (96 per cent).

mp: about 113 °C.

Thioacetamide Reagent

To 0.2 mL of *thioacetamide solution R* add 1 mL of a mixture of 5 mL of *water R*, 15 mL of *1 M sodium hydroxide* and 20 mL of *glycerol (85 per cent) R*. Heat in a water-bath for 20 s. Prepare immediately before use.

Thioacetamide Solution

A 40 g/L solution of *thioacetamide R*.

Thiobarbituric Acid 4,6-Dihydroxy-2-sulfanylpyrimidine; $C_4H_4N_2O_2S$ = 144.2 (*504-17-6*)

Thiodiglycol 2,2′-thiodiethanol; Thiodiethylene glycol; $C_4H_{10}O_2S$ = 122.2 (*111-48-8*)

Colourless or yellow, viscous liquid.

Content: minimum 99.0 per cent.

d_{20}^{20}: about 1.18.

Thiomalic Acid (2*RS*)-2-Sulfanylbutanedioic acid; $C_4H_6O_4S$ = 150.2 (*70-49-5*)

mp: 150 °C to 152 °C.

Thiomersal Sodium mercurothiolate; Sodium 2-[(ethylmercurio)thio]benzoate; $C_9H_9HgNaO_2S$ = 404.8 (*54-64-8*)

Light, yellowish-white, crystalline powder, very soluble in water, freely soluble in ethanol (96 per cent).

Thiourea CH_4N_2S = 76.1 (*62-56-6*)

White or almost white, crystalline powder or crystals, soluble in water and in ethanol (96 per cent).

mp: about 178 °C.

L-Threonine Threonine; (*72-19-5*)

See *Threonine (1049)*.

Thrombin Thrombin, human; (*9002-04-4*)

Dried human thrombin. A preparation of the enzyme which converts human fibrinogen into fibrin. It is obtained from liquid human plasma and may be prepared by precipitation with suitable salts and organic solvents under controlled conditions of pH, ionic strength and temperature.

Yellowish-white powder, freely soluble in a 9 g/L solution of sodium chloride forming a cloudy, pale yellow solution.

Storage: in a sealed, sterile container under nitrogen, protected from light, at a temperature below 25 °C.

Thrombin, Bovine (*9002-04-4*)

A preparation of the enzyme, obtained from bovine plasma, that converts fibrinogen into fibrin.

A yellowish-white powder.

Storage: at a temperature below 0 °C.

Thrombin Solution Thrombin solution, human

Reconstitute *human thrombin R* as directed by the manufacturer and dilute to 5 IU/mL with *tris(hydroxymethyl) aminomethane sodium chloride buffer solution pH 7.4 R*.

Thrombin Solution, Human R1

Reconstitute *human thrombin R* as directed by the manufacturer and dilute to 2.5 IU/mL with *phosphate buffer solution pH 6.5 R*.

Thrombin Solution, Human R2

Reconstitute *human thrombin R* as directed by the manufacturer and dilute to 5 IU/mL with *tris(hydroxymethyl) aminomethane-EDTA buffer solution pH 8.4 R1*.

Thromboplastin

A preparation containing the membrane glycoprotein tissue factor and phospholipid, either purified from animal brain (usually rabbit) or human placenta or manufactured using recombinant DNA technology with added phospholipid. The preparation is formulated for routine use in the prothrombin time test and may contain calcium.

Thromboplastin Reagent

Thrombokinase extract

Extract 1.5 g of acetone-dried ox brain with 60 mL of water for 10 to 15 minutes at 50°, centifuge for 2 minutes at 1500 revolutions per minute and decant the supernatant liquid. This extract will retain its activity for several days

when stored in a refrigerator. It may contain 0.3% w/v of o-cresol as an antimicrobial preservative.

Thujone 4-Methyl-1-(1-methylethyl)bicyclo[3.1.0]hexan-3-one; $C_{10}H_{16}O$ = 152.2 (*76231-76-0*)

Colourless or almost colourless liquid, practically insoluble in water, soluble in ethanol (96 per cent) and in many other organic solvents.

Thymidine 1-(2-Deoxy-β-D-*erythro*-pentofuranosyl)-5-methylpyrimidine-2,4(1*H*,3*H*)-dione; $C_{10}H_{14}N_2O_5$ = 242.2

Needles, soluble in water, in hot ethanol (96 per cent) and in glacial acetic acid.

Thymine 5-Methylpyrimidine-2,4(1*H*,3*H*)-dione; $C_5H_6N_2O_2$ = 126.1 (*65-71-4*)

Short needles or plates, slightly soluble in cold water, soluble in hot water. It dissolves in dilute solution of alkali hydroxides.

Thyminose 2-Deoxy-D-erythro-pentose; 2-Deoxy-D-ribose; $C_5H_{10}O_4$ = 134.1 (*533-67-5*)

Thymol 2-Isopropyl-5-methylphenol; (*89-83-8*)

Thymol used in gas chromatography complies with the following additional test.

Assay. Gas chromatography (*2.2.28*) as prescribed in the monograph *Peppermint oil (0405)*.

Test solution. Dissolve 0.1 g in about 10 mL of *acetone R*.

Content: minimum 95.0 per cent, calculated by the normalisation procedure.

Thymol Blue Thymolsulfonphthalein; 4,4′-(3*H*-2,1-Benzoxathiol-3-ylidene)bis(2-isopropyl-5-methylphenol) *S*,*S*-dioxide; $C_{27}H_{30}O_5S$ = 466.6 (*76-61-9*)

Brownish-green or greenish-blue, crystalline powder, slightly soluble in water, soluble in ethanol (96 per cent) and in dilute solutions of alkali hydroxides.

Thymol Blue Solution

Dissolve 0.1 g of *thymol blue R* in a mixture of 2.15 mL of *0.1 M sodium hydroxide* and 20 mL of *ethanol (96 per cent) R* and dilute to 100 mL with *water R*.

Test for sensitivity. To 0.1 mL of the thymol blue solution add 100 mL of *carbon dioxide-free water R* and 0.2 mL of *0.02 M sodium hydroxide*. The solution is blue. Not more than 0.15 mL of *0.02 M hydrochloric acid* is required to change the colour to yellow.

Colour change: pH 1.2 (red) to pH 2.8 (yellow); pH 8.0 (olive-green) to pH 9.6 (blue).

Thymolphthalein 3,3-Bis(4-hydroxy-5-isopropyl-2-methylphenyl)phthalide; $C_{28}H_{30}O_4$ = 430.5 (*125-20-2*)

White or yellowish-white powder, practically insoluble in water, soluble in ethanol (96 per cent) and in dilute solutions of alkali hydroxides.

Thymolphthalein Solution

A 1 g/L solution of *thymolphthalein R* in *ethanol (96 per cent) R*.

Test for sensitivity. To 0.2 mL of the thymolphthalein solution add 100 mL of *carbon dioxide-free water R*. The solution is colourless. Not more than 0.05 mL of *0.1 M sodium hydroxide* is required to change the colour to blue.

Colour change: pH 9.3 (colourless) to pH 10.5 (blue).

Tin Granulated tin; Sn = 118.7 (*7440-31-5*)

Silvery-white granules, soluble in hydrochloric acid with release of hydrogen.

Arsenic (*2.4.2, Method A*): maximum 10 ppm, determined on 0.1 g.

Tin(II) Chloride Tin dichloride dehydrate; Stannous chloride; $SnCl_2,2H_2O$ = 225.6 (*10025-69-1*)

Content: minimum 97.0 per cent of $SnCl_2,2H_2O$.

Colourless crystals, very soluble in water, freely soluble in ethanol (96 per cent), in glacial acetic acid and in dilute and concentrated hydrochloric acid.

Assay. Dissolve 0.500 g in 15 mL of *hydrochloric acid R* in a ground-glass-stoppered flask. Add 10 mL of *water R* and 5 mL of *chloroform R*. Titrate rapidly with *0.05 M potassium iodate* until the chloroform layer is colourless.

1 mL of *0.05 M potassium iodate* is equivalent to 22.56 mg of $SnCl_2,2H_2O$.

Tin(II) Chloride Solution Stannous chloride solution

Heat 20 g of *tin R* with 85 mL of *hydrochloric acid R* until no more hydrogen is released. Allow to cool.

Storage: over an excess of *tin R*, protected from air.

Tin(II) Chloride Solution AsT

Stannous chloride solution, low in arsenic, of commerce, or prepare from *tin(II) chloride solution* by adding an equal volume of *hydrochloric acid*, reducing the original volume by boiling and filtering through a fine-grain filter paper.

Complies with the following test.

To 10 mL add 6 mL of *water* and 10 mL of *hydrochloric acid*, distil and collect 16 mL. To the distillate add 50 mL of *water*, 0.1 mL of the solution, 5 mL of 0.1M *potassium iodide* and 5 g of *activated zinc*. Using the apparatus and procedure described for the *limit test for arsenic*, Appendix VII, the colour obtained in the test-tube with the test solution is not more intense than that produced when the test is repeated with the addition of 1 mL of *arsenic standard solution (1 ppm As)*.

Tin(II) Chloride Solution R1 Stannous chloride solution R1

Immediately before use, dilute 1 volume of *stannous chloride solution R* with 10 volumes of *dilute hydrochloric acid R*.

Tin(II) Chloride Solution R2 Stannous chloride solution R2

To 8 g of *stannous chloride R* add 100 mL of a 20 per cent *V/V* solution of *hydrochloric acid R*. Shake until dissolved, heating, if necessary, on a water-bath at 50 °C. Pass a current of *nitrogen R* for 15 min. Prepare immediately before use.

Tin Test Kit, Semi-quantitative

Commercially available set of reagents consisting of tin test strips and a reagent mixture for the determination of tin in aqueous solutions, in a range of 10-200 µg/mL.

Tiron 4,5-Dihydroxy-1,3-benzenedisulfonic acid disodium salt monohydrate; $C_6H_4Na_2O_8S_2,H_2O$ = 332 (*270573-71-2*)

General reagent grade of commerce.

Tiron Indicator Solution

2% w/v solution of *tiron* in *water*.

Titan Yellow Thiazol yellow; Disodium 2,2′-[(1-triazene-1,3-diyl)di-4,1-phenylene]bis-[6-methylbenzothiazole-7-sulfonate]; $C_{28}H_{19}N_5Na_2O_6S_4$ = 696 (*1829-00-1*)

Schultz No. 280

Colour Index No. 19540

A yellowish-brown powder, freely soluble in water and in ethanol (96 per cent).

Titan Yellow Paper

Immerse strips of filter paper in *titan yellow solution R* and leave for a few minutes. Allow to dry at room temperature.

Titan Yellow Solution

A 0.5 g/L solution of *titan yellow R*.

Test for sensitivity. To 0.1 mL of the titan yellow solution add 10 mL of *water R*, 0.2 mL of *magnesium standard solution (10 ppm Mg) R* and 1.0 mL of *1 M sodium hydroxide*. A distinct pink colour is visible by comparison with a reference solution prepared in a similar manner omitting the magnesium.

Titanium Ti = 47.88 (*7440-32-6*)

Content: minimum 99 per cent.

Metal powder, fine wire (diameter not more than 0.5 mm), sponge.

mp: about 1668 °C.

Density: about 4.507 g/cm^3.

Titanium(III) Chloride Titanium trichloride; $TiCl_3$ = 154.3 (*7705-07-9*)

Reddish-violet crystals, deliquescent, soluble in water and in ethanol (96 per cent).

mp: about 440 °C.

Storage: in an airtight container.

Titanium(III) Chloride Solution Titanium trichloride solution

d_{20}^{20}: about 1.19.

A 150 g/L solution of *titanium trichloride R* in hydrochloric acid (100 g/L HCl).

Titanium(III) Chloride–Sulfuric Acid Reagent Titanium(III) chloride-sulphuric acid reagent; Titanium trichloride–sulphuric acid reagent; Titanium trichloride-sulfuric acid reagent

Carefully mix 20 mL of *titanium trichloride solution R* with 13 mL of *sulfuric acid R*. Add sufficient *strong hydrogen peroxide solution R* to give a yellow colour. Heat until white fumes are evolved. Allow to cool. Dilute with *water R* and repeat the evaporation and addition of *water R* until a colourless solution is obtained. Dilute to 100 mL with *water R*.

Titanium Dioxide Titanium(IV) oxide; (*13463-67-7*)

See *Titanium dioxide (0150)*.

TLC Aluminium Oxide G Plate

Support of metal, glass or plastic, coated with a layer of aluminium oxide (particle size 5-40 µm) containing about 10 per cent of calcium sulfate hemihydrate as a binder.

TLC Cellulose Plate

Support of glass, metal or plastic, coated with a layer of cellulose.

TLC Octadecylsilyl Silica Gel Plate

Support of glass, metal or plastic coated with a layer of octadecylsilyl silica gel. The plate may contain an organic binder.

TLC Octadecylsilyl Silica Gel F_{254} Plate

Support of glass, metal or plastic coated with a layer of octadecylsilyl silica gel.

It contains a fluorescent indicator having a maximum absorbance in ultraviolet light at 254 nm.

TLC Performance Test Solution

Prepare a mixture of 1.0 mL of each of the following solutions and dilute to 10.0 mL with *acetone R*: a 0.5 g/L solution of *Sudan red G R* in *toluene R*, a 0.5 g/L solution of *methyl orange R* in *ethanol R* prepared immediately before use, a 0.5 g/L solution of *bromocresol green R* in *acetone R* and a 0.25 g/L solution of *methyl red R* in *acetone R*.

TLC Silica Gel Plate Silica gel

Support of glass, metal or plastic, coated with a layer of silica gel of a suitable thickness and particle size (usually 2 µm to 10 µm for fine particle size [High Performance Thin-Layer Chromatography, HPTLC] plates and 5 µm to 40 µm for normal TLC plates). If necessary, the particle size is indicated after the name of the reagent in the tests where it is used.

The plate may contain an organic binder.

Chromatographic separation. Apply to the plate an appropriate volume (10 µL for a normal TLC plate and 1 µL to 2 µL for a fine particle size plate) of *TLC performance test solution R*. Develop over a pathlength two-thirds of the plate height, using a mixture of 20 volumes of *methanol R* and 80 volumes of *toluene R*. The plate is not satisfactory, unless the chromatogram shows four clearly separated spots, the spot of bromocresol green with an R_F value less than 0.15, the spot of methyl orange with an R_F value in the range of 0.1 to 0.25, the spot of methyl red with an R_F value in the range of 0.35 to 0.55 and the spot of Sudan red G with an R_F value in the range of 0.75 to 0.98.

TLC Silica Gel F_{254} Plate

Complies with the requirements prescribed for *TLC silica gel plate R* with the following modification.

It contains a fluorescent indicator having a maximum absorbance at 254 nm.

Fluorescence suppression. Apply separately to the plate at five points increasing volumes (1 µL to 10 µL for normal TLC plates and 0.2 µL to 2 µL for fine particle size plates) of a 1 g/L solution of *benzoic acid R* in a mixture of 15 volumes of *anhydrous ethanol R* and 85 volumes of *cyclohexane R*. Develop over a pathlength half of the plate height with the same mixture of solvents. After evaporating the solvents examine the chromatogram in ultraviolet light at 254 nm. For normal TLC plates the benzoic acid appears as dark spots on a fluorescent background approximately in the middle of the chromatogram for quantities of 2 µg and greater. For fine particle size plates the benzoic acid appears as dark spots on a fluorescent background approximately in the middle of the chromatogram for quantities of 0.2 µg and greater.

TLC Silica Gel F_{254} Silanised Plate

It complies with the requirements prescribed for *TLC silanised silica gel plate R* with the following modification.

It contains a fluorescent indicator having a maximum absorbance at 254 nm.

TLC Silica Gel G Plate

Complies with the requirements prescribed for *TLC silica gel plate R* with the following modification.

It contains calcium sulfate hemihydrate as binder.

TLC Silica Gel GF_{254} Plate

Complies with the requirements prescribed for *TLC silica gel plate R* with the following modifications.

It contains calcium sulfate hemihydrate as binder and a fluorescent indicator having a maximum absorbance at 254 nm.

Fluorescence suppression. Complies with the test prescribed for *TLC silica gel F_{254} plate R*.

TLC Silica Gel Plate for Aminopolyether Test

Immerse a *TLC silica gel plate R* in *iodoplatinate reagent R1* for 5-10 s. Dry at room temperature for 12 h, protected from light.

Storage: protected from light, in an open container; use within 30 days after preparation.

TLC Silica Gel Plate for Chiral Separations, Octadecylsilyl

Support of glass, metal or plastic, coated with a layer of octadecylsilyl silica gel, impregnated with Cu^{2+} ions and enantiomerically pure hydroxyproline. The plate may contain an organic binder.

TLC Silica Gel Silanised Plate

Support of glass, metal or plastic, coated with a layer of silanised silica gel of a suitable thickness and particle size (usually 2 µm to 10 µm for fine particle size [High Performance Thin-Layer Chromatography, HPTLC] plates and 5 µm to 40 µm for normal TLC plates). If necessary, the particle size is indicated after the name of the reagent in the tests where it is used.

The plate may contain an organic binder.

Chromatographic separation. Introduce 0.1 g each of *methyl laurate R, methyl myristate R, methyl palmitate R* and *methyl stearate R* into a 250 mL conical flask. Add 40 mL of *alcoholic potassium hydroxide solution R* and heat under a reflux condenser on a water-bath for 1 h. Allow to cool, transfer the solution to a separating funnel by means of 100 mL of *water R*, acidify (pH 2 to 3) with *dilute hydrochloric acid R* and shake with three quantitites each of 10 mL of *methylene chloride R*. Dry the combined methylene chloride extracts over *anhydrous sodium sulfate R*, filter and evaporate to dryness on a water-bath. Dissolve the residue in 50 mL of *methylene chloride R*. Examine by thin-layer chromatography (*2.2.27*), using *TLC silanised silica gel plate R*. Apply an appropriate quantity (about 10 µL for normal TLC plates and about 1 µL to 2 µL for fine particle size plates) of the methylene chloride solution at each of three separate points. Develop over a pathlength two-thirds of the plate height with a mixture of 10 volumes of *glacial acetic acid R*, 25 volumes of *water R* and 65 volumes of *dioxan R*. Dry the plate at 120 °C for 30 min. Allow to cool, spray with a 35 g/L solution of *phosphomolybdic acid R* in *2-propanol R* and heat at 150 °C until the spots become visible. Treat the plate with ammonia vapour until the background is white.
The chromatograms show four clearly separated, well-defined spots.

α-Tocopherol (*10191-41-0*)

See *all-rac-α-Tocopherol (0692)*.

α-Tocopheryl Acetate (*7695-91-2*)

See *all-rac-α-Tocopheryl acetate (0439)*.

***o*-Tolidine** 3,3′-Dimethylbenzidine; $C_{14}H_{16}N_2$ = 212.3 (*119-93-7*)

Content: minimum 97.0 per cent.

Light brownish, crystalline power.

mp: about 130 °C.

***o*-Tolidine Solution**

Dissolve 0.16 g of *o-tolidine R* in 30.0 mL of *glacial acetic acid R*, add 1.0 g of *potassium iodide R* and dilute to 500.0 mL with *water R*.

Toluene Methylbenzene; C_7H_8 = 92.1 (*108-88-3*)

Clear, colourless, flammable liquid, very slightly soluble in water, miscible with ethanol (96 per cent).

d_{20}^{20}: 0.865 to 0.870.

bp: about 110 °C.

Toluene, Sulfur-free Toluene, sulphur-free

Complies with the requirements prescribed for *toluene R* with the following additional requirements.

Sulfur compounds. To 10 mL add 1 mL of *anhydrous ethanol R* and 3 mL of *potassium plumbite solution R* and boil under a reflux condenser for 15 min. Allow to stand for 5 min. No darkening is produced in the aqueous layer.

Thiophen-related substances. Shake 2 mL with 5 mL of *isatin reagent R* for 5 min and allow to stand for 15 min. No blue colour is produced in the lower layer.

***p*-Toluenesulfonamide** (*70-55-3*)

See *toluenesulfonamide R*.

Toluene-*o*-sulfonamide Toluene-*o*-sulphonamide; *o*-Toluenesulphonamide; 2-Methylbenzenesulfonamide; 2-Methylbenzenesulphonamide; *o*-Toluenesulfonamide; $C_7H_9NO_2S$ = 171.2 (*88-19-7*)

White or almost white, crystalline powder, slightly soluble in water, soluble in ethanol (96 per cent) and in solutions of alkali hydroxides.

mp: about 156 °C.

Toluene-*p*-sulfonamide *p*-Toluenesulfonamide; Toluene sulfonamide; 4-Methylbenzenesulfonamide; Toluenesulfonamide; $C_7H_9NO_2S$ = 171.2 (*70-55-3*)

Content: minimum 99.0 per cent.

White or almost white, crystalline powder, slightly soluble in water, soluble in ethanol (96 per cent) and in solutions of alkali hydroxides.

mp: about 136 °C.

Toluene-*p*-sulfonic Acid Toluene-*p*-sulphonic acid; Toluenesulphonic acid; 4-Methylbenzenesulfonic acid; 4-Methylbenzenesulphonic acid; Toluenesulfonic acid; $C_7H_8O_3S,H_2O$ = 190.2 (*6192-52-5*)

Content: minimum 87.0 per cent of $C_7H_8O_3S$.

White or almost white, crystalline powder or crystals, freely soluble in water, soluble in ethanol (96 per cent).

Toluenesulfonylurea 4-Methylbenzenesulfonylurea; *p*-Toluenesulfonylurea; (4-Methylphenyl)sulfonylurea; $C_8H_{10}N_2O_3S$ = 214.2 (*1694-06-0*)

White or almost white, crystalline powder.

mp: 196 to 198 °C.

***o*-Toluic Acid** $C_8H_8O_2$ = 136.2 (*118-90-1*)

mp: about 104°.

General reagent grade of commerce.

A white, crystalline powder.

***o*-Toluidine** 2-Methylaniline; C_7H_9N = 107.2 (*95-53-4*)

Pale-yellow liquid becoming reddish-brown on exposure to air and light, slightly soluble in water, soluble in ethanol (96 per cent) and in dilute acids.

d_{20}^{20}: about 1.01.

n_D^{20}: about 1.569.

bp: about 200 °C.

Storage: in an airtight container, protected from light.

***p*-Toluidine** 4-Methylaniline; C_7H_9N = 107.2 (*106-49-0*)

Lustrous plates or flakes, slightly soluble in water, freely soluble in acetone and in ethanol (96 per cent).

mp: about 44 °C.

Toluidine Blue Toluidine Blue O; 3-Amino-7-dimethylamino-2-methylphenothiazin-5-ium chloride; $C_{15}H_{16}ClN_3S$ = 305.8 (*92-31-9*)

Schultz No. 1041

Colour Index No. 52040

Dark-green powder, soluble in water, slightly soluble in ethanol (96 per cent).

o-Toluidine Hydrochloride 2-Methylaniline hydrochloride; 2-Methylbenzenamine hydrochloride; $C_7H_{10}ClN$ = 143.6 (*636-21-5*)

Content: minimum 98.0 per cent.

mp: 215 °C to 217 °C.

Tosylarginine Methyl Ester Hydrochloride *N*-Tosyl-L-arginine methyl ester hydrochloride; Methyl (*S*)-5-guanidino-2-(4-methylbenzenesulfonamido)valerate hydrochloride; $C_{14}H_{23}ClN_4O_4S$ = 378.9 (*1784-03-8*)

$[\alpha]_D^{20}$: −12 to −16, determined on a 40 g/L solution.

mp: about 145 °C.

Tosylarginine Methyl Ester Hydrochloride Solution

To 98.5 mg of *tosylarginine methyl ester hydrochloride R* add 5 mL of *tris(hydroxymethyl)aminomethane buffer solution pH 8.1 R* and shake to dissolve. Add 2.5 mL of *methyl red mixed solution R* and dilute to 25.0 mL with *water R*.

Tosyl-lysyl-chloromethane Hydrochloride (3S)-7-Amino-1-chloro-3-tosylamido-2-heptanone hydrochloride; $C_{14}H_{22}Cl_2N_2O_3S$ = 369.3 (*4238-41-9*)

$[\alpha]_D^{20}$: −7 to −9, determined on a 20 g/L solution.

mp: about 155 °C, with decomposition.

$A_{1\,cm}^{1\%}$: 310 to 340, determined at 230 nm in *water R*.

Tosylphenylalanylchloromethane L-tosylaminophenethyl chloromethyl ketone; $C_{17}H_{18}ClNO_3S$ = 351.9 (*402-71-1*)

$[\alpha]_D^{20}$: −85 to −89, determined on a 10 g/L solution in *ethanol (96 per cent) R*.

mp: about 105 °C.

$A_{1\,cm}^{1\%}$: 290 to 320, determined at 228.5 nm in *ethanol (96 per cent) R*.

Toxaphene (*8001-35-2*)

A mixture of polychloro derivatives.

mp: 65 °C to 90 °C.

A suitable certified reference solution (10 ng/µL in iso-octane) may be used.

Tragacanth (*9000-65-1*)

See *Tragacanth (0532)*.

Triacetin Propane-1,2,3-triyl triacetate; Glycerol triacetate; $C_9H_{14}O_6$ = 218.2 (*102-76-1*)

Almost clear, colourless to yellowish liquid, soluble in water, miscible with ethanol (96 per cent).

d_{20}^{20}: about 1.16.

n_D^{20}: about 1.43.

bp: about 260 °C.

Triamcinolone 9-Fluoro-11β,16α,17,21-tetrahydroxypregna-1,4-diene-3,20-dione; $C_{21}H_{27}FO_6$ = 394.4 (*124-94-7*)

A crystalline powder.

mp: 262 °C to 263 °C.

Triamcinolone Acetonide (*76-25-5*)

See *Triamcinolone acetonide (0533)*.

Tribromophenol 2,4,6-Tribromophenol; $C_6H_3Br_3O$ = 330.8 (*118-79-6*)

Tributyl Citrate Tributyl 2-hydroxypropane-1,2,3-tricarboxylate; $C_{18}H_{32}O_7$ = 360.4 (*77-94-1*)

d_4^{20}: about 1.043.

n_D^{20}: about 1.445.

Tributyl Orthophosphate Tributoxyphosphine oxide; Tributoxyphosphane oxide; Tributyl phosphate; $C_{12}H_{27}O_4P$ = 266.3 (*126-73-8*)

Colourless liquid, slightly soluble in water, soluble in the usual organic solvents.

d_{25}^{25}: about 0.976.

n_D^{25}: about 1.422.

bp: about 289 °C, with decomposition.

Tributylphosphine $C_{12}H_{27}P$ = 202.3 (*998-40-3*)

Clear, colourless liquid.

bp: about 240 °C.

mp: about −60 °C.

Trichlorethylene Trichloroethylene; C_2HCl_3 = 131.4 (*79-01-6*)

Colourless liquid, practically insoluble in water, miscible with ethanol (96 per cent).

d_{20}^{20}: about 1.46.

n_D^{20}: about 1.477.

Trichloroacetic Acid $C_2HCl_3O_2$ = 163.4 (*76-03-9*)

Colourless crystals or a crystalline mass, very deliquescent, very soluble in water and in ethanol (96 per cent).

Storage: in an airtight container.

Trichloroacetic Acid Solution

Dissolve 40.0 g of *trichloroacetic acid R* in *water R* and dilute to 1000.0 mL with the same solvent. Verify the concentration by titration with *0.1 M sodium hydroxide* and adjust if necessary to 40 ± 1 g/L.

1,1,1-Trichloroethane Methylchloroform; $C_2H_3Cl_3$ = 133.4 (*71-55-6*)

Non-flammable liquid, practically insoluble in water, soluble in acetone and in methanol.

d_{20}^{20}: about 1.34.

n_D^{20}: about 1.438.

bp: about 74 °C.

Trichlorotrifluoroethane 1,1,2-Trichloro-1,2,2-trifluoroethane; $C_2Cl_3F_3$ = 187.4 (*76-13-1*)

Colourless, volatile liquid, practically insoluble in water, miscible with acetone.

d_{20}^{20}: about 1.58.

Distillation range (*2.2.11*). Not less than 98 per cent distils between 47 °C and 48 °C.

Tricine *N*-[2-Hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; $C_6H_{13}NO_5$ = 179.2 (*5704-04-1*)

Use electrophoresis-grade reagent.

mp: about 183 °C.

Tricosane $C_{23}H_{48}$ = 324.6 (*638-67-5*)

White or almost white crystals, practically insoluble in water, soluble in hexane.

mp: about 48 °C.

Tridecyl Alcohol Tridecanol; $C_{13}H_{28}O$ = 200.4 (*112-70-9*)

Tridocosahexaenoin Triglyceride of docosahexaenoic acid (C22:6); Glycerol tridocosahexaenoate; Propane-1,2,3-triyl tri-(*all-Z*)-docosa-4,7,10,13,16,19-hexaenoate; $C_{69}H_{98}O_6$ = 1023.5 (*124596-98-1*)

The reagent from Nu-Chek Prep, Inc. has been found suitable.

Triethanolamine (*102-71-6*)

See *Trolamine (1577)*.

Triethyl Phosphonoformate Ethyl (diethoxyphosphoryl) formate; $C_7H_{15}O_5P$ = 210.2 (*1474-78-8*)

Colourless liquid.

$bp_{12\ mm}$: about 135 °C.

Triethylamine *N,N*-Diethylethanamine; $C_6H_{15}N$ = 101.2 (*121-44-8*)

Colourless liquid, slightly soluble in water at a temperature below 18.7 °C, miscible with ethanol (96 per cent).

d_{20}^{20}: about 0.727.

n_D^{20}: about 1.401.

bp: about 90 °C.

Triethylamine R1 *N,N*-Diethylethanamine; $C_6H_{15}N$ = 101.2 (*121-44-8*)

Complies with the requirements prescribed for *triethylamine R* with the following additional requirements.

Content: minimum 99.5 per cent, determined by gas chromatography.

Water: maximum 0.1 per cent.

Use freshly distilled or from a freshly opened container.

Triethylamine R2 *N,N*-Diethylethanamine; $C_6H_{15}N$ = 101.2 (*121-44-8*)

Complies with the requirements prescribed for *triethylamine R* and with the following additional requirements.

Content: minimum 99.5 per cent, determined by gas chromatography.

Water: maximum 0.2 per cent.

It is suitable for gradient elution in liquid chromatography.

Use freshly distilled or from a freshly opened container.

Triethylamine Hydrogen Carbonate Solution

Pass a gentle current of *carbon dioxide* through a 5% w/v solution of *triethylamine* for 16 hours.

Triethylenediamine 1,4-Diazabicyclo[2.2.2]octane; $C_6H_{12}N_2$ = 112.2

Crystals, very hygroscopic, sublimes readily at room temperature, freely soluble in water, in acetone and in anhydrous ethanol.

bp: about 174 °C.

mp: about 158 °C.

Storage: in an airtight container.

Triflumuron 1-(2-Chlorobenzoyl)-3-(4-triflumoromethoxyphenyl)urea; $C_{15}H_{10}ClF_3N_2O_3$ = 358.7 (*64628-44-0*)

White or almost white crystalline powder, practically insoluble in water, sparingly soluble in acetone and in methylene chloride.

Trifluoroacetic Acid $C_2HF_3O_2$ = 114.0 (*76-05-1*)

Content: minimum 99 per cent.

Liquid, miscible with acetone and with ethanol (96 per cent).

d_{20}^{20}: about 1.53.

bp: about 72 °C.

Use a grade suitable for protein sequencing.

Storage: in an airtight container.

Trifluoroacetic Anhydride $C_4F_6O_3$ = 210.0 (*407-25-0*)

Colourless liquid.

d_{20}^{20}: about 1.5.

3-Trifluoromethylaniline 3-(Trifluoromethyl)aniline; α,α,α-Trifluoro-*m*-toluidine; 3-(Trifluoromethyl)benzenamide; $C_7H_6F_3N$ = 161.1 (*98-16-8*)

Colourless liquid.

Density: 1.30 g/cm^3 (20 °C).

4-Trifluoromethylphenol $C_7H_5F_3O$ = 162.1 (*402-45-9*)

White or light yellow, crystalline solid or powder.

mp: about 46 °C.

Trifluoropropylmethylpolysiloxane

Polysiloxane substituted with trifluoropropyl groups and methyl groups.

Triglycine 2-[[2-[(2-Aminoacetyl)amino]acetyl]amino] acetic acid; Glycylglycylglycine; $C_6H_{11}N_3O_4$ = 189.2 (*556-33-2*)

Trigonelline Hydrochloride 1-Methylpyridinium-3-carboxylate hydrochloride; $C_7H_8ClNO_2$ = 173.6 (*6138-41-6*)

Crystalline powder, very soluble in water, soluble in ethanol (96 per cent).

mp: about 258 °C.

1,2,4-Trimethylbenzene Pseudocumene; C_9H_{12} = 120.2 (*95-63-6*)

Trimethylchlorosilane Chlorotrimethylsilane; C_3H_9ClSi = 108.6 (*75-77-4*)

Clear, colourless liquid, fuming in air.

d_{20}^{20}: about 0.86.

n_D^{20}: about 1.388.

bp: about 57 °C.

2,2,4-Trimethylpentane Iso-octane; Trimethylpentane; C_8H_{18} = 114.2 (*540-84-1*)

Colourless, flammable liquid, practically insoluble in water, soluble in anhydrous ethanol.

d_{20}^{20}: 0.691 to 0.696.

n_D^{20}: 1.391 to 1.393.

Distillation range (*2.2.11*). Not less than 95 per cent distils between 98 °C and 100 °C.

Trimethylpentane used in spectrophotometry complies with the following additional test.

Absorbance (*2.2.25*): maximum 0.01 from 250 nm to 420 nm, determined using *water R* as compensation liquid.

Trimethylpentane for Chromatography

Complies with the requirements prescribed for *trimethylpentane R* with the following additional requirement.

Residue on evaporation: maximum 2 mg/L.

Trimethylpentane R1

Complies with the requirements prescribed for *trimethylpentane R* with the following modification.

Absorbance (2.2.25). Not more than 0.07 from 220 nm to 360 nm, determined using *water R* as the compensation liquid.

***N*-Trimethylsilylimidazole** 1-Trimethylsilylimidazole; $C_6H_{12}N_2Si$ = 140.3 (*18156-74-6*)

Colourless, hygroscopic liquid.

d_{20}^{20}: about 0.96.

n_D^{20}: about 1.48.

Storage: in an airtight container.

3-Trimethylsilyl-1-propanesulfonic Acid, Sodium Salt Sodium 3-(trimethylsilyl)-1-propanesulfonate; sodium 3-(trimethylsilyl)-1-propanesulphonate; 3-trimethylsilyl-1-propanesulphonic acid, sodium salt; $C_6H_{15}NaO_3SSi$ = 218.3 (*2039-96-5*)

mp: about 165°.

Beige powder; content, minimum 97.0 %.

Trimethylsulfonium Hydroxide Trimethylsulfonium hydroxide; $C_3H_{10}OS$ = 94.2 (*17287-03-5*)

d_4^{20}: about 0.81.

Trimethyltin Chloride Chlorotrimethylstannane; C_3H_9ClSn = 199.3 (*1066-45-1*)

2,4,6-Trinitrobenzenesulfonic Acid Picrylsulfonic acid; Picrylsulphonic acid; 2,4,6-Trinitrobenzenesulphonic acid; $C_6H_3N_3O_9S,3H_2O$ = 347.2 (*2508-19-2*)

White or almost white, crystalline powder, soluble in water.

mp: 190 °C to 195 °C.

Triolein Propane-1,2,3-triyl tris[(9*Z*)-octadec-9-enoate]; *sn*-Glyceryl trioleate; Glycerol trioleate; Oleyl triglyceride; $C_{57}H_{104}O_6$ = 885.4 (*122-32-7*)

Content: minimum 99.0 per cent.

Triphenylamine $C_{18}H_{15}N$ = 245.3 (*603-34-9*)

mp: about 126°.

General reagent grade of commerce.

A white, crystalline solid.

Triphenylethylene $C_{20}H_{16}$ = 256.4 (*58-72-0*)

mp: about 70°.

General reagent grade of commerce.

Triphenylmethanol Triphenylcarbinol; $C_{19}H_{16}O$ = 260.3 (*76-84-6*)

Colourless crystals, practically insoluble in water, freely soluble in ethanol (96 per cent).

2,3,5-Triphenyltetrazolium Chloride Tetrazolium Salt; Triphenyltetrazolium Chloride; $C_{19}H_{15}ClN_4$ = 334.8 (*298-96-4*)

Pale or dull-yellow powder, soluble in water, in acetone and in ethanol (96 per cent).

mp: about 240 °C, with decomposition.

Storage: protected from light.

Analytical reagent grade of commerce containing not less than 98.0 per cent of $C_{19}H_{15}ClN_4$

Assay Dissolve 1 g in a mixture of 5 mL of *dilute nitric acid* and 45 mL of *water*, add 50 mL of *0.1M silver nitrate VS* and heat to boiling. Allow to cool, add 3 mL of *dibutyl phthalate*, shake vigorously and titrate with *0.1M ammonium thiocyanate VS* using 2 mL of *ammonium iron(III) sulfate solution R2* as indicator. Each mL of *0.1M silver nitrate VS* is equivalent to 33.48 mg of $C_{19}H_{15}ClN_4$.

Triphenyltetrazolium Chloride Solution

A 0.5% w/v solution of *2,3,5-triphenyltetrazolium chloride* in *aldehyde-free ethanol (96%)*.

Store protected from light.

Tripotassium Phosphate Trihydrate $K_3PO_4,3H_2O$ = 266.3 (*22763-03-7*)

White or almost white crystalline powder, freely soluble in water.

Triscyanoethoxypropane 1,2,3-Tris(2-cyanoethoxy) propane; $C_{12}H_{17}N_3O_3$ = 251.3

Viscous, brown-yellow liquid, soluble in methanol. Used as a stationary phase in gas chromatography.

d_{20}^{20}: about 1.11.

Viscosity (*2.2.9*): about 172 mPa·s.

1,3,5-Tris(3,5-di-(1,1-dimethylethyl)-4-hydroxybenzyl)-1H,3H,5H-1,3,5-triazine-2,4,6-trione Tris(3,5-di-*tert*-butyl-4-hydroxybenzyl isocyanurate; 1,3,5-Tris[3,5-di(1,1-dimethylethyl)-4-hydroxybenzyl]-1,3,5-triazine-2,4,6(1H,3H,5H)-trione; $C_{48}H_{69}O_6N_3$ = 784.1 (*27676-62-6*)

White or almost white, crystalline powder.

mp: 218 °C to 222 °C.

Tris(2,4-di-(1,1-dimethylethyl)phenyl) Phosphite $C_{42}H_{63}O_3P$ = 647 (*31570-04-4*)

White or almost white powder.

mp: 182 °C to 186 °C.

Tris(hydroxymethyl)nitromethane $C_4H_9NO_5$ = 151 (*126-11-4*)

General reagent of commerce.

Tris(hydroxymethyl)aminomethane Hydrochloride 2-Amino-2-(hydroxymethyl)-1,3-propanediol hydrochloride; $NH_2C(CH_2OH)_3,HCl$ = 157.60 (*1185-53-1*)

Analytical reagent grade of commerce.

Tris(hydroxymethyl)aminomethane MB $C_4H_{11}NO_3$ = 121.14 (*77-86-1*)

Molecular biology grade.

Tris(hydroxymethyl)aminomethane Solution R1

Dissolve 60.6 mg of *tris(hydroxymethyl)aminomethane R* and 0.234 g of *sodium chloride R* in *water R* and dilute to 100 mL with the same solvent.

Storage: at 2 °C to 8 °C; use within 3 days.

Tris(hydroxymethyl)methylamine Tris(hydroxymethyl) aminomethane; (*77-86-1*)

See *Trometamol (1053)*.

Tris(hydroxymethyl)methylamine Solution Tris(hydroxymethyl)aminomethane solution

A solution containing the equivalent of 24.22 g of $C_4H_{11}NO_3$ in 1000.0 mL.

Tris(hydroxymethyl)methylamine Solution, Methanolic

Dissolve 6.07 g of *tris(hydroxymethyl)methylamine* in 900 mL of *methanol*, add 50 mL of 0.5M *hydrochloric acid* and dilute to 1000 mL with *water*.

Trisodium Orthophosphate Trisodium phosphate dodecahydrate; $Na_3PO_4,12H_2O$ = 380.1 (*10101-89-0*)

Colourless or white or almost white crystals, freely soluble in water.

Trometamol (*77-86-1*)

See *Tris(hydroxymethyl)aminomethane R*.

Tropic Acid (2*RS*)-3-Hydroxy-2-phenylpropanoic acid; $C_9H_{10}O_3$ = 166.17 (*529-64-6*)

Tropine (1*R*,3*r*,5*S*)-Tropan-3-ol; $C_8H_{15}NO$ = 141.2 (*120-29-6*)

mp: about 54°.

General reagent grade of commerce.

Colourless crystals.

Troxerutin Trihydroxyethylrutin; 3′,4′,7-Tris[*O*-(2-hydroxyethyl)]rutin; 2-[3,4-Bis(2-hydroxyethoxy)phenyl]-3-[[6-*O*-(6-deoxy-α-L-mannopyranosyl)-β-D-glucopyranosyl] oxy]-5-hydroxy-7-(2-hydroxyethoxy)-4*H*-1-benzopyran-4-one; $C_{33}H_{42}O_{19}$ = 743 (*7085-55-4*)

mp: 168 °C to 176 °C.

Trypsin *(9002-07-7)*

A proteolytic enzyme obtained by activation of trypsinogen extracted from the pancreas of beef (*Bos taurus* L.).

White or almost white, crystalline or amorphous powder, sparingly soluble in water.

Trypsin for Peptide Mapping *(9002-07-7)*

Trypsin of high purity treated to eliminate chymotryptic activity.

Tryptophan $C_{11}H_{12}N_2O_2$ = 204.2 *(73-22-3)*

White or yellowish-white, crystalline powder or colourless crystals, slightly soluble in water, very slightly soluble in ethanol (96 per cent).

$[\alpha]_D^{20}$: about −30, determined on a 10 g/L solution.

Typhaneoside 3-[6-Deoxy-α-L-mannopyranosyl-(1→2)-[6-deoxy-α-L-mannopyranosyl-(1→6)]-β-D-glucopyranosyloxy]-5,7-dihydroxy-2-(4-hydroxy-3-methoxyphenyl)-4*H*-1-benzopyran-4-one; $C_{34}H_{42}O_{20}$ = 771 *(104472-68-6)*

Tyramine 4-(2-Aminoethyl)phenol; $C_8H_{11}NO$ = 137.2 *(51-67-2)*

Crystals, sparingly soluble in water, soluble in boiling anhydrous ethanol.

mp: 164 °C to 165 °C.

L-Tyrosine 2-Amino-3-(4-hydroxyphenyl)propionic acid; Tyrosine; $C_9H_{11}NO_3$ = 181.2 *(60-18-4)*

White or almost white, crystalline powder, or colourless or white or almost white crystals, slightly soluble in water, practically insoluble in acetone and in anhydrous ethanol, soluble in dilute hydrochloric acid and in solutions of alkali hydroxides.

Umbelliferone 7-Hydroxycoumarin; 7-Hydroxy-2*H*-1-benzopyran-2-one; $C_9H_6O_3$ = 162.1 *(93-35-6)*

Needles from water.

mp: 225 °C to 228 °C.

Undecanoic Acid Hendecanoic acid; Undecylic acid; $C_{11}H_{22}O_2$ = 186.29 *(112-37-8)*

mp: about 30 °C.

Content: minimum 97.0 per cent of $C_{11}H_{22}O_2$.

Uracil $C_4H_4N_2O_2$ = 112.1 *(66-22-8)*

Content: minimum 95.0 per cent.

Urea *(57-13-6)*

See *Urea (0743)*.

Urease-active Meal *(9002-13-5)*

General reagent grade of commerce.

Activity Each mg of urease-active meal hydrolyses 3 mg of urea in 30 minutes at 37°.

Uridine 1-β-D-Ribofuranosyluracil; $C_9H_{12}N_2O_6$ = 244.2 *(58-96-8)*

White or almost white, crystalline powder, soluble in water.

mp: about 165 °C.

Ursolic Acid 3β-Hydroxyurs-12-en-28-oic acid; $C_{30}H_{48}O_3$ = 456.7 *(77-52-1)*

White or almost white powder, practically insoluble in water, sparingly soluble in methanol, slightly soluble in ethanol (96 per cent).

$[\alpha]_D^{21}$: about 67.50, determined on a 10 g/L solution in a 56.1 g/L solution of *potassium hydroxide R* in *ethanol (96 per cent) R*.

mp: 285 °C to 288 °C.

Urushiol I 3-Pentadecyl-1,2-benzenediol; $C_{21}H_{36}O_2$ = 320.51 *(492-89-7)*

General reagent grade of commerce.

Urushiol II 3-(8-Pentadecenyl)-1,2-benzenediol; $C_{21}H_{34}O_2$ = 318.26 *(2764-91-2)*

General reagent grade of commerce.

Valencene 4β*H*,5α-Eremophila-1(10),11-diene; (1*R*,7*R*,8a*S*)-1,8a-Dimethyl-7-(1-methylethenyl)-1,2,3,5,6,7,8,8a-octahydronaphthalene; $C_{15}H_{24}$ = 204.4 *(4630-07-3)*

Oily, colourless or pale yellow liquid, with a characteristic odour, practically insoluble in water, soluble in ethanol (96 per cent).

d_4^{20}: about 0.918.

n_D^{20}: about 1.508.

bp: about 123 °C.

Valencene used in gas chromatography complies with the following additional test.

Assay. Gas chromatography *(2.2.28)* as prescribed in the monograph *Sweet orange oil (1811)*.

Content: minimum 80 per cent, calculated by the normalisation procedure.

Valerenic Acid (2*E*)-3-[(4*S*,7*R*,7a*R*)-3,7-Dimethyl-2,4,5,6,7,7a-hexahydro-1*H*-inden-4-yl]-2-methylprop-2-enoic acid; $C_{15}H_{22}O_2$ = 234.3 *(3569-10-6)*

mp: 134 °C to 138 °C.

Valeric Acid *N*-Valeric acid; $C_5H_{10}O_2$ = 102.1 *(109-52-4)*

Colourless liquid, soluble in water, freely soluble in ethanol (96 per cent).

d_{20}^{20}: about 0.94.

n_D^{20}: about 1.409.

bp: about 186 °C.

Valine *(72-18-4)*

See *Valine (0796)*.

Vanillin 4-Hydroxy-3-methoxybenzaldehyde; *(121-33-5)*

See *Vanillin (0747)*.

Vanillin Reagent

Carefully add, dropwise, 2 mL of *sulfuric acid R* to 100 mL of a 10 g/L solution of *vanillin R* in *ethanol (96 per cent) R*.

Storage: use within 48 h.

Vanillin Solution, Phosphoric

Dissolve 1.0 g of *vanillin R* in 25 mL of *ethanol (96 per cent) R*. Add 25 mL of *water R* and 35 mL of *phosphoric acid R*.

Veratric Acid 3,4-Dimethoxybenzoic acid; $C_9H_{10}O_4$ = 182.2 *(93-07-2)*

mp: about 180°.

General reagent grade of commerce.

Veratrole 1,2-Dimethoxybenzene; $C_8H_{10}O_2$ = 138.2 *(91-16-7)*

d_4^{20}: 1.085.

n_D^{20}: 1.534.

bp: about 206 °C.

mp: about 22 °C.

Verbenone (1*S*,5*S*)-4,6,6-Trimethylbicyclo[3.1.1]hept-3-en-2-one; $C_{10}H_{14}O$ = 150.2 *(1196-01-6)*

Oil with a characteristic odour, practically insoluble in water, miscible with organic solvents.

d_{20}^{20}: about 0.978.

n_D^{18}: about 1.49.
$[\alpha]_D^{18}$: about + 249.6.
bp: 227 °C to 228 °C.
mp: about 6.5 °C.
Verbenone used in gas chromatography complies with the following additional test.
Assay. Gas chromatography (*2.2.28*) as prescribed in the monograph *Rosemary oil (1846)*.
Content: minimum 99 per cent, calculated by the normalisation procedure.

Vinyl Acetate Ethenyl acetate; $C_4H_6O_2$ = 86,10 (*108-05-4*)
d_{20}^{20}: about 0.930.
bp: about 72 °C.

Vinyl Chloride C_2H_3Cl = 62.5 (*75-01-4*)
Colourless gas, slightly soluble in organic solvents.

Vinyl Polymer for Chromatography, Amino Alkyl
Spherical particles (5 µm) of a vinyl alcohol copolymer chemically modified by bonding of amino alkyl groups.

Vinyl Polymer for Chromatography, Octadecyl
Spherical particles (5 µm) of a vinyl alcohol copolymer chemically modified by bonding of octadecyl groups on the hydroxyl groups.

Vinyl Polymer for Chromatography, Octadecylsilyl
Spherical particles (5 µm) of a vinyl alcohol copolymer bonded to an octadecylsilane. Carbon content of 17 per cent.

Vinyl(1)phenyl(5)methyl(94)polysiloxane
Polysiloxane substituted with 1 per cent of vinyl groups, 5 per cent of phenyl groups and 94 per cent of methyl groups.

2-Vinylpyridine C_7H_7N = 105.1 (*100-69-6*)
Yellow liquid, miscible in water.
d_{20}^{20}: about 0.97.
n_D^{20}: about 1.549.

4-Vinylpyridine 4-Ethenylpyridine; C_7H_7N = 105.1 (*100-43-6*)
Clear, deep yellowish-brown liquid.
bp: 58-61 °C.

1-Vinylpyrrolidin-2-one 1-Ethenylpyrrolidin-2-one; C_6H_9NO = 111.1 (*88-12-0*)
Content: minimum 99.0 per cent.
Clear colourless liquid.
Water (*2.5.12*): maximum 0.1 per cent, determined on 2.5 g. Use as the solvent, a mixture of 50 mL of *anhydrous methanol R* and 10 mL of *butyrolactone R*.
Assay. Gas chromatography (*2.2.28*): use the normalisation procedure.
Column:
— *material*: fused-silica;
— *size*: *l* = 30 m, Ø = 0.5 mm;
— *stationary phase*: *macrogol 20 000 R*.
Carrier gas: *helium for chromatography R*.
Temperature:

	Time (min)	Temperature (°C)
Column	0 - 1	80
	1 - 12	80 → 190
	12 - 27	190
Injection port		190

Detection: flame-ionisation.
Injection: 0.3 µL of the substance to be examined.
Adjust the flow rate of the carrier gas so that the retention time of the peak corresponding to 1-vinylpyrrolidin-2-one is about 17 min.

Vitexin Apigenin 8-glucoside; $C_{21}H_{20}O_{10}$ = 432.4 (*3681-93-4*)
Yellow powder.
Storage: in an airtight container, protected from light.

Vitexin-2''-O-rhamnoside 8-[2-*O*-(6-Deoxy-α-L-mannopyranosyl)-β-D-glucopyranosyl]-5,7-dihydroxy-2-(4-hydroxyphenyl)-4*H*-1-benzopyran-4-one; $C_{27}H_{30}O_{14}$. (M_r 578.5) (*64820-99-1*)

Water (*7732-18-5*)
See *Purified water (0008)*.

Water MB
Deionised, filtered and autoclaved water.

Water R1
Prepared from *distilled water R* by multiple distillation. Remove carbon dioxide by boiling for at least 15 min before use in a boiling flask of fused silica or borosilicate glass and cool. Any other suitable method may be used. The boiling flask has been already used for the test or has been filled with *water R* and kept in an autoclave at 121 °C for at least 1 h prior to first use. When tested immediately before use, *water R1* is neutral to *methyl red solution R*, i.e. it shall produce an orange-red (not a violet-red or yellow) colour corresponding to pH 5.5 ± 0.1 when 0.05 mL of *methyl red solution R* is added to 50 mL of the water to be examined.
Conductivity: maximum 1 µS·cm^{-1}, determined at 25 °C by an in-line conductivity meter (see *Purified water (0008)*).

Water, Ammonia-free Water, ammonium-free
To 100 mL of *water R* add 0.1 mL of *sulfuric acid R*. Distil using the apparatus described for the determination of *Distillation range* (*2.2.11*). Reject the first 10 mL and collect the following 50 mL.

Water, Carbon Dioxide-free
Water R which has been boiled for a few minutes and protected from the atmosphere during cooling and storage or deionised *water R* with a resistivity of not less than 0.18 MΩ·m, determined at 25 °C.

Water, Distilled
Water R prepared by distillation.

Water, Distilled, Deionised
Deionised *water R* prepared by distillation with a resistivity of not less than 0.18 MΩ·m, determined at 25 °C.

Water for Chromatography
Deionised water with a resistivity of not less than 0.18 MΩ·m, determined at 25 °C, prepared by distillation, ion exchange, reverse osmosis or any other suitable method, using water that complies with the regulations on water intended for human consumption, as laid down by the competent authority.
Its quality is such that no significant interfering peaks or loss of sensitivity are observed when used in chromatography. Isocratic elution with UV detection at low wavelengths (i.e. less than 230 nm), with evaporative detectors (e.g. light scattering detector, particle counter detector, charged aerosol detector) or mass detectors, or gradient elution, may require the use of water with a total organic carbon content of maximum 5 ppb.

Water for Injections See *Water for injections (0169)*.

Water, Nitrate-free

To 100 mL of *water R* add a few milligrams of *potassium permanganate R* and of *barium hydroxide R*. Distil using the apparatus described for the determination of *Distillation range (2.2.11)*. Reject the first 10 mL and collect the following 50 mL.

Water, Particle-free

Filter *water R* through a membrane with a pore size of 0.22 µm.

Water, Standard Solution for the Micro Determination of

Commercially available standard solution for the coulometric titration of water, containing a certified content of water in a suitable solvent.

Wedelolactone 1,8,9-Trihydroxy-3-methoxy-6*H*-benzofuro [3,2-*c*][1]benzopyran-6-one; $C_{16}H_{10}O_7$ = 314.3 (*524-12-9*)

White Beeswax See *White beeswax (0069)*.

Xanthine 2,6-Dihydroxypurine; $C_5H_4N_4O_2$ = 152.11 (*69-89-6*)

Xanthydrol 9-Hydroxyxanthene; Xanthen-9-ol; $C_{13}H_{10}O_2$ = 198.2 (*90-46-0*)

Content: minimum 90.0 per cent.

White or pale-yellow powder, very slightly soluble in water, soluble in ethanol (96 per cent) and in glacial acetic acid.

It is also available as a methanolic solution containing 90 g/L to 110 g/L of xanthydrol.

mp: about 123 °C.

Assay. In a 250 mL flask dissolve 0.300 g in 3 mL of *methanol R* or use 3.0 mL of solution. Add 50 mL of *glacial acetic acid R* and, dropwise with shaking, 25 mL of a 20 g/L solution of *urea R*. Allow to stand for 12 h, collect the precipitate on a sintered-glass filter (16) (*2.1.2*), wash with 20 mL of *ethanol (96 per cent) R*, dry in an oven at 100 °C to 105 °C and weigh.

1 g of precipitate is equivalent to 0.9429 g of xanthydrol.

Storage: protected from light. If a methanolic solution is used, store in small sealed ampoules and filter before use if necessary.

Xanthydrol R1

Complies with the requirements prescribed for *xanthydrol R* with the following requirement.

Content: minimum 98.0 per cent of $C_{13}H_{10}O_2$.

Xanthydrol Reagent

Dissolve about 0.125 g of *xanthydrol* in 100 mL of *anhydrous acetic acid*. Add 1 mL of *hydrochloric acid* immediately before use.

Xanthydrol Solution

To 0.1 mL of a 100 g/L solution of *xanthydrol R* in *methanol R* add 100 mL of *anhydrous acetic acid R* and 1 mL of *hydrochloric acid R*. Allow to stand for 24 h before using.

Xylene A mixture of o-, m- and p- isomers; C_8H_{10} = 106.2 (*1330-20-7*)

Mixture of isomers. Clear, colourless, flammable liquid, practically insoluble in water, miscible with ethanol (96 per cent).

d_{20}^{20}: about 0.867.

n_D^{20}: about 1.497.

bp: about 138 °C.

***m*-Xylene** 1,3-Dimethylbenzene; C_8H_{10} = 106.2 (*108-38-3*)

Clear, colourless, flammable liquid, practically insoluble in water, miscible with ethanol (96 per cent).

d_{20}^{20}: about 0.884.

n_D^{20}: about 1.497.

bp: about 139 °C.

mp: about −47 °C.

***o*-Xylene** 1,2-Dimethylbenzene; C_8H_{10} = 106.2 (*95-47-6*)

Clear, colourless, flammable liquid, practically insoluble in water, miscible with ethanol (96 per cent).

d_{20}^{20}: about 0.881.

n_D^{20}: about 1.505.

bp: about 144 °C.

mp: about −25 °C.

Xylene Cyanol FF (*2650-17-1*)

Colour Index No. 42135

A blue, alcohol-soluble dye used as a screening agent in *methyl orange–xylene cyanol FF solution.*

Xylenol Orange [3H-2,1-Benzoxathiol-3-ylidenebis(6-hydroxy-5-methyl-*m*-phenylene)methylenenitrilo]tetra-acetic acid S,S-dioxide tetrasodium salt; $C_{31}H_{28}N_2Na_4O_{13}S$ = 761 (*3618-43-7*)

Reddish-brown crystalline powder, soluble in water.

Xylenol Orange Solution

Dissolve 50.8 mg of *xylenol orange R* in *water R* and dilute to 100.0 mL with the same solvent.

Xylenol Orange Triturate

Triturate 1 part of *xylenol orange R* with 99 parts of *potassium nitrate R*.

Test for sensitivity. To 50 mL of *water R* add 1 mL of *dilute acetic acid R*, 50 mg of the xylenol orange triturate and 0.05 mL of *lead nitrate solution R*.
Add *hexamethylenetetramine R* until the colour changes from yellow to violet-red. After addition of 0.1 mL of *0.1 M sodium edetate* the colour changes to yellow.

Xylitol $C_5H_{12}O_5$ = 152.1 (*87-99-0*)

White or almost white, crystalline powder or crystals.

Content: minimum 96.0 per cent.

D-Xylose Xylose; (*58-86-6*)

See *Xylose (1278)*.

Zinc Zn = 65.4 (*7440-66-6*)

Content: minimum 99.5 per cent.

Silver-white cylinders, granules, pellets or filings with a blue sheen.

Arsenic (2.4.2, Method A): maximum 0.2 ppm.

Dissolve 5.0 g in a mixture of the 15 mL of *hydrochloric acid R* and 25 mL of *water R* prescribed.

Zinc Acetate Zinc acetate dihydrate; $(C_2H_3O_2)_2Zn,2H_2O$ = 219.5 (*5970-45-6*)

Bright white or almost white crystals, slightly efflorescent, freely soluble in water, soluble in ethanol (96 per cent). It loses its crystallisation water at 100 °C.

d_{20}^{20}: about 1.735.

mp: about 237 °C.

Zinc Acetate Solution

Mix 600 mL of *water R* with 150 mL of *glacial acetic acid R*, 54.9 g of *zinc acetate R* and stir to dissolve. Continue stirring while adding 150 mL of *concentrated ammonia R*. Cool to

room temperature and adjust with *ammonia R* to pH 6.4. Dilute the mixture to 1 L with *water R*.

Zinc, Activated

Place the zinc cylinders or pellets to be activated in a conical flask and add a sufficient quantity of a 50 ppm solution of *chloroplatinic acid R* to cover the metal. Allow the metal to remain in contact with the solution for 10 min, wash, drain and dry immediately.

Arsenic (2.4.2, Method A). To 5 g of the activated zinc add 15 mL of *hydrochloric acid R*, 25 mL of *water R*, 0.1 mL of *stannous chloride solution R* and 5 mL of *potassium iodide solution R*. No colour is produced during the test.

Activity. The requirements of the suitability test for arsenic (*2.4.2, Method A*) are met.

Zinc Chloride (*7646-85-7*)

See *Zinc chloride (0110)*.

Zinc Chloride–Formic Acid Solution

Dissolve 20 g of *zinc chloride R* in 80 g of an 850 g/L solution of *anhydrous formic acid R*.

Zinc Chloride Solution, Iodinated

Dissolve 20 g of *zinc chloride R* and 6.5 g of *potassium iodide R* in 10.5 mL of *water R*. Add 0.5 g of *iodine R* and shake for 15 min. Filter if necessary.

Storage: protected from light.

Zinc Iodide ZnI_2 = 319.2 (*10139-47-6*)

General reagent grade of commerce.

Zinc Iodide and Starch Solution

To a solution of 2 g of *zinc chloride R* in 10 mL of *water R* add 0.4 g of *soluble starch R* and heat until the starch has dissolved. After cooling to room temperature add 1.0 mL of a colourless solution containing 0.10 g *zinc R* as filings and 0.2 g of *iodine R* in *water R*. Dilute the solution to 100 mL with *water R* and filter.

Storage: protected from light.

Test for sensitivity. Dilute 0.05 mL of *sodium nitrite solution R* to 50 mL with *water R*. To 5 mL of this solution add 0.1 mL of *dilute sulfuric acid R* and 0.05 mL of the zinc iodide and starch solution and mix. The solution becomes blue.

Zinc Oxide (*1314-13-2*)

See *Zinc oxide (0252)*.

Zinc Powder Zn = 65.4 (*7440-66-6*)

Content: minimum 90.0 per cent.

Very fine, grey powder, soluble in *dilute hydrochloric acid R*.

Zinc Shot Zn = 65.38

Analytical reagent grade of commerce.

Shot, 0.5 to 2.0 mm (about 8 to 30 mesh).

Zinc Sulfate Zinc sulphate; Zinc Sulfate Heptahydrate; (*7446-20-0*)

See *Zinc sulfate (0111)*.

Zirconyl Chloride $ZrOCl_2,8H_2O$ = 322.3 (*15461-27-5*)

Content, minimum 96.0% of $ZrCl_2O,8H_2O$.

White or almost white, crystalline powder or crystals, freely soluble in water and in ethanol (96%).

Assay Dissolve 0.600 g in a mixture of 5 mL of *nitric acid* and 50 mL of *water*. Add 50.0 mL of *0.1 M silver nitrate* and 3 mL of *dibutyl phthalate* and shake. Using 2 mL of *ferric ammonium sulfate solution R2* as indicator, titrate with *0.1 M ammonium thiocyanate* until a reddish-yellow colour is obtained.

1 mL of *0.1 M silver nitrate* is equivalent to 16.11 mg of $ZrCl_2O,8H_2O$.

Zirconyl Nitrate A basic salt corresponding approximately to the formula $ZrO(NO_3)_2,2H_2O$ (*14985-18-3*)

A white or almost white powder or crystals, hygroscopic, soluble in water. The aqueous solution is a clear or at most slightly opalescent liquid.

Storage: in an airtight container.

Zirconyl Nitrate Solution

A 1 g/L solution in a mixture of 40 mL of *water R* and 60 mL of *hydrochloric acid R*.

B. Volumetric Reagents and Solutions

Terminology

Volumetric solutions are prepared according to the usual chemical analytical methods. The accuracy of the apparatus used is verified to ensure that it is appropriate for the intended use.

The concentration of volumetric solutions is indicated in terms of molarity. Molarity expresses, as the number of moles, the amount of substance dissolved in 1 L of solution. A solution which contains x moles of substance per litre is said to be x M.

Volumetric solutions do not differ from the prescribed strength by more than 10 per cent. The molarity of the volumetric solutions is determined by an appropriate number of titrations. The repeatability does not exceed 0.2 per cent (relative standard deviation).

Volumetric solutions are standardised by the methods described below. When a volumetric solution is to be used in an assay in which the end-point is determined by an electrochemical process (for example, amperometry or potentiometry) the solution is standardised by the same method. The composition of the medium in which a volumetric solution is standardised should be the same as that in which it is to be used.

Solutions more dilute than those described below are either prepared by adapting the quantities stated or by dilution, with *carbon dioxide-free water R* (unless otherwise prescribed), of a more concentrated solution that has been previously standardised. In the first case, the correction factor is determined on the volumetric solution to be used in the monograph. In the latter case, the correction factor of the dilute solution is the same as that of the standardised solution from which it was prepared.

Commercially available volumetric solutions traceable to a primary standard may be used provided their titre is determined or verified prior to first use.

Titres of volumetric solutions are verified at appropriate intervals that are defined in the quality system procedures.

British Pharmacopoeia monographs In assays and other quantitative tests, solutions to be standardised before use; include the letters VS after the name of the reagent.

Primary Standards

The following materials, after drying under the specified conditions, are recommended for use as primary standards in the standardisation of volumetric solutions. Analytical reagent grade materials of commerce must be used.

For primary standards from commercial sources, a pre-treatment step may be necessary. Follow the supplier's instructions.

A secondary standard may be used provided its traceability to a primary standard has been demonstrated.

For monographs of the European Pharmacopoeia, primary standards are indicated by the suffix RV.

Arsenic Trioxide Arsenious trioxide; As_2O_3 = 197.8 (*1327-53-3*)

Sublime *arsenious trioxide R* in a suitable apparatus.

Storage: over *anhydrous silica gel R*.

Benzoic Acid $C_7H_6O_2$ = 122.1 (*65-85-0*)

Sublime *benzoic acid R* in a suitable apparatus.

Ferrous Ethylenediammonium Sulfate Ethylenediammonium iron(II) disulfate tetrahydrate; Ethylenediammonium tetraaquabis(sulfato)iron(II); $Fe(C_2H_{10}N_2)(SO_4)_2,4H_2O$ = 382.1 (*113193-60-5*)

Content: minimum 99.5 per cent.

Potassium Bromate $KBrO_3$ = 167.0 (*7758-01-2*)

Crystallise *potassium bromate R* from boiling *water R*. Collect the crystals and dry to constant mass in an oven at 180 ± 10 °C (*2.2.32*).

Potassium Dichromate

Dry to constant weight at 150°.

Potassium Hydrogen Phthalate Potassium 2-carboxybenzoate; $C_8H_5KO_4$ = 204.2 (*877-24-7*)

Recrystallise *potassium hydrogen phthalate R* from boiling *water R*, collect the crystals at a temperature above 35 °C and dry to constant mass at 110 °C.

Potassium Iodate

Dry to constant weight at 130°.

Sodium Carbonate, Anhydrous Sodium Carbonate; Na_2CO_3 = 106.0 (*497-19-8*)

Filter at room temperature a saturated solution of *sodium carbonate R*. Introduce slowly into the filtrate a stream of *carbon dioxide R* with constant cooling and stirring. After about 2 h, collect the precipitate on a sintered-glass filter (*2.1.2*). Wash the filter with iced *water R* containing carbon dioxide. After drying at 100 °C to 105 °C, heat to constant mass at 270-300 °C, stirring from time to time.

Sodium Chloride NaCl = 58.44 (*7647-14-5*)

To 1 volume of the *saturated sodium chloride solution R* add 2 volumes of *hydrochloric acid R*. Collect the crystals formed and wash with *hydrochloric acid R1*. Remove the hydrochloric acid by heating on a water-bath and dry the crystals to constant mass at 300 °C.

Sulfanilic Acid 4-Aminobenzenesulfonic acid; $C_6H_7NO_3S$ = 173.2 (*121-57-3*)

Recrystallise *sulfanilic acid R* from boiling *water R*. Filter and dry to constant mass at 100-105 °C.

Trometamol 2-Amino-2-(hydroxymethyl)propane-1,3-diol; Tris(hydroxymethyl)aminomethane; $C_4H_{11}NO_3$ = 121.1 (*77-86-1*)

Content: minimum 99.5 per cent.

Zinc Zn = 65.4 (*7440-66-6*)

Content: minimum 99.9 per cent.

Preparation and Standardisation

For each solution the preparation and standardisation of the most commonly used strengths are described. Solutions more concentrated than those described are prepared and standardised using proportionate amounts of the reagents. Aqueous solutions less concentrated than those described are prepared by making an exact dilution of a more concentrated solution with *carbon dioxide-free water*. The correction factors of these solutions are the same as those from which the dilutions were prepared. Aqueous solutions of molarity below 0.1M are freshly prepared using *carbon dioxide-free water*.

The water used in preparing volumetric solutions complies with the requirements of the monograph for Purified Water. When used for the preparation of unstable solutions such as potassium permanganate and sodium thiosulfate, it should be freshly boiled and cooled. When a solution is to be used in an assay in which the end point is determined by an electrochemical process, the exact concentration of the

solution must be determined in the same way.
The composition of the medium in which a volumetric solution is standardised should be the same as that in which it is to be used.

All volumetric solutions should, if practicable, be prepared, standardised and used at 20°; if a titration is carried out at a markedly different temperature from that at which the standardisation took place, a suitable temperature correction should be made.

Acetic Acid VS $C_2H_4O_2$ = 60.1

Dilute 6.0 g of *glacial acetic acid R* to 1000.0 mL with *water R*.

Standardisation. To 25.0 mL of acetic acid add 0.5 mL of *phenolphthalein solution R* and titrate with 0.1M *sodium hydroxide*.

0.1M Ammonium Cerium(IV) Nitrate VS 0.1M ammonium and cerium nitrate

Shake for 2 min a solution containing 56 mL of *sulfuric acid R* and 54.82 g of *ammonium and cerium nitrate R*, then add 5 successive quantities, each of 100 mL, of *water R*, shaking after each addition. Dilute the clear solution to 1000.0 mL with *water R*. Standardise the solution after 10 days.

Standardisation. Dissolve 0.300 g of *ferrous ethylenediammonium sulfate RV* in 50 mL of a diluted solution of *sulfuric acid R* (49 g/L H_2SO_4). Titrate with the ammonium and cerium nitrate solution, determining the end-point potentiometrically (*2.2.20*) or using 0.1 mL of *ferroin R* as indicator.

1 mL of *0.1 M ammonium and cerium nitrate* is equivalent to 38.21 mg of $Fe(C_2H_{10}N_2)(SO_4)_2,4H_2O$.

Storage: protected from light.

0.1M Ammonium Cerium(IV) Sulfate VS 0.1M ammonium and cerium sulfate

Dissolve 65.0 g of *ammonium and cerium sulfate R* in a mixture of 500 mL of *water R* and 30 mL of *sulfuric acid R*. Allow to cool and dilute to 1000.0 mL with *water R*.

Standardisation. Dissolve 0.300 g of *ferrous ethylenediammonium sulfate RV* in 50 mL of a diluted solution of *sulfuric acid R* (49 g/L H_2SO_4). Titrate with the ammonium and cerium sulfate solution, determining the end-point potentiometrically (*2.2.20*) or using 0.1 mL of *ferroin R* as indicator.

1 mL of *0.1 M ammonium and cerium sulfate* is equivalent to 38.21 mg of $Fe(C_2H_{10}N_2)(SO_4)_2,4H_2O$.

Dilution. Use a diluted solution of *sulfuric acid R* (59 g/L H_2SO_4) while cooling the solution.

0.01M Ammonium Cerium(IV) Sulfate VS Ammonium and cerium sulfate

To 100 mL of *0.1M ammonium cerium(IV) sulfate VS* add, while cooling, 30 mL of *sulfuric acid* and sufficient water to produce 1000 mL.

Ammonium Iron(II) Sulfate VS Ammonium Iron(II) Sulphate; Ferrous Ammonium Sulfate; $(NH_4)_2Fe(SO_4)_2,6H_2O$ = 392.1

For a 0.1M solution Dissolve 40 g of *ammonium iron(II) sulfate* in 100 mL of 2M *sulfuric acid* and dilute with sufficient freshly boiled and cooled *water* to produce 1000 mL.

Ascertain its exact concentration in the following manner. To 25 mL add 10 mL of 1M *sulfuric acid* and 1 mL of *orthophosphoric acid* and titrate with 0.02M *potassium permanganate VS*. Each mL of 0.02M *potassium permanganate VS* is equivalent to 39.21 mg of $(NH_4)_2Fe(SO_4)_2,6H_2O$.

0.1M Ammonium Iron(III) Sulfate VS 0.1M Ferric Ammonium Sulfate

Dissolve 50.0 g of *ferric ammonium sulfate R* in a mixture of 6 mL of *sulfuric acid R* and 300 mL of *water R* and dilute to 1000.0 mL with *water R*.

Standardisation. To 10.0 mL of the ferric ammonium sulfate solution add 35 mL of *water R*, 3 mL of *hydrochloric acid R* and 1 g of *potassium iodide R*. Allow to stand for 10 min. Titrate with *0.1 M sodium thiosulfate*, determining the end-point potentiometrically (*2.2.20*) or using 1 mL of *starch solution R* as indicator.

1 mL of *0.1 M sodium thiosulfate* is equivalent to 48.22 mg of $FeNH_4(SO_4)_2,12H_2O$.

0.1M Ammonium Thiocyanate VS

Dissolve 7.612 g of *ammonium thiocyanate R* in *water R* and dilute to 1000.0 mL with the same solvent.

Standardisation. To 20.0 mL of *0.1 M silver nitrate* add 25 mL of *water R*, 2 mL of *dilute nitric acid R* and 2 mL of *ferric ammonium sulfate solution R2*. Titrate with the ammonium thiocyanate solution until a reddish-yellow colour is obtained.

0.1M Barium Chloride VS

Dissolve 24.4 g of *barium chloride R* in *water R* and dilute to 1000.0 mL with the same solvent.

Standardisation. To 10.0 mL of the barium chloride solution add 60 mL of *water R*, 3 mL of *concentrated ammonia R* and 0.5-1 mg of *phthalein purple R*. Titrate with *0.1 M sodium edetate*. When the solution begins to decolorise, add 50 mL of *ethanol (96 per cent) R* and continue the titration until the blue-violet colour disappears.

0.05M Barium Perchlorate VS

Dissolve 15.8 g of *barium hydroxide R* in a mixture of 7.5 mL of *perchloric acid R* and 75 mL of *water R*, adjust the solution to pH 3 by adding *perchloric acid R* and filter if necessary. Add 150 mL of *ethanol (96 per cent) R* and dilute to 250 mL with *water R*. Dilute to 1000.0 mL with *buffer solution pH 3.7 R*.

Standardisation. To 5.0 mL of *0.05 M sulfuric acid* add 5 mL of *water R*, 50 mL of *buffer solution pH 3.7 R* and 0.5 mL of *alizarin S solution R*. Titrate with the barium perchlorate solution until an orange-red colour appears. Standardise immediately before use.

Dilution. Use *buffer solution pH 3.7 R*.

0.025M Barium Perchlorate VS

Dilute 500 mL of *0.05M barium perchlorate VS* to 1000 mL with *buffer solution pH 3.7*.

0.005M Barium Perchlorate VS

Dilute 10.0 mL of *0.05 M barium perchlorate* to 100.0 mL with a buffer solution prepared as follows: to 15.0 mL of *acetic acid R* add 60.0 mL of *2-propanol R*. Adjust to pH 3.7 with *ammonia R* and dilute to 100.0 mL with *water R*.

0.004M Benzethonium Chloride VS

Dissolve in *water R* 1.792 g of *benzethonium chloride R*, previously dried to constant mass at 100-105 °C, and dilute to 1000.0 mL with the same solvent.

Standardisation. Dissolve 0.350 g of the dried substance in 35 mL of a mixture of 30 volumes of *anhydrous acetic acid R* and 70 volumes of *acetic anhydride R*. Titrate with *0.1 M perchloric acid*, using 0.05 mL of *crystal violet solution R* as indicator. Carry out a blank titration.

1 mL of *0.1 M perchloric acid* is equivalent to 44.81 mg of $C_{27}H_{42}ClNO_2$.

0.01M Bismuth Nitrate VS

Dissolve 4.86 g of *bismuth nitrate pentahydrate R* in 60 mL of *dilute nitric acid R* and dilute to 1000.0 mL with *water R*.

Standardisation. To 25.0 mL of the bismuth nitrate solution, add 50 mL of *water R* and titrate with *0.01 M sodium edetate* using 0.05 mL of a 1 g/L solution of *xylenol orange R* as indicator.

0.05M Bromine VS 0.0167M bromide-bromate

Dissolve 2.7835 g of *potassium bromate RV* and 13 g of *potassium bromide R* in *water R* and dilute to 1000.0 mL with the same solvent.

0.1M Cerium(IV) Sulfate VS 0.1M Cerium Sulfate

Dissolve 40.4 g of *cerium sulfate R* in a mixture of 500 mL of *water R* and 50 mL of *sulfuric acid R*. Allow to cool and dilute to 1000.0 mL with *water R*.

Standardisation. Dissolve 0.300 g of *ferrous ethylenediammonium sulfate RV* in 50 mL of a diluted solution of *sulfuric acid R* (49 g/L H_2SO_4). Titrate with the cerium sulfate solution, determining the end-point potentiometrically (*2.2.20*) or using 0.1 mL of *ferroin R* as indicator.

1 mL of *0.1 M cerium sulfate* is equivalent to 38.21 mg of $Fe(C_2H_{10}N_2)(SO_4)_2,4H_2O$.

Cetylpyridinium Chloride VS $C_{21}H_{38}ClN,H_2O$ = 358.0

For a 0.005M solution Dissolve 1.8 g of *cetylpyridinium chloride* in 10 mL of *ethanol (96%)* and dilute to 1000 mL with *water*.

Ascertain its exact concentration in the following manner. Transfer 25 mL to a separating funnel, add 25 mL of *chloroform*, 10 mL of 0.01M *sodium hydroxide* and 10 mL of a freshly prepared 0.5% w/v solution of *potassium iodide*. Shake well, allow to separate and discard the chloroform layer. Shake the aqueous layer with three further 10-mL quantities of *chloroform* and discard the chloroform solutions. Add 40 mL of *hydrochloric acid*, cool and titrate with *0.005M potassium iodate VS* until the solution becomes pale brown in colour. Add 2 mL of *chloroform* and continue the titration, shaking vigorously and allowing the layers to separate after each addition, until the chloroform becomes colourless. Titrate a mixture of 20 mL of *water*, 10 mL of the potassium iodide solution and 40 mL of *hydrochloric acid* with *0.005M potassium iodate VS* in the same manner. The difference between the titrations represents the amount of potassium iodate required. Each mL of *0.005M potassium iodate VS* is equivalent to 3.580 mg of $C_{21}H_{38}ClN,H_2O$.

0.02M Copper Sulfate VS 0.02M Copper Sulphate VS

Dissolve 5.0 g of *copper sulfate pentahydrate R* in *water R* and dilute to 1000.0 mL with the same solvent.

Standardisation. To 20.0 mL of the copper sulfate solution add 2 g of *sodium acetate R* and 0.1 mL of *pyridylazonaphthol solution R*. Titrate with *0.02 M sodium edetate* until the colour changes from violet-blue to bright green. Titrate slowly towards the end of the titration.

Cupriethylenediamine Hydroxide Solution Use a 1M solution in which the molar ratio of ethylenediamine to copper is 2.00 ± 0.04.

Dioctyl Sodium Sulfosuccinate VS Dioctyl Sodium Sulphosuccinate VS; $C_{20}H_{37}NaO_7S$ = 444.6

For a 0.01M solution Dissolve 4.5 g of *dioctyl sodium sulfosuccinate* in warm *water*, cool and dilute to 1000 mL with *water*.

Ascertain its exact concentration in the following manner. To 25 mL add 25 mL of a solution containing 20% w/v of *anhydrous sodium sulfate* and 2% w/v of *sodium carbonate*, 50 mL of *chloroform* and 1.5 mL of *bromophenol blue solution* and mix. Titrate with 0.01M *tetrabutylammonium iodide VS* until about 1 mL from the end point. Stopper the flask, shake vigorously for 2 minutes and continue the titration, in 0.05-mL increments, shaking vigorously and allowing the flask to stand for about 10 seconds after each addition. Continue the titration until a blue colour just appears in the chloroform layer. Each mL of 0.01M *tetrabutylammonium iodide VS* is equivalent to 4.446 mg of $C_{20}H_{37}NaO_7S$.

0.1M Disodium Edetate VS 0.1M Sodium Edetate VS

Dissolve 37.5 g of *sodium edetate R* in 500 mL of *water R*, add 100 mL of *1 M sodium hydroxide* and dilute to 1000.0 mL with *water R*.

Standardisation. Dissolve 0.120 g of *zinc RV* in 4 mL of *hydrochloric acid R1*. Add *dilute sodium hydroxide solution R* until the solution is weakly acid and carry out the assay of zinc by complexometry (*2.5.11*).

1 mL of *0.1 M sodium edetate* is equivalent to 6.538 mg of Zn.

Storage: in a polyethylene container.

0.05M Disodium Edetate VS 0.05M Sodium Edetate

Dissolve 18.6 g of *disodium edetate* in sufficient water to produce 1000 mL.

Ascertain its exact concentration in the following manner.

Dissolve 0.100 g of *zinc*, in granules, in 4 mL of 7M *hydrochloric acid* and add 0.1 mL of *bromine water*. Boil to remove excess bromine, cool and dilute to 100 mL with *water*. Dilute 25 mL of this solution to 200 mL with *water*, add about 50 mg of *xylenol orange triturate* and *hexamine* until the solution becomes violet-pink and add 2 g of *hexamine* in excess. Titrate with the disodium edetate solution until the violet-pink colour changes to yellow. Each mL of 0.05M disodium edetate VS is equivalent to 3.269 mg of Zn.

0.02M Disodium Edetate VS 0.02M Sodium Edetate

Dissolve 7.444 g of *disodium edetate* in sufficient water to produce 1000 mL.

Ascertain its exact concentration in the following manner.

Dissolve 0.100 g of *zinc*, in granules, in 4 mL of 7M *hydrochloric acid* and add 0.1 mL of *bromine water*. Boil to remove excess bromine, cool and dilute to 100 mL with *water*. Dilute 25 mL of this solution to 200 mL with *water*, add about 50 mg of *xylenol orange triturate* and *hexamine* until the solution becomes violet-pink and add 2 g of *hexamine* in excess. Titrate with the disodium edetate solution until the violet-pink colour changes to yellow. Each mL of 0.02M disodium edetate VS is equivalent to 1.308 mg of Zn.

0.01M Disodium Edetate VS 0.01M Sodium Edetate

Dissolve 3.722 g of *disodium edetate* in sufficient water to produce 1000 mL.

Ascertain its exact concentration in the following manner.

Dissolve 0.100 g of *zinc*, in granules, in 4 mL of 7M *hydrochloric acid* and add 0.1 mL of *bromine water*. Boil to remove excess bromine, cool and dilute to 100 mL with *water*. Dilute 10 mL of this solution to 200 mL with *water*, add about 50 mg of *xylenol orange triturate* and *hexamine* until the solution becomes violet-pink and add 2 g of *hexamine* in excess. Titrate with the disodium edetate solution until the violet-pink colour changes to yellow. Each mL of 0.01M disodium edetate VS is equivalent to 0.6538 mg of Zn.

6M Hydrochloric Acid VS

Dilute 618.0 g of *hydrochloric acid* to 1000 mL with *water*.

3M Hydrochloric Acid VS

Dilute 309.0 g of *hydrochloric acid* to 1000 mL with *water*.

2M Hydrochloric Acid VS

Dilute 206.0 g of *hydrochloric acid* to 1000 mL with *water*.

1M Hydrochloric Acid VS

Dilute 103.0 g of *hydrochloric acid R* to 1000.0 mL with *water R*.

Standardisation. Dissolve 0.950 g of *trometamol RV* in 50 mL of *water R*. Titrate with the hydrochloric acid solution, determining the end-point potentiometrically (*2.2.20*) or using 0.1 mL of *methyl orange solution R* as indicator until a yellowish-red colour is obtained.

1 mL of *1 M hydrochloric acid* is equivalent to 121.1 mg of $C_4H_{11}NO_3$.

0.1M Hydrochloric Acid VS

Dilute 100.0 mL of *1 M hydrochloric acid* to 1000.0 mL with *carbon dioxide-free water R*.

Standardisation. Carry out the titration described for *1 M hydrochloric acid* using 95 mg of *trometamol RV* dissolved in 50 mL of *water R*.

1 mL of *0.1 M hydrochloric acid* is equivalent to 12.11 mg of $C_4H_{11}NO_3$.

0.5M Iodine VS

Dissolve 127 g of *iodine R* and 200 g of *potassium iodide R* in *water R* and dilute to 1000.0 mL with the same solvent.

Standardisation. To 2.0 mL of the iodine solution add 1 mL of *dilute acetic acid R* and 50 mL of *water R*. Titrate with *0.1 M sodium thiosulfate,* using *starch solution R* as indicator.

Storage: protected from light.

0.05M Iodine VS

Dissolve 12.7 g of *iodine R* and 20 g of *potassium iodide R* in *water R* and dilute to 1000.0 mL with the same solvent.

Standardisation. To 10.0 mL of the iodine solution add 1 mL of *dilute acetic acid R* and 40 mL of *water R*. Titrate with *0.1 M sodium thiosulfate,* determining the end-point potentiometrically (*2.2.20*) or using *starch solution R* as indicator.

Storage: protected from light.

0.01M Iodine VS

Add 0.3 g of *potassium iodide R* to 20.0 mL of *0.05 M iodine* and dilute to 100.0 mL with *water R*.

0.1M Iron(II) Sulfate VS 0.1M Ferrous sulfate; 0.1M Iron(II) Sulphate VS

Dissolve 27.80 g of *ferrous sulfate R* in 500 mL of *dilute sulfuric acid R* and dilute to 1000.0 mL with *water R*.

Standardisation. To 25.0 mL of the ferrous sulfate solution add 3 mL of *phosphoric acid R* and titrate immediately with *0.02 M potassium permanganate.* Standardise immediately before use.

Karl Fischer Reagent VS

Iodosulfurous reagent

The apparatus, which must be kept closed and dry during the preparation, consists of a 3000- to 4000-mL round-bottomed flask with inlets for a thermometer and a stirrer and fitted with a drying tube. To 700 mL of *anhydrous pyridine* and 700 mL of *2-methoxyethanol* add, stirring constantly, 220 g of finely powdered *iodine,* previously dried over *phosphorus pentoxide.* Continue stirring until the iodine has completely dissolved (about 30 minutes), cool to −10° and add quickly, while stirring, 190 g of *sulfur dioxide.* Do not allow the temperature to exceed 30°; cool.

Determine the water-equivalent of the reagent immediately before use in the following manner. Transfer 20 mL of *anhydrous methanol* to the titration vessel and titrate to the electrometric end point with the reagent, Appendix IX C. Add in an appropriate form a suitable amount of water, accurately weighed, and titrate to the end point. Calculate the water-equivalent of the reagent in mg per mL.

The minimum water equivalent is 3.5 mg of water per mL of reagent. Work protected from humidity.

The composition of commercially available Karl Fischer reagents often differs from that above by the replacement of pyridine with other basic compounds. The use of these reagents must be validated in order to verify in each individual case the stoichiometry and the absence of incompatibility between the substance under test and the reagent.

0.1M Lanthanum Nitrate VS

Dissolve 43.30 g of *lanthanum nitrate R* in *water R* and dilute to 1000.0 mL with the same solvent.

Standardisation. To 20.0 mL of the lanthanum nitrate solution, add 15 mL of *water R* and 25 mL of *0.1 M sodium edetate.* Add about 50 mg of *xylenol orange triturate R* and about 2 g of *hexamethylenetetramine R*. Titrate with *0.1 M zinc sulfate* until the colour changes from yellow to violet-pink.

1 mL of *0.1 M sodium edetate* is equivalent to 43.30 mg of $La(NO_3)_3,6H_2O$.

0.1M Lead Nitrate VS

Dissolve 33 g of *lead nitrate R* in *water R* and dilute to 1000.0 mL with the same solvent.

Standardisation. Take 20.0 mL of the lead nitrate solution and carry out the determination of lead by complexometry (*2.5.11*).

0.05M Lead Nitrate VS

Dissolve 16.5 g of *lead(II) nitrate* in sufficient *water* to produce 1000 mL.

Ascertain its exact concentration in the following manner.

To 50 mL of the solution add 300 mL of *water* and carry out the method for the complexometric titration of lead, *Appendix VIII D*. Each mL of *0.1M disodium edetate VS* is equivalent to 33.12 mg of $Pb(NO_3)_2$.

0.1M Lithium Methoxide VS

Dissolve 0.694 g of *lithium R* in 150 mL of *anhydrous methanol R* and dilute to 1000.0 mL with *toluene R*.

Standardisation. To 10 mL of *dimethylformamide R* add 0.05 mL of a 3 g/L solution of *thymol blue R* in *methanol R* and titrate with the lithium methoxide solution until a pure blue colour is obtained. Immediately add 0.100 g of *benzoic acid RV*. Stir to effect solution and titrate with the lithium methoxide solution until the pure blue colour is again obtained. Protect the solution from atmospheric carbon dioxide throughout the titration. From the volume of titrant used in the second titration ascertain the exact strength of the lithium methoxide solution. Standardise immediately before use.

1 mL of *0.1 M lithium methoxide* is equivalent to 12.21 mg of $C_7H_6O_2$.

0.1M Magnesium Chloride VS

Dissolve 20.33 g of *magnesium chloride R* in *water R* and dilute to 1000.0 mL with the same solvent.

Standardisation. Carry out the determination of magnesium by complexometry (*2.5.11*).

Magnesium Sulfate VS Magnesium Sulphate VS; $MgSO_4,7H_2O$ = 246.5

For a 0.05M solution Dissolve 12.5 g of *magnesium sulfate* in sufficient *water* to produce 1000 mL.

Ascertain its exact concentration by carrying out the method for the *complexometric titration of magnesium*, Appendix VIII D, using 40 mL of the magnesium sulfate solution. Each mL of 0.1M *disodium edetate VS* is equivalent to 24.65 mg of $MgSO_4,7H_2O$.

Mercury(II) Nitrate VS Mercuric nitrate

$Hg(NO_3)_2$+ aq

For a 0.02M solution Dissolve 6.85 g of *mercury(II) nitrate* in 20 mL of 1M *nitric acid* and add sufficient *water* to produce 1000 mL.

Ascertain its exact concentration in the following manner. Dissolve 15 mg of *sodium chloride* in 50 mL of *water* and titrate with the mercury nitrate solution determining the end point potentiometrically, using a platinum or mercury indicator electrode and a mercury–mercury(I) sulfate reference electrode. Each mL of 0.02M *mercury(II) nitrate VS* is equivalent to 2.338 mg of NaCl.

1M Nitric Acid VS

Dilute 96.6 g of *nitric acid R* to 1000.0 mL with *water R*.

Standardisation. Dissolve 0.950 g of *trometamol RV* in 50 mL of *water R*. Titrate with the nitric acid solution, determining the end-point potentiometrically (*2.2.20*) or using 0.1 mL of *methyl orange solution R* as indicator until a reddish-yellow colour is obtained.

1 mL of *1 M nitric acid* is equivalent to 121.1 mg of $C_4H_{11}NO_3$.

0.1M Perchloric Acid VS

Place 8.5 mL of *perchloric acid R* in a volumetric flask containing about 900 mL of *glacial acetic acid R* and mix. Add 30 mL of *acetic anhydride R*, dilute to 1000.0 mL with *glacial acetic acid R*, mix and allow to stand for 24 h. Determine the water content (*2.5.12*) without addition of methanol and, if necessary, adjust the water content to 0.1-0.2 per cent by adding either *acetic anhydride R* or *water R*. Allow to stand for 24 h.

Standardisation. Dissolve 0.170 g of *potassium hydrogen phthalate RV* in 50 mL of *anhydrous acetic acid R*, warming gently if necessary. Allow to cool protected from air, and titrate with the perchloric acid solution, determining the end-point potentiometrically (*2.2.20*) or using 0.05 mL of *crystal violet solution R* as indicator. Note the temperature of the perchloric acid solution at the time of the titration. If the temperature at which an assay is carried out is different from that at which the *0.1 M perchloric acid* has been standardised, the volume used in the assay becomes:

$$V_c = V[1 + (t_1 - t_2)0.0011]$$

t_1 = temperature during standardisation,
t_2 = temperature during the assay,
V_c = corrected volume,
V = observed volume.

1 mL of *0.1 M perchloric acid* is equivalent to 20.42 mg of $C_8H_5KO_4$.

Dilution. Use *anhydrous acetic acid R*.

Other strengths of perchloric acid should be prepared by diluting *0.1M perchloric acid VS* appropriately with anhydrous acetic acid.

0.02M Perchloric Acid VS

Dilute 20.0 mL of 0.1M perchloric acid to 100.0 mL with anhydrous acetic acid.

Other strengths of perchloric acid should be prepared by diluting 0.1M perchloric acid VS appropriately with anhydrous acetic acid.

0.033M Potassium Bromate VS

Dissolve 5.5670 g of *potassium bromate RV* in *water R* and dilute to 1000.0 mL with the same solvent.

0.02M Potassium Bromate VS

Dissolve 3.340 g of *potassium bromate* in sufficient *water* to produce 1000 mL.

0.0167M Potassium Bromate VS

Dissolve 2.783 g of *potassium bromate* in sufficient *water* to produce 1000 mL.

0.0083M Potassium Bromate VS

Prepare by appropriate dilution of *0.033M Potassium Bromate VS*.

Potassium Dichromate VS $K_2Cr_2O_7$ = 294.2

For a 0.0167M solution Dissolve 4.9 g of *potassium dichromate* in sufficient *water* to produce 1000 mL.

Ascertain its exact concentration in the following manner. To 20 mL of the solution add 1 g of *potassium iodide* and 7 mL of 2M *hydrochloric acid*. Add 250 mL of *water* and titrate with 0.1M *sodium thiosulfate VS*, using 3 mL of *starch solution* as indicator, until the colour changes from blue to light green. Each mL of 0.1M *sodium thiosulfate VS* is equivalent to 4.9 mg of $K_2Cr_2O_7$.

0.1M Potassium Hydrogen Phthalate VS

In a conical flask containing about 800 mL of *anhydrous acetic acid R*, dissolve 20.42 g of *potassium hydrogen phthalate RV*. Heat on a water-bath until completely dissolved, protected from humidity. Cool to 20 °C and dilute to 1000.0 mL with *anhydrous acetic acid R*.

0.5M Potassium Hydroxide in Ethanol (60%) VS 0.5M Potassium hydroxide in alcohol (60 per cent V/V)

Dissolve 3 g of *potassium hydroxide R* in *aldehyde-free alcohol R* (60 per cent *V/V*) and dilute to 100.0 mL with the same solvent.

Standardisation. Dissolve 0.500 g of *benzoic acid RV* in 10 mL of *water R* and 40 mL of *ethanol (96 per cent) R*. Titrate with the potassium hydroxide solution, determining the end-point potentiometrically (*2.2.20*) or using 0.1 mL of *phenolphthalein solution R* as indicator.

1 mL of *0.5 M potassium hydroxide in alcohol (60 per cent V/V)* is equivalent to 61.06 mg of $C_7H_6O_2$.

Potassium Hydroxide in Ethanol (90%) VS

For a 1M solution Dissolve 60 g of *potassium hydroxide* in sufficient *aldehyde-free ethanol (90%)* to produce 1000 mL, allow the solution to stand for 24 hours, decant the clear solution and ascertain its exact concentration by the method described under *0.5M ethanolic potassium hydroxide VS*.

1M Potassium Hydroxide VS

Dissolve 60 g of *potassium hydroxide* in sufficient *carbon dioxide-free water* to produce 1000 mL. Ascertain its exact concentration in the following manner. Titrate 20 mL of the solution with 1M *hydrochloric acid VS* using 0.5 mL of *phenolphthalein solution* as indicator. Each mL of 1M *hydrochloric acid VS* is equivalent to 56.11 mg of KOH.

0.1M Potassium Hydroxide VS

Dissolve 6 g of *potassium hydroxide R* in *carbon dioxide-free water R* and dilute to 1000.0 mL with the same solvent.

Standardisation. Dissolve 0.150 g of *potassium hydrogen phthalate RV* in 50 mL of *water R*. Titrate with the potassium hydroxide solution, determining the end-point potentiometrically (*2.2.20*) or using 0.1 mL of *phenolphthalein solution R* as indicator.

1 mL of *0.1 M potassium hydroxide* is equivalent to 20.42 mg of $C_8H_5KO_4$.

0.5M Potassium Hydroxide, Ethanolic VS 0.5M Potassium hydroxide, alcoholic

Dissolve 3 g of *potassium hydroxide R* in 5 mL of *water R* and dilute to 100.0 mL with *aldehyde-free alcohol R*.

Standardisation. Dissolve 0.500 g of *benzoic acid RV* in 10 mL of *water R* and 40 mL of *ethanol (96 per cent) R*. Titrate with the potassium hydroxide solution, determining the end-point potentiometrically (*2.2.20*) or using 0.1 mL of *phenolphthalein solution R* as indicator.

1 mL of *0.5 M alcoholic potassium hydroxide* is equivalent to 61.06 mg of $C_7H_6O_2$.

Dilution. Use *aldehyde-free alcohol R*.

0.1M Potassium Hydroxide, Ethanolic VS 0.1M Potassium hydroxide, alcoholic

Dissolve 6 g of *potassium hydroxide* in 50 mL of *water* and dilute to 1000 mL with *aldehyde-free ethanol (96%)*.

0.01M Potassium Hydroxide, Ethanolic VS 0.01M Potassium hydroxide, alcoholic

Dilute 2.0 mL of 0.5M *ethanolic potassium hydroxide* to 100.0 mL with *aldehyde-free ethanol (96%)*).

0.05M Potassium Iodate VS

Dissolve 10.70 g of *potassium iodate R* in *water R* and dilute to 1000.0 mL with the same solvent.

Standardisation. To 3.0 mL of the potassium iodate solution add 40.0 mL of *water R*, 1 g of *potassium iodide R* and 5 mL of *dilute sulfuric acid R*. Titrate with *0.1 M sodium thiosulfate*, determining the end-point potentiometrically (*2.2.20*) or using 1 mL of *starch solution R*, added towards the end of the titration, as indicator.

1 mL of *0.1 M sodium thiosulfate* is equivalent to 3.567 mg of KIO_3.

0.001M Potassium Iodide VS

Dilute 10.0 mL of *potassium iodide solution R* to 100.0 mL with *water R*. Dilute 5.0 mL of this solution to 500.0 mL with *water R*.

0.02M Potassium Permanganate VS

Dissolve 3.2 g of *potassium permanganate R* in *water R* and dilute to 1000.0 mL with the same solvent. Heat the solution for 1 h on a water-bath, allow to cool and filter through a sintered-glass filter (*2.1.2*).

Standardisation. Dissolve 0.300 g of *ferrous ethylenediammonium sulfate RV* in 50 mL of a diluted solution of *sulfuric acid R* (49 g/L H_2SO_4). Titrate with the potassium permanganate solution, determining the end-point potentiometrically (*2.2.20*) or by the colour of the solution changing to pink. Standardise immediately before use.

1 mL of *0.02 M potassium permanganate* is equivalent to 38.21 mg of $Fe(C_2H_{10}N_2)(SO_4)_2,4H_2O$.

Storage: protected from light.

0.1M Silver Nitrate VS

Dissolve 17.0 g of *silver nitrate R* in *water R* and dilute to 1000.0 mL with the same solvent.

Standardisation. Dissolve 50 mg of *sodium chloride RV* in *water R*, add 5 mL of *dilute nitric acid R* and dilute to 50 mL with *water R*. Titrate with the silver nitrate solution, determining the end-point potentiometrically (*2.2.20*).

1 mL of *0.1 M silver nitrate* is equivalent to 5.844 mg of NaCl.

Storage: protected from light.

0.001M Silver Nitrate VS

Dilute 5 mL of *0.1M silver nitrate VS* to 500 mL with water.

0.1M Sodium Arsenite VS

Dissolve *arsenious trioxide RV* equivalent to 4.946 g of As_2O_3 in a mixture of 20 mL of *strong sodium hydroxide solution R* and 20 mL of *water R*, dilute to 400 mL with *water R* and add *dilute hydrochloric acid R* until the solution is neutral to *blue litmus paper R*. Dissolve 2 g of *sodium hydrogen carbonate R* in the solution and dilute to 500.0 mL with *water R*.

Sodium Dodecyl Sulfate VS Sodium Dodecyl Sulphate VS; $C_{12}H_{25}NaO_4S$ = 288.4

For a 0.001M solution Dissolve 0.2884 g of *sodium dodecyl sulfate*, calculated with reference to the substance dried at 105° for 2 hours, in sufficient *water* to produce 1000 mL.

Ascertain its exact concentration in the following manner. To 50 mL add 15 mL of *chloroform*, 10 mL of 1M *sulfuric acid* and 1 mL of a solution containing 0.003% w/v of each of *dimethyl yellow* and *oracet blue 2R* in *chloroform* and titrate with 0.004M *benzethonium chloride VS*, shaking vigorously and allowing the layers to separate after each addition, until the chloroform layer acquires a permanent clear green colour. Each mL of 0.004M *benzethonium chloride VS* is equivalent to 1.154 mg of $C_{12}H_{25}NaO_4S$.

1M Sodium Hydroxide VS

Dissolve 42 g of *sodium hydroxide R* in *carbon dioxide-free water R* and dilute to 1000.0 mL with the same solvent.

Standardisation. Dissolve 1.50 g of *potassium hydrogen phthalate RV* in 50 mL of *water R*. Titrate with the sodium hydroxide solution, determining the end-point potentiometrically (*2.2.20*) or using 0.1 mL of *phenolphthalein solution R* as indicator.

1 mL of *1 M sodium hydroxide* is equivalent to 204.2 mg of $C_8H_5KO_4$.

If sodium hydroxide free from carbonate is prescribed, prepare it as follows. Dissolve *sodium hydroxide R* in *water R* to give a concentration of 400-600 g/L and allow to stand. Decant the clear supernatant, taking precautions to avoid the introduction of carbon dioxide, and dilute with *carbon dioxide-free water R* to the required molarity. The solution complies with the following test. Titrate 20.0 mL of hydrochloric acid of the same molarity with the solution of sodium hydroxide, using 0.1 mL of *phenolphthalein solution R* as indicator. At the end-point add just sufficient of the acid to discharge the pink colour and concentrate the solution to 20 mL by boiling. During boiling add just sufficient acid to discharge the pink colour, which should not reappear after prolonged boiling. The volume of acid used does not exceed 0.1 mL.

0.1M Sodium Hydroxide VS

Dilute 100.0 mL of *1 M sodium hydroxide* to 1000.0 mL with *carbon dioxide-free water R*.

Standardisation. Carry out the titration described for *1 M sodium hydroxide* using 0.150 g of *potassium hydrogen phthalate RV* in 50 mL of *water R.*

1 mL of *0.1 M sodium hydroxide* is equivalent to 20.42 mg of $C_8H_5KO_4$.

Standardisation (for use in the assay of halide salts of organic bases). Dissolve 0.100 g of *benzoic acid RV* in a mixture of 5 mL of *0.01 M hydrochloric acid* and 50 mL of *ethanol (96 per cent) R.* Carry out the titration (*2.2.20*), using the sodium hydroxide solution. Note the volume added between the 2 points of inflexion.

1 mL of *0.1 M sodium hydroxide* is equivalent to 12.21 mg of $C_7H_6O_2$.

0.1M Sodium Hydroxide, Ethanolic VS

To 250 mL of *anhydrous ethanol R* add 3.3 g of *strong sodium hydroxide solution R.*

Standardisation. Dissolve 0.100 g of *benzoic acid RV* in 10 mL of *water R* and 40 mL of *ethanol (96 per cent) R.* Titrate with the ethanolic sodium hydroxide solution, determining the end-point potentiometrically (*2.2.20*) or using 0.2 mL of *thymolphthalein solution R* as indicator. Standardise immediately before use.

1 mL of *0.1 M ethanolic sodium hydroxide* is equivalent to 12.21 mg of $C_7H_6O_2$.

0.1M Sodium Methoxide VS

Cool 175 mL of *anhydrous methanol R* in iced *water R* and add, in small portions, about 2.5 g of freshly cut *sodium R.* When the metal has dissolved, dilute to 1000.0 mL with *toluene R.*

Standardisation. To 10 mL of *dimethylformamide R* add 0.05 mL of a 3 g/L solution of *thymol blue R* in *methanol R,* and titrate with the sodium methoxide solution until a pure blue colour is obtained. Immediately add 0.100 g of *benzoic acid RV.* Stir until dissolution and titrate with the sodium methoxide solution until the pure blue colour is again obtained. Protect the solution from atmospheric carbon dioxide throughout the titration. From the volume of titrant used in the second titration ascertain the exact strength of the sodium methoxide solution. Standardise immediately before use.

1 mL of *0.1 M sodium methoxide* is equivalent to 12.21 mg of $C_7H_6O_2$.

0.1M Sodium Nitrite VS

Dissolve 7.5 g of *sodium nitrite R* in *water R* and dilute to 1000.0 mL with the same solvent.

Standardisation. Dissolve 0.150 g of *sulfanilic acid RV* in 50 mL of *dilute hydrochloric acid R* and carry out the determination of primary aromatic amino-nitrogen (*2.5.8*), using the sodium nitrite solution and determining the end-point electrometrically. Standardise immediately before use.

1 mL of *0.1 M sodium nitrite* is equivalent to 17.32 mg of $C_6H_7NO_3S$.

0.1M Sodium Periodate VS

Dissolve 21.4 g of *sodium periodate R* in about 500 mL of *water R* and dilute to 1000.0 mL with the same solvent.

Standardisation. In a stoppered flask, introduce 5.0 mL of the sodium periodate solution and add 100 mL of *water R.* Add 10 mL of *potassium iodide solution R* and 5 mL of *hydrochloric acid R1,* close, shake and allow to stand for 2 min. Titrate with *0.1 M sodium thiosulfate* until the yellow colour almost disappears. Determine the end-point potentiometrically (*2.2.20*) or add 2 mL of *starch solution R* and titrate slowly until the colour is completely discharged.

1 mL of *0.1 M sodium thiosulfate* is equivalent to 2.674 mg of $NaIO_4$ or 0.125 mL of *0.1 M sodium periodate.*

Sodium Tetraphenylborate VS $B(C_6H_5)_4Na = 342.2$

For a 0.01M solution Dissolve 3.5 g of *sodium tetraphenylborate* in 50 mL of *water,* shake for 20 minutes with 0.5 g of *aluminium hydroxide gel,* add 250 mL of *water* and 16.6 g of *sodium chloride* and allow to stand for 30 minutes. Filter, add 600 mL of *water,* adjust the pH to 8.0 to 9.0 with 0.1M *sodium hydroxide* and dilute to 1000 mL with *water.*

Ascertain its exact concentration in the following manner. Dissolve 7 mg of *potassium chloride,* previously dried at 150° for 1 hour, in 5 mL of *acetate buffer pH 3.7* and 5 mL of *water,* add 15 mL of the sodium tetraphenylborate solution, allow to stand for 5 minutes and filter through a dry, sintered-glass filter. To 20 mL of the filtrate add 0.5 mL of *bromophenol blue solution* and titrate the excess of sodium tetraphenylborate with 0.005M *cetylpyridinium chloride VS* to the blue colour of the indicator. Repeat the procedure without the potassium chloride. The molarity of the solution is given by the expression:

$$aw/[15(a - b)0.07455]$$

where a is the volume of 0.005M *cetylpyridinium chloride VS* required when the potassium chloride is omitted, b is the volume of 0.005M *cetylpyridinium chloride VS* required when the potassium chloride is present and w is the weight, in g, of potassium chloride taken.

0.1M Sodium Thiosulfate VS

Dissolve 25 g of *sodium thiosulfate R* and 0.2 g of *sodium carbonate R* in *carbon dioxide-free water R* and dilute to 1000.0 mL with the same solvent.

Standardisation. To 10.0 mL of *0.033 M potassium bromate,* add 40 mL of *water R,* 10 mL of *potassium iodide solution R* and 5 mL of *hydrochloric acid R1.* Titrate with the sodium thiosulfate solution, using 1 mL of *starch solution R,* added towards the end of the titration, as indicator.

1 mL of *0.1 M sodium thiosulfate* is equivalent to 2.783 mg of $KBrO_3$ or 0.5 mL of *0.033 M potassium bromate.*

0.5M Sulfuric Acid VS 0.5M Sulphuric Acid VS

Dissolve 28 mL of *sulfuric acid R* in *water R* and dilute to 1000.0 mL with the same solvent.

Standardisation. Dissolve 0.950 g of *trometamol RV* in 50 mL of *water R.* Titrate with the sulfuric acid solution, determining the end-point potentiometrically (*2.2.20*) or using 0.1 mL of *methyl orange solution R* as indicator until the solution turns reddish-yellow.

1 mL of *0.5 M sulfuric acid* is equivalent to 121.1 mg of $C_4H_{11}NO_3$.

0.05M Sulfuric Acid VS 0.05M Sulphuric Acid VS

Dilute 100 mL of *0.5M Sulfuric Acid VS* to 1000 mL with water.

Use the exact concentration as ascertained for *0.5M Sulfuric Acid VS.*

0.1M Tetrabutylammonium Hydroxide VS

Dissolve 40 g of *tetrabutylammonium iodide R* in 90 mL of *anhydrous methanol R,* add 20 g of finely powdered *silver oxide R* and shake vigorously for 1 h. Centrifuge a few millilitres of the mixture and test the supernatant for iodides. If a positive reaction is obtained, add an additional 2 g of *silver oxide R* and shake for a further 30 min. Repeat this procedure until the liquid is free from iodides, filter the mixture through a fine sintered-glass filter (*2.1.2*) and rinse

the reaction vessel and filter with three quantities, each of 50 mL, of *toluene R*. Add the washings to the filtrate and dilute to 1000.0 mL with *toluene R*. Pass dry carbon dioxide-free nitrogen through the solution for 5 min.

Standardisation. To 10 mL of *dimethylformamide R* add 0.05 mL of a 3 g/L solution of *thymol blue R* in *methanol R* and titrate with the tetrabutylammonium hydroxide solution until a pure blue colour is obtained. Immediately add 0.100 g of *benzoic acid RV*. Stir to effect solution, and titrate with the tetrabutylammonium hydroxide solution until the pure blue colour is again obtained. Protect the solution from atmospheric carbon dioxide throughout the titration. From the volume of titrant used in the second titration ascertain the exact strength of the tetrabutylammonium hydroxide solution. Standardise immediately before use.

1 mL of *0.1 M tetrabutylammonium hydroxide* is equivalent to 12.21 mg of $C_7H_6O_2$.

0.1M Tetrabutylammonium Hydroxide in 2-propanol VS

Prepare as described for *0.1 M tetrabutylammonium hydroxide* using *2-propanol R* instead of *toluene R* and standardise as described.

Tetrabutylammonium Iodide VS $C_{16}H_{36}IN = 369.4$

For a 0.01M solution Dissolve 4 g of *tetrabutylammonium iodide* in sufficient *water* to produce 1000 mL.

Ascertain its exact concentration in the following manner. To 25 mL add 50 mL of 0.01M *silver nitrate VS* and 0.5 mL of 2M *nitric acid* and titrate the excess of silver nitrate with *0.01M ammonium thiocyanate VS* using *ammonium iron(III) sulfate solution R1* as indicator. Each mL of 0.01M *silver nitrate VS* is equivalent to 3.694 mg of $C_{16}H_{36}IN$.

Titanium(III) Chloride VS $TiCl_3 = 154.3$

For a 0.1M solution Dilute 100 mL of *titanium(III) chloride solution* with 200 mL of *hydrochloric acid* and add sufficient freshly boiled and cooled *water* to produce 1000 mL.

Ascertain its exact concentration immediately before use by titrating with it, in an atmosphere of carbon dioxide, 25 mL of 0.1M *ammonium iron(III) sulfate VS* acidified with *sulfuric acid*, using *ammonium thiocyanate solution*, added just before the end point, as indicator. Each mL of 0.1M *ammonium iron(III) sulfate VS* is equivalent to 15.43 mg of $TiCl_3$.

0.05M Zinc Chloride VS

Dissolve 6.82 g of *zinc chloride R*, weighed with appropriate precautions, in *water R*. If necessary, add dropwise *dilute hydrochloric acid R* until the opalescence disappears. Dilute to 1000.0 mL with *water R*.

Standardisation. To 20.0 mL of the zinc chloride solution add 5 mL of *dilute acetic acid R* and carry out the determination of zinc by complexometry (*2.5.11*).

0.1M Zinc Sulfate VS 0.1M Zinc Sulphate VS

Dissolve 29 g of *zinc sulfate R* in *water R* and dilute to 1000.0 mL with the same solvent.

Standardisation. To 20.0 mL of the zinc sulfate solution add 5 mL of *dilute acetic acid R* and carry out the determination of zinc by complexometry (*2.5.11*).

C. Standard Solutions

The following solutions are used as reference standards in limit tests and should, unless experience has shown it to be unnecessary, be prepared immediately before use.

In monographs of the European Pharmacopoeia, the symbol '%' may be replaced by the words 'per cent'. In such cases the reagent described below is to be used.

Acetaldehyde Standard Solution (100 ppm C_2H_4O)

Dissolve 1.0 g of *acetaldehyde R* in *2-propanol R* and dilute to 100.0 mL with the same solvent. Dilute 5.0 mL of the solution to 500.0 mL with *2-propanol R*. Prepare immediately before use.

Acetaldehyde Standard Solution (100 ppm C_2H_4O) R1

Dissolve 1.0 g of *acetaldehyde R* in *water R* and dilute to 100.0 mL with the same solvent. Dilute 5.0 mL of the solution to 500.0 mL with *water R*. Prepare immediately before use.

Aluminium Standard Solution (200 ppm Al)

Dissolve in *water R* a quantity of *aluminium potassium sulfate R* equivalent to 0.352 g of $AlK(SO_4)_2,12H_2O$. Add 10 mL of *dilute sulfuric acid R* and dilute to 100.0 mL with *water R*.

Aluminium Standard Solution (100 ppm Al)

Immediately before use, dilute with *water R* to 10 times its volume a solution containing 8.947 g of *aluminium chloride R* in 1000.0 mL of *water R*.

Aluminium Standard Solution (25 ppm Al)

Immediately before use, dilute with *water* to 40 times its volume a solution containing 8.947 g of *aluminium chloride* in 1000.0 mL of *water*.

Aluminium Standard Solution (10 ppm Al)

Immediately before use, dilute with *water R* to 100 times its volume in a solution containing *aluminium nitrate R* equivalent to 1.39 g of $Al(NO_3)_3,9H_2O$ in 100.0 mL.

Aluminium Standard Solution (5 ppm Al)

Immediately before use, dilute with *water R* to 100 times its volume in a solution containing *aluminium nitrate R* equivalent to 0.695 g of $Al(NO_3)_3,9H_2O$ in 100.0 mL. Alternatively, use a commercially available standard solution containing a known amount of aluminium (5 ppm Al).

Aluminium Standard Solution (2 ppm Al)

Immediately before use, dilute with *water R* to 100 times its volume a solution containing *aluminium potassium sulfate R* equivalent to 0.352 g of $AlK(SO_4)_2,12H_2O$ and 10 mL of *dilute sulfuric acid R* in 100.0 mL.

Ammonium Standard Solution (100 ppm NH4)

Immediately before use, dilute to 25 mL with *water R* 10 mL of a solution containing *ammonium chloride R* equivalent to 0.741 g of NH_4Cl in 1000 mL.

Ammonium Standard Solution (3 ppm NH4)

Immediately before use, dilute with *water R* to 100 times its volume a solution containing *ammonium chloride R* equivalent to 0.889 g of NH_4Cl in 1000.0 mL.

Ammonium Standard Solution (2.5 ppm NH4)

Immediately before use, dilute with *water R* to 100 times its volume a solution containing *ammonium chloride R* equivalent to 0.741 g of NH_4Cl in 1000.0 mL.

Ammonium Standard Solution (1 ppm NH4)

Immediately before use, dilute *ammonium standard solution (2.5 ppm NH_4) R* to 2.5 times its volume with *water R*.

Antimony Standard Solution (100 ppm Sb)

Dissolve *antimony potassium tartrate R* equivalent to 0.274 g of $C_8H_4K_2O_{12}Sb_2,3H_2O$ in 500 mL of *1 M hydrochloric acid* and dilute the clear solution to 1000 mL with *water R*.

Antimony Standard Solution (1 ppm Sb)

Dissolve *antimony potassium tartrate R* equivalent to 0.274 g of $C_8H_4K_2O_{12}Sb_2,3H_2O$ in 20 mL of *hydrochloric acid R1* and dilute the clear solution to 100.0 mL with *water R*. To 10.0 mL of this solution add 200 mL of *hydrochloric acid R1* and dilute to 1000.0 mL with *water R*. To 100.0 mL of this solution add 300 mL of *hydrochloric acid R1* and dilute to 1000.0 mL with *water R*. Prepare the dilute solutions immediately before use.

Arsenic Standard Solution (10 ppm As)

Immediately before use, dilute with *water R* to 100 times its volume a solution prepared by dissolving *arsenious trioxide R* equivalent to 0.330 g of As_2O_3 in 5 mL of *dilute sodium hydroxide solution R* and diluting to 250.0 mL with *water R*.

Arsenic Standard Solution (1 ppm As)

Immediately before use, dilute *arsenic standard solution (10 ppm As) R* to 10 times its volume with *water R*.

Arsenic Standard Solution (0.1 ppm As)

Immediately before use, dilute *arsenic standard solution (1 ppm As) R* to 10 times its volume with *water R*.

Barium Standard Solution (0.1% Ba)

Dissolve *barium chloride R* equivalent to 0.178 g of $BaCl_2,2H_2O$ in *distilled water R* and dilute to 100.0 mL with the same solvent.

Barium Standard Solution (50 ppm Ba)

Immediately before use, dilute with *distilled water R* to 20 times its volume a solution in *distilled water R* containing *barium chloride R* equivalent to 0.178 g of $BaCl_2,2H_2O$ in 100.0 mL.

Barium Standard Solution (2 ppm Ba)

Immediately before use, dilute *barium standard solution (50 ppm Ba) R* to 25 times its volume with *distilled water R*.

Bismuth Standard Solution (100 ppm Bi)

Dissolve *bismuth subnitrate R* equivalent to 0.500 g of Bi in 50 mL of *nitric acid R* and dilute to 500.0 mL with *water R*. Dilute the solution to 10 times its volume with *dilute nitric acid R* immediately before use.

Cadmium Standard Solution (0.1% Cd)

Dissolve *cadmium R* equivalent to 0.100 g of Cd in the smallest necessary amount of a mixture of equal volumes of *hydrochloric acid R* and *water R* and dilute to 100.0 mL with a 1 per cent *V/V* solution of *hydrochloric acid R*.

Cadmium Standard Solution (10 ppm Cd)

Immediately before use, dilute *cadmium standard solution (0.1 per cent Cd) R* to 100 times its volume with a 1 per cent *V/V* solution of *hydrochloric acid R*.

Calcium Standard Solution (1000 ppm Ca)

Dissolve 2.5 g of *calcium carbonate* in 23 mL of 1M *hydrochloric acid* and add sufficient *distilled water* to produce 100 mL. Dilute 1 volume of this solution to 10 volumes with *distilled water* immediately before use.

Calcium Standard Solution (400 ppm Ca)

Immediately before use, dilute with *distilled water R* to 10 times its volume a solution in *distilled water R* containing *calcium carbonate R* equivalent to 1.000 g of $CaCO_3$ and 23 mL of *1 M hydrochloric acid* in 100.0 mL.

Calcium Standard Solution (100 ppm Ca)

Immediately before use, dilute with *distilled water R* to 10 times its volume a solution in *distilled water R* containing *calcium carbonate R* equivalent to 0.624 g of $CaCO_3$ and 3 mL of *acetic acid R* in 250.0 mL.

Calcium Standard Solution (100 ppm Ca), Alcoholic

Immediately before use, dilute with *ethanol (96 per cent) R* to 10 times its volume a solution in *distilled water R* containing *calcium carbonate R* equivalent to 2.50 g of $CaCO_3$ and 12 mL of *acetic acid R* in 1000.0 mL.

Calcium Standard Solution (100 ppm Ca) R1

Immediately before use, dilute with *water R* to 10 times its volume a solution containing *anhydrous calcium chloride R* equivalent to 2.769 g of $CaCl_2$ in 1000.0 mL of *dilute hydrochloric acid R*.

Calcium Standard Solution (10 ppm Ca)

Immediately before use, dilute with *distilled water R* to 100 times its volume a solution in *distilled water R* containing *calcium carbonate R* equivalent to 0.624 g of $CaCO_3$ and 3 mL of *acetic acid R* in 250.0 mL.

Chloride Standard Solution (50 ppm Cl)

Immediately before use, dilute with *water R* to 10 times its volume a solution containing *sodium chloride R* equivalent to 0.824 g of NaCl in 1000.0 mL.

Chloride Standard Solution (8 ppm Cl)

Immediately before use, dilute with *water R* to 100 times its volume a solution containing *sodium chloride R* equivalent to 1.32 g of NaCl in 1000.0 mL.

Chloride Standard Solution (5 ppm Cl)

Immediately before use, dilute with *water R* to 100 times its volume a solution containing *sodium chloride R* equivalent to 0.824 g of NaCl in 1000.0 mL.

Chromium Liposoluble Standard Solution (1000 ppm Cr)

A chromium (metal) organic compound in an oil.

Chromium Standard Solution (0.1% Cr)

Dissolve *potassium dichromate R* equivalent to 2.83 g of $K_2Cr_2O_7$ in *water R* and dilute to 1000.0 mL with the same solvent.

Chromium Standard Solution (100 ppm Cr)

Dissolve *potassium dichromate R* equivalent to 0.283 g of $K_2Cr_2O_7$ in *water R* and dilute to 1000.0 mL with the same solvent.

Chromium Standard Solution (0.1 ppm Cr)

Immediately before use, dilute *chromium standard solution (100 ppm Cr) R* to 1000 times its volume with *water R*.

Cobalt Standard Solution (100 ppm Co)

Dissolve *cobalt nitrate R* equivalent to 0.494 g of $Co(NO_3)_2,6H_2O$ in 500 mL of *1 M nitric acid* and dilute the clear solution to 1000 mL with *water R*.

Copper Liposoluble Standard Solution (1000 ppm Cu)

A copper (metal) organic compound in an oil.

Copper Standard Solution (0.1% Cu)

Dissolve *copper sulfate pentahydrate R* equivalent to 0.393 g of $CuSO_4,5H_2O$ in *water R* and dilute to 100.0 mL with the same solvent.

Copper Standard Solution (10 ppm Cu)

Immediately before use, dilute *copper standard solution (0.1 per cent Cu) R* to 100 times its volume with *water R*.

Copper Standard Solution (0.1 ppm Cu)

Immediately before use, dilute *copper standard solution (10 ppm Cu) R* to 100 times its volume with *water R*.

Copper Standard Solution (0.1 per cent Cu) for ICP

A copper standard solution (1000 mg/L) suitable for inductively coupled plasma (ICP) applications and traceable to national or international standards.

Elementary Standard Solutions for Atomic Spectrometry, 1.000 g/L

This solution is prepared, generally in acid conditions, from the element or a salt of the element whose minimum content is not less than 99.0 per cent. The quantity per litre of solution is greater than 0.995 g throughout the guaranteed period, as long as the vial has not been opened. The starting material (element or salt) and the characteristics of the final solvent (nature and acidity, etc.) are mentioned on the label.

Ferricyanide Standard Solution (50 ppm $Fe(CN)_6$)

Immediately before use, dilute with *water R* to 100 times its volume a solution containing *potassium ferricyanide R* equivalent to 0.78 g of $K_3Fe(CN)_6$ in 100.0 mL.

Ferrocyanide Standard Solution (100 ppm $Fe(CN)_6$)

Immediately before use, dilute with *water R* to 10 times its volume a solution containing *potassium ferrocyanide R* equivalent to 0.20 g of $K_4Fe(CN)_6,3H_2O$ in 100.0 mL.

Fluoride Standard Solution (10 ppm F)

Dissolve in *water R sodium fluoride R* previously dried at 300 °C for 12 h, equivalent to 0.442 g of NaF, and dilute to 1000.0 mL with the same solvent (1 mL = 0.2 mg F). Store in a polyethylene container. Immediately before use, dilute the solution to 20 times its volume with *water R*.

Fluoride Standard Solution (1 ppm F)

Immediately before use, dilute *fluoride standard solution (10 ppm F) R* to 10 times its volume with *water R*.

Formaldehyde Standard Solution (5 ppm CH_2O)

Immediately before use, dilute with *water R* to 200 times its volume a solution containing 1.0 g of CH_2O per litre prepared from *formaldehyde solution R*.

Germanium Standard Solution (100 ppm Ge)

Dissolve *ammonium hexafluorogermanate(IV) R* equivalent to 0.307 g of $(NH_4)_2GeF_6$ in a 0.01 per cent *V/V* solution of *hydrofluoric acid R*. Dilute the clear solution to 1000 mL with *water R*.

Glucose Standard Solution

Dissolve 0.10 g of *glucose* in a saturated solution of *benzoic acid* in *water*, dilute to 100 mL with the saturated benzoic acid solution and dilute 2 mL of this solution to 100 mL with *water*.

Glucose Standard Solution contains 20 µg of glucose per mL.

Glyoxal Standard Solution (20 ppm $C_2H_2O_2$)

In a 100 mL graduated flask weigh a quantity of *glyoxal solution R* corresponding to 0.200 g of $C_2H_2O_2$ and make up to volume with *anhydrous ethanol R*. Immediately before use dilute the solution to 100 times its volume with the same solvent.

Glyoxal Standard Solution (2 ppm $C_2H_2O_2$)

Immediately before use, dilute *glyoxal standard solution (20 ppm $C_2H_2O_2$) R* to 10 times its volume with *anhydrous ethanol R*.

Hydrogen Peroxide Standard Solution (2 ppm H_2O_2)

Dilute 10.0 mL of *dilute hydrogen peroxide solution R* to 300.0 mL with *water R*. Dilute 2.0 mL of this solution to 1000.0 mL with *water R*. Prepare immediately before use.

Iodide Standard Solution (20 ppm I)

Dilute 10.0 mL of a 0.026% w/v solution of *potassium iodide* to 100.0 mL with *water*.

Iodide Standard Solution (10 ppm I)

Immediately before use, dilute with *water R* to 100 times its volume a solution containing *potassium iodide R* equivalent to 0.131 g of KI in 100.0 mL.

Iron Standard Solution (0.1% Fe)

Dissolve 0.100 g of Fe in the smallest amount necessary of a mixture of equal volumes of *hydrochloric acid R* and *water R* and dilute to 100.0 mL with *water R*.

Iron Standard Solution (250 ppm Fe)

Immediately before use, dilute with *water R* to 40 times its volume a solution containing 4.840 g of *ferric chloride R* in a 150 g/L solution of *hydrochloric acid R* diluted to 100.0 mL.

Iron Standard Solution (20 ppm Fe)

Immediately before use, dilute with *water R* to 10 times its volume a solution containing *ferric ammonium sulfate R* equivalent to 0.863 g of $FeNH_4(SO_4)_2,12H_2O$ and 25 mL of *dilute sulfuric acid R* in 500.0 mL.

Iron Standard Solution (10 ppm Fe)

Immediately before use, dilute with *water R* to 100 times its volume a solution containing *ferrous ammonium sulfate R* equivalent to 7.022 g of $Fe(NH_4)_2(SO_4)_2,6H_2O$ and 25 mL of *dilute sulfuric acid R* in 1000.0 mL.

Iron Standard Solution (8 ppm Fe)

Immediately before use, dilute with *water R* to 10 times its volume a solution containing 80 mg of *iron R* and 50 mL of *hydrochloric acid R* (220 g/L HCl) in 1000.0 mL.

Iron Standard Solution (2 ppm Fe)

Immediately before use, dilute *iron standard solution (20 ppm Fe) R* to 10 times its volume with *water R*.

Iron Standard Solution (1 ppm Fe)

Immediately before use, dilute *iron standard solution (20 ppm Fe) R* to 20 times its volume with *water R*.

Lead Liposoluble Standard Solution (1000 ppm Pb)

A lead (metal) organic compound in an oil.

Lead Standard Solution (0.1% Pb)

Dissolve *lead nitrate R* equivalent to 0.400 g of $Pb(NO_3)_2$ in *water R* and dilute to 250.0 mL with the same solvent.

Lead Standard Solution (100 ppm Pb)

Immediately before use, dilute *lead standard solution (0.1 per cent Pb) R* to 10 times its volume with *water R*.

Lead Standard Solution (20 ppm Pb)

Dissolve 0.80 g of *lead(II) nitrate* in *water* containing 2 mL of *nitric acid* and add sufficient *water* to produce 250 mL. Dilute 1 volume to 100 volumes with *water* immediately before use.

Lead Standard Solution (10 ppm Pb)

Immediately before use, dilute *lead standard solution (100 ppm Pb) R* to 10 times its volume with *water R*.

Lead Standard Solution (10 ppm Pb) R1

Immediately before use, dilute with *water R* to 10 times its volume a solution containing 0.160 g of *lead nitrate R* in 100 mL of *water R*, to which is added 1 mL of *lead-free nitric acid R* and dilute to 1000.0 mL.

Lead Standard Solution (2 ppm Pb)

Immediately before use, dilute *lead standard solution (10 ppm Pb) R* to 5 times its volume with *water R*.

Lead Standard Solution (1 ppm Pb)

Immediately before use, dilute *lead standard solution (10 ppm Pb) R* to 10 times its volume with *water R*.

Lead Standard Solution (0.25 ppm Pb)

Immediately before use, dilute *lead standard solution (1 ppm Pb) R* to 4 times its volume with *water R*.

Lead Standard Solution (0.1 ppm Pb)

Immediately before use, dilute *lead standard solution (1 ppm Pb) R* to 10 times its volume with *water R*.

Lithium Standard Solution (100 ppm Li)

Dissolve 0.6109 g of *lithium chloride* in sufficient *water* to produce 1000 mL.

Lutetium Standard Solution (20 ppm Lu)

Immediately before use, dissolve 0.445 g of *lutetium chloride hexahydrate R* in a mixture of equal volumes of *heavy metal-free nitric acid R* and *water R*, and dilute to 100.0 mL with the same mixture of solvents.

Dilute 1.0 mL of this solution to 100.0 mL with *water R*.

Magnesium Standard Solution (0.1% Mg)

Dissolve *magnesium sulfate R* equivalent to 1.010 g of $MgSO_4,7H_2O$ in *distilled water R* and dilute to 100.0 mL with the same solvent.

Magnesium Standard Solution (1000 ppm Mg)

Dissolve 5.275 g of *magnesium nitrate R* in 16 mL of *dilute nitric acid R* and dilute to 500.0 mL with *water R*.

Standardisation: carry out the determination of magnesium by complexometry (*2.5.11*).

Magnesium Standard Solution (100 ppm Mg)

Immediately before use, dilute with *water R* to 10 times its volume a solution containing *magnesium sulfate R* equivalent to 1.010 g of $MgSO_4,7H_2O$ in 100.0 mL.

Magnesium Standard Solution (10 ppm Mg)

Immediately before use, dilute *magnesium standard solution (100 ppm Mg) R* to 10 times its volume with *water R*.

Magnesium Standard Solution (10 ppm Mg) R1

Immediately before use, dilute with *water R* to 100 times its volume a solution containing 8.365 g of *magnesium chloride R* in 1000.0 mL of *dilute hydrochloric acid R*.

Manganese Standard Solution (1000 ppm Mn)

Dissolve *manganese sulfate R* equivalent to 3.08 g of $MnSO_4$, H_2O in 500 mL of *1 M nitric acid* and dilute the solution to 1000 mL with *water R*.

Manganese Standard Solution (100 ppm Mn)

Dissolve *manganese sulfate R* equivalent to 0.308 g of $MnSO_4,H_2O$ in 500 mL of *1 M nitric acid* and dilute the clear solution to 1000 mL with *water R*.

Mercury Standard Solution (1000 ppm Hg)

Dissolve *mercuric chloride R* equivalent to 1.354 g of $HgCl_2$ in 50 mL of *dilute nitric acid R* and dilute to 1000.0 mL with *water R*.

Mercury Standard Solution (100 ppm Hg)

Dissolve 1.080 g of *yellow mercury(II) oxide* in the minimum volume of 2M *hydrochloric acid* and add sufficient *water* to produce 1000 mL.

Mercury Standard Solution (10 ppm Hg)

Immediately before use, dilute with water to 100 times its volume a solution containing *mercuric chloride R* equivalent to 0.338 g of $HgCl_2$ in 250.0 mL.

Mercury Standard Solution (5 ppm Hg)

Dilute 1.0 mL of a 0.0675% w/v solution of *mercury(II) chloride* to 100.0 mL with *water*.

Nickel Liposoluble Standard Solution (1000 ppm Ni)

A nickel (metal) organic compound in an oil.

Nickel Standard Solution (10 ppm Ni)

Immediately before use, dilute with *water R* to 100 times its volume a solution containing *nickel sulfate R* equivalent to 4.78 g of $NiSO_4,7H_2O$ in 1000.0 mL.

Nickel Standard Solution (5 ppm Ni)

Immediately before use dilute *nickel standard solution (10 ppm Ni) R* to twice its volume with *water for chromatography R*.

Nickel Standard Solution (0.2 ppm Ni)

Immediately before use, dilute *nickel standard solution (10 ppm Ni) R* to 50 times its volume with *water R*.

Nickel Standard Solution (0.1 ppm Ni)

Immediately before use, dilute *nickel standard solution (10 ppm Ni) R* to 100 times its volume with *water R*.

Nitrate Standard Solution (100 ppm NO_3)

Immediately before use, dilute with *water R* to 10 times its volume a solution containing *potassium nitrate R* equivalent to 0.815 g of KNO_3 in 500.0 mL.

Nitrate Standard Solution (10 ppm NO_3)

Immediately before use, dilute *nitrate standard solution (100 ppm NO_3) R* to 10 times its volume with *water R*.

Nitrate Standard Solution (2 ppm NO_3)

Immediately before use, dilute *nitrate standard solution (10 ppm NO_3) R* to 5 times its volume with *water R*.

Nitrite Standard Solution (20 ppm NO_2)

Dissolve 0.6 g of *sodium nitrite* in sufficient *water* to produce 100 mL and dilute 1 mL of this solution to 200 mL with *water*.

Palladium Standard Solution (500 ppm Pd)

Dissolve 50.0 mg of *palladium R* in 9 mL of *hydrochloric acid R* and dilute to 100.0 mL with *water R*.

Palladium Standard Solution (20 ppm Pd)

Dissolve 0.333 g of *palladium chloride R* in 2 mL of warm *hydrochloric acid R*. Dilute the solution to 1000.0 mL with a mixture of equal volumes of *dilute hydrochloric acid R* and *water R*. Immediately before use dilute to 10 times its volume with *water R*.

Palladium Standard Solution (0.5 ppm Pd)

Dilute 1 mL of *palladium standard solution (500 ppm Pd) R* to 1000 mL with a mixture of 0.3 volumes of *nitric acid R* and 99.7 volumes of *water R*.

Phosphate Standard Solution (200 ppm PO_4)

Dissolve *potassium dihydrogen phosphate R* equivalent to 0.286 g of KH_2PO_4 in *water R* and dilute to 1000.0 mL with the same solvent.

Phosphate Standard Solution (100 ppm PO_4)

Dilute 10.0 mL of a 0.143% w/v solution of *potassium dihydrogen orthophosphate* to 100.0 mL with *water* immediately before use.

Phosphate Standard Solution (5 ppm PO_4)

Immediately before use, dilute with *water R* to 100 times its volume a solution containing *potassium dihydrogen phosphate R* equivalent to 0.716 g of KH_2PO_4 in 1000.0 mL.

Platinum Standard Solution (30 ppm Pt)

Immediately before use, dilute with *1 M hydrochloric acid* to 10 times its volume a solution containing 80 mg of *chloroplatinic acid R* in 100.0 mL of *1 M hydrochloric acid.*

Potassium Standard Solution (0.2 % K)

Dissolve *dipotassium sulfate R* equivalent to 0.446 g of K_2SO_4 in *distilled water R* and dilute to 100.0 mL with the same solvent.

Potassium Standard Solution (600 ppm K)

Immediately before use, dilute with *water R* to 20 times its volume a solution containing *dipotassium sulfate R* equivalent to 2.676 g of K_2SO_4 in 100.0 mL.

Potassium Standard Solution (100 ppm K)

Immediately before use, dilute with *water R* to 20 times its volume a solution containing *dipotassium sulfate R* equivalent to 0.446 g of K_2SO_4 in 100.0 mL.

Potassium Standard Solution (20 ppm K)

Immediately before use, dilute *potassium standard solution (100 ppm K) R* to 5 times its volume with *water R.*

Scandium Standard Solution (0.1 per cent Sc) for ICP

A scandium standard solution (1000 mg/L) suitable for inductively coupled plasma (ICP) applications and traceable to national or international standards.

Selenium Standard Solution (100 ppm Se)

Dissolve 0.100 g of *selenium R* in 2 mL of *nitric acid R.* Evaporate to dryness. Take up the residue in 2 mL of *water R* and evaporate to dryness; carry out three times. Dissolve the residue in 50 mL of *dilute hydrochloric acid R* and dilute to 1000.0 mL with the same acid.

Selenium Standard Solution (1 ppm Se)

Immediately before use, dilute with *water R* to 40 times its volume a solution containing *selenious acid R* equivalent to 6.54 mg of H_2SeO_3 in 100.0 mL.

Silver Standard Solution (5 ppm Ag)

Immediately before use, dilute with *water R* to 100 times its volume a solution containing *silver nitrate R* equivalent to 0.790 g of $AgNO_3$ in 1000.0 mL.

Sodium Standard Solution (1000 ppm Na)

Dissolve a quantity of *anhydrous sodium carbonate R* equivalent to 2.305 g of Na_2CO_3 in a mixture of 25 mL of *water R* and 25 mL of *nitric acid R* and dilute to 1000.0 mL with *water R.*

Sodium Standard Solution (200 ppm Na)

Immediately before use, dilute with *water R* to 10 times its volume a solution containing *sodium chloride R* equivalent to 0.509 g of NaCl in 100.0 mL.

Sodium Standard Solution (50 ppm Na)

Dilute the *sodium standard solution (200 ppm Na) R* to four times its volume with *water R.*

Strontium Standard Solution (1.0 per cent Sr)

Cover with *water R, strontium carbonate R* equivalent to 1.6849 g of $SrCO_3$. Cautiously add *hydrochloric acid R* until all the solid has dissolved and there is no sign of further effervescence. Dilute to 100.0 mL with *water R.*

Sulfate Standard Solution (100 ppm SO_4)

Immediately before use, dilute with *distilled water R* to 10 times its volume a solution in *distilled water R* containing *dipotassium sulfate R* equivalent to 0.181 g of K_2SO_4 in 100.0 mL.

Sulfate Standard Solution (10 ppm SO_4)

Immediately before use, dilute with *distilled water R* to 100 times its volume a solution in *distilled water R* containing *dipotassium sulfate R* equivalent to 0.181 g of K_2SO_4 in 100.0 mL.

Sulfate Standard Solution (10 ppm SO_4) R1

Immediately before use, dilute with *ethanol (30 per cent V/V) R* to 100 times its volume a solution containing *dipotassium sulfate R* equivalent to 0.181 g of K_2SO_4 in 100.0 mL of *ethanol (30 per cent V/V) R.*

Sulfite Standard Solution (80 ppm SO_2)

Dissolve 3.150 g of *anhydrous sodium sulfite R* in freshly prepared *distilled water R* and dilute to 100.0 mL with the same solvent. Dilute 0.5 mL to 100.0 mL with freshly prepared *distilled water R.*

Sulfite Standard Solution (1.5 ppm SO_2)

Dissolve *sodium metabisulfite R* equivalent to 0.152 g of $Na_2S_2O_5$ in *water R* and dilute to 100.0 mL with the same solvent. Dilute 5.0 mL of this solution to 100.0 mL with *water R.* To 3.0 mL of the resulting solution, add 4.0 mL of *0.1 M sodium hydroxide* and dilute to 100.0 mL with *water R.*

Thallium Standard Solution (10 ppm Tl)

Dissolve *thallous sulfate R* equivalent to 0.1235 g of Tl_2SO_4 in a 9 g/L solution of *sodium chloride R* and dilute to 1000.0 mL with the same solution. Dilute 10.0 mL of the solution to 100.0 mL with the 9 g/L solution of *sodium chloride R.*

Tin Liposoluble Standard Solution (1000 ppm Sn)

A tin (metal) organic compound in an oil.

Tin Standard Solution (5 ppm Sn)

Dissolve *tin R* equivalent to 0.500 g of Sn in a mixture of 5 mL of *water R* and 25 mL of *hydrochloric acid R* and dilute to 1000.0 mL with *water R.* Dilute the solution to 100 times its volume with a 2.5 per cent *V/V* solution of *hydrochloric acid R* immediately before use.

Tin Standard Solution (0.1 ppm Sn)

Immediately before use, dilute *tin standard solution (5 ppm Sn) R* to 50 times its volume with *water R.*

Titanium Standard Solution (100 ppm Ti)

Dissolve 100.0 mg of *titanium R* in 100 mL of *hydrochloric acid R* diluted to 150 mL with *water R,* heating if necessary. Allow to cool and dilute to 1000 mL with *water R.*

Vanadium Standard Solution (1 g/L V)

Dissolve in *water R ammonium vanadate R* equivalent to 0.230 g of NH_4VO_3 and dilute to 100.0 mL with the same solvent.

Zinc Standard Solution (5 mg/mL Zn)

Dissolve 3.15 g of *zinc oxide R* in 15 mL of *hydrochloric acid R* and dilute to 500.0 mL with *water R.*

Zinc Standard Solution (100 ppm Zn)

Immediately before use, dilute with *water R* to 10 times its volume a solution containing *zinc sulfate R* equivalent to 0.440 g of $ZnSO_4,7H_2O$ and 1 mL of *acetic acid R* in 100.0 mL.

Zinc Standard Solution (25 ppm Zn)

Dilute 25.0 mL of *zinc standard solution (100 ppm Zn)* to 100.0 mL with *water* immediately before use.

Zinc Standard Solution (10 ppm Zn)

Immediately before use, dilute *zinc standard solution (100 ppm Zn) R* to 10 times its volume with *water R*.

Zinc Standard Solution (5 ppm Zn)

Immediately before use, dilute *zinc standard solution (100 ppm Zn) R* to 20 times its volume with *water R*.

Zirconium Standard Solution (1 g/L Zr)

Dissolve *zirconyl nitrate R* equivalent to 0.293 g of $ZrO(NO_3)_2,2H_2O$ in a mixture of 2 volumes of *hydrochloric acid R* and 8 volumes of *water R* and dilute to 100.0 mL with the same mixture of solvents.

D. Buffer Solutions

Buffer solutions should be prepared using *carbon dioxide-free water*.

Acetate Buffer pH 2.45

Mix 200 mL of 1M *hydrochloric acid* with 200 mL of 1M *sodium acetate* and dilute to 1000 mL with *water*. Immediately before use adjust the pH to 2.45 by the addition of 1M *hydrochloric acid* or 1M *sodium acetate*, as required.

Acetate Buffer pH 2.8

Dissolve 4 g of *anhydrous sodium acetate* in about 840 mL of *water*, add sufficient *glacial acetic acid* to adjust the pH to 2.8 (about 155 mL) and dilute to 1000 mL with *water*.

Acetate Buffer pH 3.4

Mix 5 volumes of 0.1M *sodium acetate* with 95 volumes of 0.1M *acetic acid*.

Acetate Buffer pH 3.5

Buffer solution pH 3.5

Dissolve 25 g of *ammonium acetate* in 25 mL of *water* and add 38 mL of 7M *hydrochloric acid*. Adjust the pH to 3.5 with either 2M *hydrochloric acid* or 6M *ammonia* and dilute to 100 mL with *water*.

Acetate Buffer pH 3.7

Dissolve 10 g of *anhydrous sodium acetate* in 300 mL of *water*, adjust to pH 3.7 with *glacial acetic acid* and dilute to 1000 mL with *water*. If necessary, readjust to pH 3.7 with *glacial acetic acid* or *anhydrous sodium acetate* as required, before use.

Acetate Buffer pH 4.4 Acetate buffer solution pH 4.4

Dissolve 136 g of *sodium acetate R* and 77 g of *ammonium acetate R* in *water R* and dilute to 1000.0 mL with the same solvent; add 250.0 mL of *glacial acetic acid R* and mix.

Acetate Buffer pH 4.6 Acetate buffer solution pH 4.6

Dissolve 5.4 g of *sodium acetate R* in 50 mL of *water R*, add 2.4 g of *glacial acetic acid R* and dilute to 100.0 mL with *water R*. Adjust the pH if necessary.

Acetate Buffer pH 5.0

Dissolve 13.6 g of *sodium acetate* and 6 mL of *glacial acetic acid* in sufficient *water* to produce 1000 mL.

Acetate Buffer pH 6.0 Acetate buffer solution pH 6.0

Dissolve 100 g of *ammonium acetate R* in 300 mL of *water R*, add 4.1 mL of *glacial acetic acid R*, adjust the pH if necessary using *ammonia R* or *acetic acid R* and dilute to 500.0 mL with *water R*.

Acetate Buffer Solution pH 4.5

Dissolve 77.1 g of *ammonium acetate R* in *water R*. Add 70 mL of *glacial acetic acid R* and dilute to 1000.0 mL with *water R*.

Acetate Buffer Solution pH 4.7

Dissolve 136.1 g of *sodium acetate R* in 500 mL of *water R*. Mix 250 mL of this solution with 250 mL of *dilute acetic acid R*. Shake twice with a freshly prepared, filtered, 0.1 g/L solution of *dithizone R* in *chloroform R*. Shake with *carbon tetrachloride R* until the extract is colourless. Filter the aqueous layer to remove traces of carbon tetrachloride.

Acetate Buffer Solution pH 4.7 R1

Dissolve 136.1 g of *sodium acetate R* in 500 mL of *water R*. Mix 250 mL of this solution with 250 mL of *dilute acetic acid R*.

Acetate–edetate Buffer Solution pH 5.5

Dissolve 250 g of *ammonium acetate R* and 15 g *sodium edetate R* in 400 mL of *water R* and add 125 mL of *glacial acetic acid R*.

Acetone Solution, Buffered

Dissolve 8.15 g of *sodium acetate R* and 42 g of *sodium chloride R* in *water R*, add 68 mL of *0.1 M hydrochloric acid* and 150 mL of *acetone R* and dilute to 500 mL with *water R*.

Ammonia Buffer pH 10.0 Ammonium chloride buffer solution pH 10.0

Dissolve 5.4 g of *ammonium chloride R* in 20 mL of *water R*, add 35.0 mL of *ammonia R* and dilute to 100.0 mL with *water R*.

Ammonia Buffer pH 10.9 Buffer solution pH 10.9

Dissolve 6.75 g of *ammonium chloride R* in *ammonia R* and dilute to 100.0 mL with the same solvent.

Ammonia Buffer pH 10.9, Dilute

Dilute 2 mL of *ammonia buffer pH 10.9* to 1000 mL with *water*.

Ammonium Acetate Buffer pH 4.5, 0.5M

Mix 14.3 mL of *glacial acetic acid R* and 470 mL of *water R* and adjust to pH 4.5 with *concentrated ammonia R*. Dilute to 500.0 mL with *water R*.

Ammonium Carbonate Buffer Solution pH 10.3, 0.1M

Dissolve 7.91 g of *ammonium carbonate R* in 800 mL of *water R*. Adjust the pH with *dilute sodium hydroxide solution R*. Dilute to 1000.0 mL with *water R*.

Ammonium Chloride Buffer Solution pH 9.5

Dissolve 33.5 g of *ammonium chloride R* in 150 mL of *water R*, add 42.0 mL of *concentrated ammonia R* and dilute to 250.0 mL with *water R*.

Storage: in a polyethylene container.

Ammonium Chloride Buffer Solution pH 10.4

Dissolve 70 g of *ammonium chloride R* in 200 mL of *water R*, add 330 mL of *concentrated ammonia R* and dilute to 1000.0 mL with *water R*. If necessary, adjust to pH 10.4 with *ammonia R*.

Ammonium Chloride Buffer Solution pH 10.7

Dissolve 67.5 g of *ammonium chloride R* in *water R*, add 570 mL of *concentrated ammonia R* and dilute to 1000.0 mL with *water R*.

Barbitone Buffer pH 7.4 Barbital buffer solution pH 7.4

Mix 50 mL of a solution in *water R* containing 19.44 g/L of *sodium acetate R* and 29.46 g/L of *barbital sodium R* with

50.5 mL of *0.1 M hydrochloric acid*, add 20 mL of an 85 g/L of *sodium chloride R* and dilute to 250 mL with *water R*.

Barbitone Buffer pH 8.4 Barbital buffer solution pH 8.4

Dissolve 8.25 g of *barbital sodium R* in *water R* and dilute to 1000.0 mL with the same solvent.

Barbitone Buffer pH 8.6 R1 Barbital buffer solution pH 8.6 R1

Dissolve in *water R* 1.38 g of *barbital R*, 8.76 g of *barbital sodium R* and 0.38 g of *calcium lactate pentahydrate R* and dilute to 1000.0 mL with the same solvent.

Borate Buffer pH 7.5 Borate buffer solution pH 7.5

Dissolve 2.5 g of *sodium chloride R*, 2.85 g of *disodium tetraborate R* and 10.5 g of *boric acid R* in *water R* and dilute to 1000.0 mL with the same solvent. Adjust the pH if necessary.

Storage: at 2 °C to 8 °C.

Borate Buffer pH 8.0

To 50 mL of a solution containing 0.6189 g of *boric acid* and 0.7456 g of *potassium chloride* add 3.97 mL of 0.2M *sodium hydroxide VS* and dilute to 200 mL with *water*.

At 20°, the solution may be used as a solution of standard pH.

Borate Buffer pH 8.4, 0.2M

Dissolve 2.0 g of *sodium hydroxide* and 12.1 g of *boric acid* in 250 mL of *water*, adjust to pH 8.4, if necessary, by adding a few granules of *boric acid*.

Borate Buffer pH 9.0

Buffer solution pH 9.0

Dissolve 6.18 g of *boric acid R* in 0.1 M *potassium chloride R* and dilute to 1000.0 mL with the same solvent.
Mix 1000.0 mL of this solution and 420.0 mL of 0.1 M *sodium hydroxide*.

Borate Buffer pH 9.6

To 50 mL of a solution containing 0.6189 g of *boric acid* and 0.7456 g of *potassium chloride* add 36.85 mL of 0.2M *sodium hydroxide VS* and dilute with *water* to 200 mL.

At 20°, the solution may be used as a solution of standard pH.

Borate Buffer Solution pH 8.0, 0.0015M

Dissolve 0.572 g of *disodium tetraborate R* and 2.94 g of *calcium chloride R* in 800 mL of *water R*. Adjust the pH with *1 M hydrochloric acid*. Dilute to 1000.0 mL with *water R*.

Borate Buffer Solution pH 10.0

Introduce 12.4 g of *boric acid R* into a 500.0 mL volumetric flask. Add 300 mL of *water R* to suspend the boric acid. Add 100 mL of a 56 g/L solution of *potassium hydroxide R* and mix to dissolve the boric acid. Adjust to pH 10.0 by slowly adding a 56 g/L solution of *potassium hydroxide R* (about 60 mL is usually needed). Mix. Dilute almost to volume with *water R*. If necessary, adjust the pH with *boric acid R* or with a 56 g/L solution of *potassium hydroxide R*. Dilute to 500.0 mL with *water R*.

Borate Buffer Solution pH 10.4

Dissolve 24.64 g of *boric acid R* in 900 mL of *distilled water R*. Adjust the pH using a 400 g/L solution of *sodium hydroxide R*. Dilute to 1000 mL with *distilled water R*.

Boric Buffer pH 9.0 Buffer solution pH 9.0 R1

Dissolve 6.20 g of *boric acid R* in 500 mL of *water R* and adjust the pH with *1 M sodium hydroxide* (about 41.5 mL). Dilute to 1000.0 mL with *water R*.

Buffer (Acetate) Solution pH 5.0

To 120 mL of a 6 g/L solution of *glacial acetic acid R* add 100 mL of *0.1 M potassium hydroxide* and about 250 mL of *water R*. Mix. Adjust the pH to 5.0 with a 6 g/L solution of *acetic acid R* or with *0.1 M potassium hydroxide* and dilute to 1000.0 mL with *water R*.

Buffer (HEPES) Solution pH 7.5

Dissolve 2.38 g of *HEPES R* in about 90 mL of *water R*. Adjust the pH to 7.5 with *sodium hydroxide solution R*. Dilute to 100 mL with *water R*.

Buffer Solution pH 2.2

Mix 6.7 mL of *phosphoric acid R* with 55.0 mL of a 40 g/L solution of *sodium hydroxide R* and dilute to 1000.0 mL with *water R*.

Buffer Solution pH 2.5

Dissolve 100 g of *potassium dihydrogen phosphate R* in 800 mL of *water R*; adjust to pH 2.5 with *hydrochloric acid R* and dilute to 1000.0 mL with *water R*.

Buffer Solution pH 2.5 R1

To 4.9 g of *dilute phosphoric acid R* add 250 mL of *water R*. Adjust the pH with *dilute sodium hydroxide solution R* and dilute to 500.0 mL with *water R*.

Buffer Solution pH 3.0

Dissolve 21.0 g of *citric acid monohydrate R* in 200 mL of *1 M sodium hydroxide* and dilute to 1000 mL with *water R*. Dilute 40.3 mL of this solution to 100.0 mL with *0.1 M hydrochloric acid*.

Buffer Solution pH 3.5

Dissolve 25.0 g of *ammonium acetate R* in 25 mL of *water R* and add 38.0 mL of *hydrochloric acid R1*. Adjust the pH if necessary with *dilute hydrochloric acid R* or *dilute ammonia R1*. Dilute to 100.0 mL with *water R*.

Buffer Solution pH 3.7

To 15.0 mL of *acetic acid R* add 60 mL of *ethanol (96 per cent) R* and 20 mL of *water R*. Adjust to pH 3.7 by the addition of *ammonia R*. Dilute to 100.0 mL with *water R*.

Buffer Solution pH 5.2

Dissolve 1.02 g of *potassium hydrogen phthalate R* in 30.0 mL of *0.1 M sodium hydroxide*. Dilute to 100.0 mL with *water R*.

Buffer Solution pH 5.5

Dissolve 54.4 g of *sodium acetate R* in 50 mL of *water R*, heating to 35 °C if necessary. After cooling, slowly add 10 mL of *anhydrous acetic acid R*. Shake and dilute to 100.0 mL with *water R*.

Buffer Solution pH 6.5

Dissolve 60.5 g of *disodium hydrogen phosphate dodecahydrate R* and 46 g of *potassium dihydrogen phosphate R* in *water R*. Add 100 mL of *0.02 M sodium edetate* and 20 mg of *mercuric chloride R* and dilute to 1000.0 mL with *water R*.

Buffer Solution pH 6.6

To 250.0 mL of *0.2 M potassium dihydrogen phosphate R* add 89.0 mL of *0.2 M sodium hydroxide*. Dilute to 1000.0 mL with *water R*.

Buffer Solution pH 7.0

To 1000 mL of a solution containing 18 g/L of *disodium hydrogen phosphate dodecahydrate R* and 23 g/L of *sodium chloride R* add sufficient (about 280 mL) of a solution containing 7.8 g/L of *sodium dihydrogen phosphate R* and 23 g/L of *sodium chloride R* to adjust the pH. Dissolve in the solution sufficient *sodium azide R* to give a 0.2 g/L solution.

Buffer Solution pH 7.2

To 250.0 mL of *0.2 M potassium dihydrogen phosphate R* add 175.0 mL of *0.2 M sodium hydroxide*. Dilute to 1000.0 mL with *water R*. Adjust the pH if necessary.

Buffer Solution pH 7.4

Dissolve 0.6 g of *potassium dihydrogen phosphate R*, 6.4 g of *disodium hydrogen phosphate dodecahydrate R* and 5.85 g of *sodium chloride R* in *water R*, and dilute to 1000.0 mL with the same solvent. Adjust the pH if necessary.

Buffer Solution pH 8.0

To 50.0 mL of *0.2 M potassium dihydrogen phosphate R* add 46.8 mL of *0.2 M sodium hydroxide*. Dilute to 200.0 mL with *water R*.

Buffer Solution pH 8.0 R1

Dissolve 20 g of *dipotassium hydrogen phosphate R* in 900 mL of *water R*. Adjust the pH with *phosphoric acid R*. Dilute to 1000 mL with *water R*.

Buffer Solution pH 9.0

Dissolve 6.18 g of *boric acid R* in *0.1 M potassium chloride R* and dilute to 1000.0 mL with the same solvent.
Mix 1000.0 mL of this solution and 420.0 mL of *0.1 M sodium hydroxide*.

Buffer Solution pH 11

Dissolve 6.21 g of *boric acid R*, 4.00 g of *sodium hydroxide R* and 3.70 g of *potassium chloride R* in 500 mL of *water R* and dilute to 1000 mL with the same solvent.

Buffered Salt Solution pH 7.2

Dissolve in *water R* 8.0 g of *sodium chloride R*, 0.2 g of *potassium chloride R*, 0.1 g of *anhydrous calcium chloride R*, 0.1 g of *magnesium chloride R*, 3.18 g of *disodium hydrogen phosphate dodecahydrate R* and 0.2 g of *potassium dihydrogen phosphate R* and dilute to 1000.0 mL with *water R*.

Carbonate Buffer pH 9.7

Dissolve 8.4 g of *sodium hydrogen carbonate* and 10.6 g of *sodium carbonate* in sufficient *water* to produce 500 mL.

Chloride Buffer pH 2.0, 0.1M Buffer solution pH 2.0

Dissolve 6.57 g of *potassium chloride R* in *water R* and add 119.0 mL of *0.1 M hydrochloric acid*. Dilute to 1000.0 mL with *water R*.

Citrate Buffer Solution pH 5.0

Prepare a solution containing 20.1 g/L of *citric acid monohydrate R* and 8.0 g/L of *sodium hydroxide R*. Adjust the pH with *dilute hydrochloric acid R*.

Citrate Buffer Solution pH 3.0, 0.25M

Dissolve 5.3 g of *citric acid monohydrate R* in 80 mL of *water R*. Adjust the pH with *1 M sodium hydroxide* and dilute to 100.0 mL with *water R*.

Citro-phosphate Buffer pH 4.5

To 30 volumes of 0.2M *disodium hydrogen orthophosphate* add sufficient 0.1M *citric acid* to give a pH of 4.5 (about 36 volumes).

Citro-phosphate Buffer pH 5.0

Mix 48.5 mL of 0.1M *citric acid* with sufficient 0.2M *disodium hydrogen orthophosphate* to produce 100 mL.

Citro-phosphate Buffer pH 6.0 Phosphate buffer solution pH 6.0

Mix 63.2 mL of a 71.5 g/L solution of *disodium hydrogen phosphate dodecahydrate R* and 36.8 mL of a 21 g/L solution of *citric acid monohydrate R*.

Citro-phosphate Buffer pH 6.5

Mix 29.0 mL of 0.1M *citric acid* with sufficient 0.2M *disodium hydrogen orthophosphate* to produce 100 mL.

Citro-phosphate Buffer pH 6.8 Phosphate buffer solution pH 6.8

Mix 77.3 mL of a 71.5 g/L solution of *disodium hydrogen phosphate dodecahydrate R* with 22.7 mL of a 21 g/L solution of *citric acid monohydrate R*.

Citro-phosphate Buffer pH 7.0 Phosphate buffer solution pH 7.0

Mix 82.4 mL of a 71.5 g/L solution of *disodium hydrogen phosphate dodecahydrate R* with 17.6 mL of a 21 g/L solution of *citric acid monohydrate R*.

Citro-phosphate Buffer pH 7.2 Phosphate buffer solution pH 7.2

Mix 87.0 mL of a 71.5 g/L solution of *disodium hydrogen phosphate dodecahydrate R* with 13.0 mL of a 21 g/L solution of *citric acid monohydrate R*.

Citro-phosphate Buffer pH 7.6

Dissolve 67.1 g of *disodium hydrogen orthophosphate* and 1.33 g of *citric acid* in sufficient *water* to produce 1000 mL.

Copper Sulfate Solution pH 2.0

Copper Sulphate Solution pH 2.0.

Mix together 53 mL of 0.2M *hydrochloric acid*, 250 mL of 0.2M *potassium chloride* and 40 mL of a 0.393% w/v solution of *copper(II) sulfate* and dilute with *water* to 1000 mL.

Copper Sulfate Solution pH 4.0, Buffered

Dissolve 0.25 g of *copper sulfate pentahydrate R* and 4.5 g of *ammonium acetate R* in *dilute acetic acid R* and dilute to 100.0 mL with the same solvent.

Copper Sulfate Solution pH 5.2, Buffered

Copper Sulphate Solution pH 5.2, Buffered

Dissolve 15.22 g of *anhydrous disodium hydrogen orthophosphate* in sufficient *water* to produce 536 mL and add a 2.1% w/v solution of *citric acid* until the pH of the solution is between 5.15 and 5.25 (about 464 mL). Mix 985 mL of the resulting solution with 15 mL of a 0.393% w/v solution of *copper(II) sulfate*.

Deuterated Sodium Phosphate Buffer Solution pH 5.0, 0.2M

Dissolve 2.76 g of *sodium dihydrogen phosphate monohydrate R* in 90 mL of *deuterium oxide R*, adjust the pH with a deuterated solution of *phosphoric acid R* or a deuterated 1 M solution of *sodium hydroxide R*, dilute to 100 mL with *deuterium oxide R* and mix.

Diethanolamine Buffer Solution pH 10.0

Dissolve 96.4 g of *diethanolamine R* in *water R* and dilute to 400 mL with the same solvent. Add 0.5 mL of an 186 g/L solution of *magnesium chloride R* and adjust the pH with *1 M hydrochloric acid*. Dilute to 500.0 mL with *water R*.

Diethylammonium Phosphate Buffer Solution pH 6.0

Dilute 68 mL of *phosphoric acid R* to 500 mL with *water R*. To 25 mL of this solution add 450 mL of *water R* and 6 mL of *diethylamine R*, adjust to pH 6 ± 0.05, if necessary, using *diethylamine R* or *phosphoric acid R* and dilute to 500.0 mL with *water R*.

Glycine Buffer pH 2.9

Dissolve 6.0 g of *glycine* and 4.68 g of *sodium chloride* in 10 litres of *water*. Adjust the pH with 1M *hydrochloric acid* (about 30 mL).

Glycine Buffer pH 11.3

Mix a solution containing 0.75% w/v of *glycine* and 0.58% w/v of *sodium chloride* with an equal volume of 0.1M *sodium hydroxide* and adjust the pH if necessary.

Glycine Buffer Solution

Mix 42 g of *sodium hydrogen carbonate* and 50 g of *potassium hydrogen carbonate* with 180 mL of *water* and add a solution containing 37.5 g of *glycine* and 15 mL of 13.5M *ammonia* in 180 mL of *water*. Dilute to 500 mL with *water* and stir until solution is complete.

Guanidine-tris(hydroxymethyl)aminomethane Buffer Solution pH 8.3

Dissolve 1.21 g of *tris(hydroxymethyl)aminomethane R* in 87.5 mL of a 764 g/L solution of *guanidine hydrochloride R*. Adjust to pH 8.3 with *hydrochloric acid R* and dilute to 100 mL with *water R*.

Guanidine-tris(hydroxymethyl)aminomethane-EDTA Buffer Solution pH 8.5

Dissolve 1.0 g of *sodium edetate R*, 12.1 g of *tris(hydroxymethyl)aminomethane R* and 57.0 g of *guanidine hydrochloride R* in 35 mL of *water R*. Adjust to pH 8.5 with *hydrochloric acid R* and dilute to 100 mL with *water R*.

Guanidine-tris(hydroxymethyl)aminomethane-EDTA Buffer Solution pH 8.6

Dissolve 0.018 g of *sodium edetate R*, 2.2 g of *tris(hydroxymethyl)aminomethane R* and 28.7 g of *guanidine hydrochloride R* in 20 mL of *water R*. Adjust to pH 8.6 with *acetic acid R* and dilute to 50 mL with *water R*.

Imidazole Buffer Solution pH 6.5

Dissolve 6.81 g of *imidazole R*, 1.23 g of *magnesium sulfate R* and 0.73 g of *calcium sulfate R* in 752 mL of *0.1 M hydrochloric acid*. Adjust the pH if necessary and dilute to 1000.0 mL with *water R*.

Imidazole Buffer Solution pH 7.3

Dissolve 3.4 g of *imidazole R* and 5.8 g of *sodium chloride R* in *water R*, add 18.6 mL of *1 M hydrochloric acid* and dilute to 1000.0 mL with *water R*. Adjust the pH if necessary.

1M Morpholinoethanesulfonate Buffer Solution pH 6.0

Dissolve 48.8 g of *2-[N-morpholino]ethanesulfonic acid R* in 160 mL of *water R* and add 25 mL of *2 M sodium hydroxide R*. Adjust to pH 6.0 with *2 M sodium hydroxide R*. Dilute to almost 250 mL with *water R*. Adjust the pH, if necessary, with *2 M sodium hydroxide R* and dilute to 250.0 mL with *water R*.

Octylamine Phosphate Buffer pH 3.0

Dilute 3.32 mL of *octylamine* to 1000 mL, adjust the pH to 3.0 using *orthophosphoric acid* and filter through a membrane filter with a nominal pore size not greater than 0.5 µm.

Phosphate Buffers

Solutions from pH 5.8 to pH 8.0 may be prepared by mixing 50 mL of 0.2M *potassium dihydrogen orthophosphate* with the quantities of 0.2M *sodium hydroxide VS* specified in the following table and diluting to 200 mL with *water*.

At 20° the solutions may be used as solutions of standard pH.

pH	5.8	6.0	6.2	6.4	6.6	6.8
ml of 0.2M *sodium hydroxide VS*	3.72	5.70	8.60	12.60	17.80	23.65
pH	7.0	7.2	7.4	7.6	7.8	8.0
ml of 0.2M *sodium hydroxide VS*	29.63	35.00	39.50	42.80	45.20	46.80

Phosphate Buffer pH 3.0

Dissolve 34 g of *potassium dihydrogen orthophosphate* in 250 mL of *water* and adjust the pH of the solution to 3.0 with *orthophosphoric acid*.

Phosphate Buffer pH 3.5 Phosphate buffer solution pH 3.5

Dissolve 68.0 g of *potassium dihydrogen phosphate R* in *water R* and dilute to 1000.0 mL with the same solvent. Adjust the pH with *phosphoric acid R*.

Phosphate Buffer pH 4.0

Dissolve 6.8 g of *potassium dihydrogen orthophosphate* in 700 mL of *water*, adjust the pH, if necessary, with a 10% v/v solution of *orthophosphoric acid* and add sufficient *water* to produce 1000 mL.

Phosphate Buffer pH 4.0, Mixed

Dissolve 5.04 g of *disodium hydrogen orthophosphate* and 3.01 g of *potassium dihydrogen orthophosphate* in sufficient *water* to produce 1000 mL and adjust the pH with *glacial acetic acid*.

Phosphate Buffer pH 4.75

Dilute 100 mL of 0.5M *potassium dihydrogen orthophosphate* to 800 mL with *water*, adjust to pH 4.75 with 0.1M *sodium hydroxide* and dilute to 1000 mL with *water*.

Phosphate Buffer pH 4.9

Dissolve 40 g of *sodium dihydrogen orthophosphate* and 1.2 g of *sodium hydroxide* in sufficient *water* to produce 100 mL. If necessary, adjust the pH with 1M *sulfuric acid* or 1M *sodium hydroxide* as required.

Phosphate Buffer pH 5.4, Mixed

Dissolve 1.76 g of *disodium hydrogen orthophosphate* and 13.61 g of *potassium dihydrogen orthophosphate* in sufficient *water* to produce 1000 mL. Adjust the pH with 0.05M *orthophosphoric acid*, if necessary.

Phosphate Buffer pH 6.8, Mixed

Dissolve 28.80 g of *disodium hydrogen orthophosphate* and 11.45 g of *potassium dihydrogen orthophosphate* in sufficient *water* to produce 1000 mL.

Phosphate Buffer pH 6.8, 0.2M Mixed Phosphate buffer solution pH 6.8 R1

To 51.0 mL of a 27.2 g/L solution of *potassium dihydrogen phosphate R* add 49.0 mL of a 71.6 g/L solution of *disodium hydrogen phosphate dodecahydrate R*. Adjust the pH if necessary.

Storage: at 2 °C to 8 °C.

Phosphate Buffer pH 7.0, Mixed

Dissolve 0.50 g of *anhydrous disodium hydrogen orthophosphate* and 0.301 g of *potassium dihydrogen orthophosphate* in sufficient *water* to produce 1000 mL.

Phosphate Buffer pH 7.0, 0.067M Mixed 0.067M Phosphate buffer solution pH 7.0

Dissolve 0.908 g of *potassium dihydrogen phosphate R* in *water R* and dilute to 100.0 mL with the same solvent (solution A). Dissolve 2.38 g of *disodium hydrogen phosphate dodecahydrate R* in *water R* and dilute to 100.0 mL with the same solvent (solution B). Mix 38.9 mL of solution A and 61.1 mL of solution B. Adjust the pH if necessary.

Phosphate Buffer pH 7.0, 0.1M Mixed 0.1M Phosphate buffer solution pH 7.0

Dissolve 1.361 g of *potassium dihydrogen phosphate R* in *water R* and dilute to 100.0 mL with the same solvent. Adjust the pH using a 35 g/L solution of *disodium hydrogen phosphate dodecahydrate R*.

Phosphate Buffer pH 7.5, 0.2M

Dissolve 27.22 g of *potassium dihydrogen phosphate R* in 930 mL of *water R*, adjust to pH 7.5 with a 300 g/L solution of *potassium hydroxide R* and dilute to 1000.0 mL with *water R*.

Phosphate Buffer pH 10, Mixed

To 100 mL of 0.2M *disodium hydrogen orthophosphate* add 6.0 mL of 0.25M *trisodium orthophosphate*.

Phosphate Buffer, 0.025M Standard

Dissolve 3.40 g of *potassium dihydrogen orthophosphate* and 3.55 g of *anhydrous disodium hydrogen orthophosphate*, both previously dried at 110° to 130° for 2 hours, in sufficient *water* to produce 1000 mL.

Phosphate Buffer Solution pH 2.0

Dissolve 8.95 g of *disodium hydrogen phosphate dodecahydrate R* and 3.40 g of *potassium dihydrogen phosphate R* in *water R* and dilute to 1000.0 mL with the same solvent. If necessary adjust the pH with *phosphoric acid R*.

0.125M Phosphate Buffer Solution pH 2.0

Dissolve 17.0 g of *potassium dihydrogen phosphate R* and 17.8 g of *anhydrous disodium hydrogen phosphate R* in *water R* and dilute to 1.0 L with the same solvent. If necessary adjust the pH with *phosphoric acid R*.

Phosphate Buffer Solution pH 2.5, 0.2M

Dissolve 27.2 g of *potassium dihydrogen phosphate R* in about 900 mL of *water R*, adjust to pH 2.5 with *phosphoric acid R* and dilute to 1.0 L with *water R*.

Phosphate Buffer Solution pH 2.8

Dissolve 7.8 g of *sodium dihydrogen phosphate R* in 900 mL of *water R*, adjust to pH 2.8 with *phosphoric acid R* and dilute to 1000 mL with the same solvent.

Phosphate Buffer Solution pH 3.0

Mix 0.7 mL of *phosphoric acid R* with 100 mL of *water R*. Dilute to 900 mL with the same solvent. Adjust to pH 3.0 with *strong sodium hydroxide solution R* and dilute to 1000 mL with *water R*.

Phosphate Buffer Solution pH 3.0, 0.1M

Dissolve 12.0 g of *anhydrous sodium dihydrogen phosphate R* in *water R*, adjust the pH with *dilute phosphoric acid R1* and dilute to 1000 mL with *water R*.

Phosphate Buffer Solution pH 3.0 R1

Dissolve 3.40 g of *potassium dihydrogen phosphate R* in 900 mL of *water R*. Adjust to pH 3.0 with *phosphoric acid R* and dilute to 1000.0 mL with *water R*.

Phosphate Buffer Solution pH 3.2

To 900 mL of a 4 g/L solution of *sodium dihydrogen phosphate R*, add 100 mL of a 2.5 g/L solution of *phosphoric acid R*. Adjust the pH if necessary.

Phosphate Buffer Solution pH 3.2 R1

Adjust a 35.8 g/L solution of *disodium hydrogen phosphate dodecahydrate R* to pH 3.2 with *dilute phosphoric acid R*. Dilute 100.0 mL of the solution to 2000.0 mL with *water R*.

Phosphate Buffer Solution pH 3.25

Dissolve about 1.36 g of *potassium dihydrogen phosphate R* in 1000 mL of *water R* and adjust to pH 3.25 ± 0.05 with *dilute phosphoric acid R*. Filter through a membrane filter (nominal pore size 0.45 µm or finer).

Phosphate Buffer Solution pH 3.4

Dissolve 68.0 g of *potassium dihydrogen phosphate R* in *water R* and dilute to 1000.0 mL with the same solvent. Adjust the pH with *phosphoric acid R*.

Phosphate Buffer Solution pH 4.5, 0.05M

Dissolve 6.80 g of *potassium dihydrogen phosphate R* in 1000.0 mL of *water R*. The pH of the solution is 4.5.

Phosphate Buffer Solution pH 5.0

Dissolve 2.72 g of *potassium dihydrogen phosphate R* in 800 mL of *water R*. Adjust the pH with a 1 M potassium hydroxide solution prepared from *potassium hydroxide R* and dilute to 1000 mL with *water R*.

Phosphate Buffer Solution pH 5.4, 0.067M

Mix appropriate volumes of a 23.99 g/L solution of *disodium hydrogen phosphate dodecahydrate R* with a 9.12 g/L solution of *sodium dihydrogen phosphate monohydrate R* to obtain pH 5.4.

Phosphate Buffer Solution pH 5.5

Dissolve 13.61 g of *potassium dihydrogen phosphate R* in *water R* and dilute to 1000.0 mL with the same solvent (solution A). Dissolve 35.81 g of *disodium hydrogen phosphate dodecahydrate R* in *water R* and dilute to 1000.0 mL with the same solvent (solution B). Mix 96.4 mL of solution A and 3.6 mL of solution B.

Phosphate Buffer Solution pH 5.6

Dissolve 0.908 g of *potassium dihydrogen phosphate R* in *water R* and dilute to 100.0 mL with the same solvent (solution A). Dissolve 1.161 g of *dipotassium hydrogen phosphate R* in *water R* and dilute to 100.0 mL with the same solvent (solution B). Mix 94.4 mL of solution A and 5.6 mL of solution B. If necessary, adjust to pH 5.6 using solution A or solution B.

Phosphate Buffer Solution pH 5.8

Dissolve 1.19 g of *disodium hydrogen phosphate dihydrate R* and 8.25 g of *potassium dihydrogen phosphate R* in *water R* and dilute to 1000.0 mL with the same solvent.

Phosphate Buffer Solution pH 6.0 R1

Dissolve 6.8 g of *sodium dihydrogen phosphate R* in *water R* and dilute to 1000.0 mL with *water R*. Adjust the pH with *strong sodium hydroxide solution R*.

Phosphate Buffer Solution pH 6.0 R2

To 250.0 mL of *0.2 M potassium dihydrogen phosphate R* add 28.5 mL of *0.2 M sodium hydroxide* and dilute to 1000.0 mL with *water R*.

Phosphate Buffer Solution pH 6.3, 0.1M

Dissolve 15.6 g of *sodium dihydrogen orthophosphate* in 900 mL of water, adjust the pH to 6.3 with 0.1M *sodium hydroxide* and add sufficient *water* to produce 1000 mL.

Phosphate Buffer Solution pH 6.4

Dissolve 2.5 g of *disodium hydrogen phosphate dodecahydrate R*, 2.5 g of *sodium dihydrogen phosphate R* and 8.2 g of *sodium chloride R* in 950 mL of *water R*. Adjust the pH of the solution to 6.4 with *1 M sodium hydroxide* or *1 M hydrochloric acid*, if necessary. Dilute to 1000.0 mL with *water R*.

Phosphate Buffer Solution pH 6.5

Dissolve 2.75 g of *sodium dihydrogen phosphate R* and 4.5 g of *sodium chloride R* in 500 mL of *water R*. Adjust the pH with *phosphate buffer solution pH 8.5 R*.

Phosphate Buffer Solution pH 6.5, 0.1M

Dissolve 13.80 g of *sodium dihydrogen phosphate monohydrate R* in 900 mL of *distilled water R*. Adjust the pH using a 400 g/L solution of *sodium hydroxide R*. Dilute to 1000 mL with *distilled water R*.

Phosphate Buffer Solution pH 6.7, 0.1M

Dissolve 15.6 g of *sodium dihydrogen phosphate R* in *water R* and dilute to 1.0 L with the same solvent. Dissolve 17.8 g of *disodium hydrogen phosphate dihydrate R* in *water R* and dilute to 1.0 L with the same solvent. Mix the solutions, check the pH and if necessary adjust to pH 6.7.

Phosphate Buffer Solution pH 7.0 R1

Mix 250.0 mL of *0.2 M potassium dihydrogen phosphate R* and 148.2 mL of a 8 g/L solution of *sodium hydroxide R*, adjust the pH if necessary. Dilute to 1000.0 mL with *water R*.

Phosphate Buffer Solution pH 7.0 R2

Mix 50.0 mL of a 136 g/L solution of *potassium dihydrogen phosphate R* with 29.5 mL of *1 M sodium hydroxide* and dilute to 100.0 mL with *water R*. Adjust the pH to 7.0 ± 0.1.

Phosphate Buffer Solution pH 7.0 R3

Dissolve 5 g of *potassium dihydrogen phosphate R* and 11 g of *dipotassium hydrogen phosphate R* in 900 mL of *water R*. Adjust to pH 7.0 with *dilute phosphoric acid R* or *dilute sodium hydroxide solution R*. Dilute to 1000 mL with *water R* and mix.

Phosphate Buffer Solution pH 7.0 R4

Dissolve 28.4 g of *anhydrous disodium hydrogen phosphate R* and 18.2 g of *potassium dihydrogen phosphate R* in *water R* and dilute to 500 mL with the same solvent.

Phosphate Buffer Solution pH 7.0 R5

Dissolve 28.4 g of *anhydrous disodium hydrogen phosphate R* in 800 mL of *water R*. Adjust the pH using a 30 per cent *m/m* solution of *phosphoric acid R* and dilute to 1000 mL with *water R*.

Phosphate Buffer Solution pH 7.0 R6

Dissolve 3.56 g of *disodium hydrogen phosphate dihydrate R* in 950 mL of *water for chromatography R*. Adjust the pH with *phosphoric acid R* and dilute to 1.0 L with *water for chromatography R*.

Phosphate Buffer Solution pH 7.0 R7

Dissolve 35 g of *dipotassium hydrogen phosphate R* in 900 mL of *water R*, adjust to pH 7.0 with *dilute phosphoric acid R* and dilute to 1.0 L with *water R*.

Phosphate Buffer Solution pH 7.0, 0.025M

Mix 1 volume of *0.063 M phosphate buffer solution pH 7.0 R* with 1.5 volumes of *water R*.

Phosphate Buffer Solution pH 7.0, 0.03M

Dissolve 5.2 g of *dipotassium hydrogen phosphate R* in 900 mL of *water for chromatography R*. Adjust the solution to pH 7.0 ± 0.1 using *phosphoric acid R* and dilute to 1000 mL with *water for chromatography R*.

Phosphate Buffer Solution pH 7.0, 0.05M

Mix 34 mL of *water R* and 100 mL of *0.067 M phosphate buffer solution pH 7.0 R*.

Phosphate Buffer Solution pH 7.0, 0.063M

Dissolve 5.18 g of *anhydrous disodium hydrogen phosphate R* and 3.65 g of *sodium dihydrogen phosphate monohydrate R* in 950 mL of *water R* and adjust the pH with *phosphoric acid R*; dilute to 1000.0 mL with *water R*.

Phosphate Buffer Solution pH 7.4

Add 250.0 mL of *0.2 M potassium dihydrogen phosphate R* to 393.4 mL of *0.1 M sodium hydroxide*.

Phosphate Buffer Solution pH 7.5, 0.05M

Dissolve 0.89 g of *disodium hydrogen phosphate dihydrate R* in about 80 mL of *water R*. Adjust to pH 7.5 with an 8.5 per cent *V/V* solution of *phosphoric acid R* and dilute to 100.0 mL with *water R*.

Phosphate Buffer Solution pH 7.5, 0.33M

Dissolve 119.31 g of *disodium hydrogen phosphate dodecahydrate R* in *water R* and dilute to 1000.0 mL with the same solvent (solution A). Dissolve 45.36 g of *potassium dihydrogen phosphate R* in *water R* and dilute to 1000.0 mL with the same solvent (solution B). Mix 85 mL of solution A and 15 mL of solution B. Adjust the pH if necessary.

Phosphate Buffer Solution pH 8.0, 0.02M

To 50.0 mL of *0.2 M potassium dihydrogen phosphate R* add 46.8 mL of *0.2 M sodium hydroxide*. Dilute to 500.0 mL with *water R*.

Phosphate Buffer Solution pH 8.0, 0.1M

Dissolve 0.523 g of *potassium dihydrogen phosphate R* and 16.73 g of *dipotassium hydrogen phosphate R* in *water R* and dilute to 1000.0 mL with the same solvent.

Phosphate Buffer Solution pH 8.0, 0.05M

Dissolve 0.31 g of *sodium dihydrogen phosphate R* in 70 mL of *water R* and adjust to pH 8.0 with *1 M sodium hydroxide*, then dilute to 100 mL with *water R*.

Phosphate Buffer Solution pH 8.0, 1M

Dissolve 136.1 g of *potassium dihydrogen phosphate R* in *water R*, adjust the pH with *1 M sodium hydroxide*. Dilute to 1000.0 mL with *water R*.

Phosphate Buffer Solution pH 8.5

Dissolve 3.5 g of *dipotassium hydrogen phosphate R* and 4.5 g of *sodium chloride R* in 500 mL of *water R*. Adjust the pH with a mixture of equal volumes of *dilute phosphoric acid R* and *water R*.

Phosphate Buffer Solution pH 9.0

Dissolve 1.74 g of *potassium dihydrogen phosphate R* in 80 mL of *water R*, adjust the pH with a 1 M potassium hydroxide solution prepared from *potassium hydroxide R* and dilute to 100.0 mL with *water R*.

Phosphate Buffer Solution pH 11.3, 0.1M

Dissolve 17.4 g of *dipotassium hydrogen phosphate R* in about 950 mL of *water R*, adjust to pH 11.3 using a 100 g/L solution of *potassium hydroxide R* and dilute to 1.0 L with *water R*. Filter through a membrane filter (nominal pore size 0.45 µm).

Phosphate–Citrate Buffer Solution pH 5.5

Mix 56.85 mL of a 28.4 g/L solution of *anhydrous disodium hydrogen phosphate R* and 43.15 mL of a 21 g/L solution of *citric acid monohydrate R*.

Phthalate Buffer pH 3.6 Buffer solution pH 3.6

To 250.0 mL of *0.2 M potassium hydrogen phthalate R* add 11.94 mL of *0.2 M hydrochloric acid.* Dilute to 1000.0 mL with *water R.*

Phthalate Buffer Solution pH 4.4

Dissolve 2.042 g of *potassium hydrogen phthalate R* in 50 mL of *water R,* add 7.5 mL of *0.2 M sodium hydroxide* and dilute to 200.0 mL with *water R.*

Phthalate Buffer Solution pH 6.4, 0.5M

Dissolve 100 g of *potassium hydrogen phthalate R* in *water R* and dilute to 1000.0 mL with the same solvent. Adjust the pH if necessary, using *strong sodium hydroxide solution R.*

Potassium Phosphate Buffer Solution pH 7.0

Dissolve 10 mg of *bovine albumin R* and 68 mg of *potassium dihydrogen phosphate R* in 30 mL of *water R.* If necessary, adjust to pH 7.0 with *potassium hydroxide R.* Dilute to 50 mL with *water R* and filter.

Saline pH 6.4, Phosphate-buffered

Dissolve 1.79 g of *disodium hydrogen orthophosphate,* 1.36 g of *potassium dihydrogen orthophosphate* and 7.02 g of *sodium chloride* in sufficient *water* to produce 1000 mL.

Saline pH 6.8, Phosphate-buffered

Dissolve 1.0 g of *potassium dihydrogen phosphate R,* 2.0 g of *dipotassium hydrogen phosphate R* and 8.5 g of *sodium chloride R* in 900 mL of *water R,* adjust the pH if necessary and dilute to 1000.0 mL with the same solvent.

Saline pH 7.2, Phosphate-albumin Buffered

Dissolve 10.75 g of *disodium hydrogen phosphate dodecahydrate R,* 7.6 g of *sodium chloride R* and 10 g of *bovine albumin R* in *water R* and dilute to 1000.0 mL with the same solvent. Immediately before use adjust the pH using *dilute sodium hydroxide solution R* or *dilute phosphoric acid R.*

Saline pH 7.2 R1, Phosphate-albumin Buffered

Dissolve 10.75 g of *disodium hydrogen phosphate dodecahydrate R,* 7.6 g of *sodium chloride R* and 1 g of *bovine albumin R* in *water R* and dilute to 1000.0 mL with the same solvent. Immediately before use adjust the pH using *dilute sodium hydroxide solution R* or *dilute phosphoric acid R.*

Saline pH 7.4, Phosphate-buffered

Dissolve 2.38 g of *disodium hydrogen phosphate dodecahydrate R,* 0.19 g of *potassium dihydrogen phosphate R* and 8.0 g of *sodium chloride R* in water. Dilute to 1000.0 mL with the same solvent. Adjust the pH if necessary.

Sodium Acetate Buffer pH 7.0.

Dissolve 1.64 g of *anhydrous sodium acetate* in 1 L of *water,* adjust to pH 7.0 using dilute acetic acid

Sodium Acetate Buffer Solution pH 4.0, 0.1M

Dissolve 822 mg of *sodium acetate R* in 100 mL of *water R* (solution A). Dilute 1.44 mL of *glacial acetic acid R* in 250 mL of *water R* (solution B). Titrate 100 mL of solution B using about 20 mL of solution A.

Sodium Acetate Buffer Solution pH 4.5

Dissolve 63 g of *anhydrous sodium acetate R* in *water R,* add 90 mL *acetic acid R* and adjust to pH 4.5, and dilute to 1000 mL with *water R.*

Sodium Acetate Buffer Solution pH 5.0

Dissolve 50.0 g of *sodium acetate R* in 10.0 mL of *glacial acetic acid R* and add *water R.* Adjust to pH 5.0 with a 4.2 g/L solution of *sodium hydroxide R* or with *glacial acetic acid R* and dilute to 1000.0 mL with *water R.*

Sodium Acetate Solution pH 6.0, Buffered

Dissolve 4.1 g of *anhydrous sodium acetate* in 1000 mL of *water* and adjust the pH to 6.0 with *glacial acetic acid.*

Sodium/calcium Acetate Buffer Solution pH 7.0

Dissolve 10 mg of *bovine albumin R* and 32 mg of *calcium acetate R* in 60 mL of *water R.* Add 580 µL of *glacial acetic acid R* and adjust to pH 7.0 with *2 M sodium hydroxide R.* Dilute to 100 mL with *water R* and filter.

Sodium Citrate Buffer Solution pH 7.8 (0.034M Sodium Citrate, 0.101M Sodium Chloride)

Dissolve 10.0 g of *sodium citrate R* and 5.90 g of *sodium chloride R* in 900 mL of *water R.* Adjust the pH by addition of *hydrochloric acid R* and dilute to 1000 mL with *water R.*

Sodium Phosphate Buffer Solution pH 7.5, 0.25M

Dissolve 3.90 g of *sodium dihydrogen phosphate R* in 70 mL of *water R,* adjust to pH 7.5 with a 300 g/L solution of *sodium hydroxide R* and dilute to 100 mL with *water R.*

Sodium Phosphate Buffer Solution pH 8.0, 0.02M

Dissolve 0.31 g of *sodium dihydrogen phosphate R* in 70 mL of *water R* and adjust to pH 8.0 with *1 M sodium hydroxide,* then dilute to 100 mL with *water R.*

Succinate Buffer Solution pH 4.6

Disssolve 11.8 g of *succinic acid R* in a mixture of 600 mL of *water R* and 82 mL of *1 M sodium hydroxide* and dilute to 1000.0 mL with *water R.*

Sulfate Buffer Solution pH 2.00

Dissolve 132.1 g of *ammonium sulfate R* in *water R* and dilute to 500.0 mL with the same solvent (Solution A). Carefully and with constant cooling stir 14 mL of *sulfuric acid R* into about 400 mL of *water R;* allow to cool and dilute to 500.0 mL with *water R* (Solution B). Mix equal volumes of solutions A and B. Adjust the pH if necessary.

Tetrabutylammonium Buffer Solution pH 7.0

Dissolve 6.16 g of *ammonium acetate R* in a mixture of 15 mL of *tetrabutylammonium hydroxide solution (400 g/L) R* and 185 mL of *water R.* Adjust the pH with *nitric acid R.*

Thiobarbituric Acid–citrate Buffer

Dissolve 5.0 g of *thiobarbituric acid* in 5 mL of 4M *sodium hydroxide* and dilute to 500 mL with *water.* Dissolve separately 37 g of *sodium citrate* in 32 mL of *hydrochloric acid* and dilute to 250 mL with *water.* Mix the two solutions and adjust the pH of the resulting solution to 2.0.

Total Ionic Strength Adjustment Buffer

Dissolve 58.5 g of *sodium chloride R,* 57.0 mL of *glacial acetic acid R,* 61.5 g of *sodium acetate R* and 5.0 g of *cyclohexylenedinitrilotetra-acetic acid R* in *water R* and dilute to 500.0 mL with the same solvent. Adjust to pH 5.0 to 5.5 with a 335 g/L solution of *sodium hydroxide R* and dilute to 1000.0 mL with *distilled water R.*

Total Ionic Strength Adjustment Buffer R1

Dissolve 210 g of *citric acid monohydrate R* in 400 mL of *distilled water R.* Adjust to pH 7.0 with *concentrated ammonia R.* Dilute to 1000.0 mL with *distilled water R* (solution A). Dissolve 132 g of *ammonium phosphate R* in *distilled water R* and dilute to 1000.0 mL with the same solvent (solution B). To a suspension of 292 g of (ethylenedinitrilo)tetra-*acetic acid R* in about 500 mL of *distilled water R,* add about 200 mL of *concentrated ammonia R* to dissolve. Adjust the pH to 6 to 7 with *concentrated ammonia R.* Dilute to 1000.0 mL with *distilled water R* (solution C). Mix equal volumes of solution A, B, and C and adjust to pH 7.5 with *concentrated ammonia R.*

Tris-acetate Buffer Solution pH 8.5

Dissolve 0.294 g of *calcium chloride R* and 12.11 g of *tris(hydroxymethyl)aminomethane R* in *water R*. Adjust the pH with *acetic acid R*. Dilute to 1000.0 mL with *water R*.

Tris-borate-EDTA Buffer Solution pH 8.4 MB

Dissolve 10.80 g of *tris(hydroxymethyl)aminomethane MB*, 5.50 g *boric acid R* and 0.584 g of *ethylenediaminetetra-acetic acid R* in 250 mL of *distilled water R*. Adjust the pH *(2.2.3)* to 8.4 using *hydrochloric acid R*. Dilute to 1000.0 mL with *distilled water R*.

Tris-chloride Buffer pH 7.4 Tris(hydroxymethyl) aminomethane sodium chloride buffer solution pH 7.4

Dissolve 6.08 g of *tris(hydroxymethyl)aminomethane R* and 8.77 g of *sodium chloride R* in 500 mL of *distilled water R*. Add 10.0 g of *bovine albumin R* or 1.0-5.0 g of *macrogol 6000 R*. Adjust the pH using *hydrochloric acid R*. Dilute to 1000.0 mL with *distilled water R*.

Tris-chloride Buffer pH 7.5 Tris(hydroxymethyl) aminomethane buffer solution pH 7.5

Dissolve 7.27 g of *tris(hydroxymethyl)aminomethane R* and 5.27 g of *sodium chloride R* in *water R*, and adjust the pH if necessary. Dilute to 1000.0 mL with *water R*.

Tris-chloride Buffer pH 7.5 R1 0.05M Tris-hydrochloride buffer solution pH 7.5

Dissolve 6.057 g of *tris(hydroxymethyl)aminomethane R* in *water R* and adjust the pH with *hydrochloric acid R*. Dilute to 1000.0 mL with *water R*.

Tris-chloride Buffer pH 8.1 Tris(hydroxymethyl) aminomethane buffer solution pH 8.1

Dissolve 0.294 g of *calcium chloride R* in 40 mL of *tris(hydroxymethyl)aminomethane solution R* and adjust the pH with *1 M hydrochloric acid*. Dilute to 100.0 mL with *water R*.

Tris-chloride Buffer pH 8.6

Dissolve 2.0 g of *tris(hydroxymethyl)methylamine* and 2.4 g of *sodium chloride* in about 100 mL of *water*, adjust the pH to 8.6 with 1M *sodium hydroxide* or 1M *hydrochloric acid* and dilute with *water* to 200 mL.

Tris-chloride Buffer Solution

Dissolve 0.606 g of *tris(hydroxymethyl)methylamine* and 2.34 g of *sodium chloride* in sufficient *water* to produce 1000 mL. Store at 2° to 8° and use within 3 days of preparation.

Tris-EDTA Buffer Solution pH 8.0 MB

Dissolve 60.57 g of *tris(hydroxymethyl)aminomethane MB* in 500 mL of *distilled water R* and adjust the pH *(2.2.3)* to 8.4 using *hydrochloric acid R* (1M Tris buffer).

Dissolve 18.60 g of *ethylenediaminetetra-acetic acid R* in100 mL of *distilled water R* and adjust the pH *(2.2.3)* to 8.0 using *sodium hydroxide R* (0.5M EDTA solution). (Use vigorous stirring and moderate heat if desired).

Mix appropriate volumes of the two solutions to achieve the required concentration. (e.g. Mix 5 mL of 1M Tris buffer with 1 mL of 0.5M EDTA solution and dilute with 496 mL of *distilled water R)*.

Tris-EDTA Buffer pH 8.4 Tris(hydroxymethyl) aminomethane-EDTA Buffer Solution pH 8.4

Dissolve 5.12 g of *sodium chloride R*, 3.03 g of *tris(hydroxymethyl)aminomethane R* and 1.40 g of *sodium edetate R* in 250 mL of *distilled water R*. Adjust the pH to 8.4 using *hydrochloric acid R*. Dilute to 500.0 mL with *distilled water R*.

Tris-EDTA BSA Buffer Solution pH 8.4

Dissolve 6.1 g of *tris(hydroxymethyl)aminomethane R*, 2.8 g of *sodium edetate R*, 10.2 g of *sodium chloride R* and 10 g of *bovine albumin R* in *water R*, adjust to pH 8.4 using *1 M hydrochloric acid* and dilute to 1000.0 mL with *water R*.

Tris-glycine Buffer Solution pH 8.3

Dissolve 6.0 g of *tris(hydroxymethyl)aminomethane R* and 28.8 g of *glycine R* in *water R* and dilute to 1000.0 mL with the same solvent. Dilute 1 volume to 10 volumes with *water R* immediately before use.

Tris-hydrochloride Buffer Solution pH 6.8, 1M

Dissolve 60.6 g of *tris(hydroxymethyl)aminomethane R* in 400 mL of *water R*. Adjust the pH with *hydrochloric acid R* and dilute to 500.0 mL with *water R*.

Tris-hydrochloride Buffer Solution pH 7.5, 0.1M

Dissolve 3.03 g of *tris(hydroxymethyl)aminomethane R* in 200 mL of *water R*, adjust to pH 7.5 with *hydrochloric acid R* and dilute to 250 mL with *water R*.

Tris-hydrochloride Buffer Solution pH 7.5, 1M

Dissolve 12.11 g of *tris(hydroxymethyl)aminomethane R* in 90 mL of *water R*, adjust to pH 7.5 with *hydrochloric acid R* and dilute to 100.0 mL with *water R*.

Tris-hydrochloride Buffer Solution pH 8.0

Dissolve 1.21 g of *tris(hydroxymethyl)aminomethane R* and 29.4 mg of *calcium chloride R* in *water R*. Adjust the pH with *1 M hydrochloric acid* and dilute to 100.0 mL with *water R*.

Tris-hydrochloride Buffer Solution pH 8.0, 1M

Dissolve 121.1 g of *tris(hydroxymethyl)aminomethane R* and 1.47 g of *calcium chloride R* in 900 mL of *water R*. Adjust the pH with *hydrochloric acid R* and dilute to 1000.0 mL with *water R*.

Tris-hydrochloride Buffer Solution pH 8.3

Dissolve 9.0 g of *tris(hydroxymethyl)aminomethane R* in 2.9 L of *water R*. Adjust the pH with *1 M hydrochloric acid*. Adjust the volume to 3 L with *water R*.

Tris-hydrochloride Buffer Solution pH 8.8, 1.5M

Dissolve 90.8 g of *tris(hydroxymethyl)aminomethane R* in 400 mL of *water R*. Adjust the pH with *hydrochloric acid R* and dilute to 500.0 mL with *water R*.

Tris-hydrochloride Buffer Solution pH 8.8, 3M

Dissolve 363.3 g of *tris(hydroxymethyl)aminomethane R* in 500 mL of *water R*. Adjust the pH with *hydrochloric acid R* and dilute to 1 L with *water R*.

Tris-hydrochloride Buffer Solution pH 9.0, 0.05M

Dissolve 0.605 g of *tris(hydroxymethyl)aminomethane R* in *water R*. Adjust the pH with *1 M hydrochloric acid* and dilute to 100.0 mL with *water R*.

Tris(hydroxymethyl)aminomethane Buffer Solution pH 7.4

Dissolve 30.3 g of *tris(hydroxymethyl)aminomethane R* in approximately 200 mL of *water R*. Add 183 mL of *1 M hydrochloric acid*. Dilute to 500.0 mL with *water R*. *Note: the pH is 7.7-7.8 at room temperature and 7.4 at 37 °C. This solution is stable for several months at 4 °C.*

Tris(hydroxymethyl)aminomethane Buffer Solution pH 7.5 R1

Dissolve 1.21 g of *tris(hydroxymethyl)aminomethane R* in 900 mL of *water R* and add 10 mL of *0.01 M calcium chloride solution R*. Adjust the pH if necessary with *sodium hydroxide solution R* or *hydrochloric acid R*, and dilute to 1000.0 mL with *water R*.

Tris(hydroxymethyl)aminomethane Buffer Solution pH 9.0

Dissolve 1.21 g of *tris(hydroxymethyl)aminomethane R* in 950 mL of *water for chromatography R*. Adjust to pH 9.0 with *acetic acid R* and dilute to 1000.0 mL with *water for chromatography R*.

Tris(hydroxymethyl)aminomethane Buffer Solution pH 9.0 R1

Dissolve 12.1 g of *tris(hydroxymethyl)aminomethane R* in 950 mL of *water R*. Adjust to pH 9.0 with *acetic acid R* and dilute to 1000.0 mL with *water R*.

Tris(hydroxymethyl)aminomethane-EDTA Buffer Solution pH 8.4 R1

Dissolve 10.20 g of *sodium chloride R*, 6.10 g of *tris(hydroxymethyl)aminomethane R*, 2.80 g of *sodium edetate R* and 1.00 g of *macrogol 6000 R* or 2.00 g of *bovine albumin R* or of *human albumin R* in 800 mL of *water R*. Adjust to pH 8.4 with *hydrochloric acid R* and dilute to 1.0 L with *water R*.

Tris(hydroxymethyl)aminomethane Sodium Chloride Buffer Solution pH 7.4 R1

Dissolve 0.1 g of *bovine albumin R* in a mixture containing 2 mL of *tris(hydroxymethyl)aminomethane buffer solution pH 7.4 R* and 50 mL of a 5.84 mg/mL solution of *sodium chloride R*. Dilute to 100.0 mL with *water R*.

Tris-sodium Acetate Buffer Solution pH 7.4

Dissolve 6.3 g of *tris(hydroxymethyl)aminomethane R* and 4.9 g of *anhydrous sodium acetate R* in 900 mL of *water R*. Adjust to pH 7.4 with *sulfuric acid R* and dilute to 1000 mL with *water R*.

Tris-sodium Acetate Buffer Solution pH 8.0

Dissolve 6.3 g of *tris(hydroxymethyl)aminomethane R* and 4.9 g of *anhydrous sodium acetate R* in 900 mL of *water R*. Adjust to pH 8.0 with *sulfuric acid R* and dilute to 1000 mL with *water R*.

Tris-sodium Acetate-sodium Chloride Buffer Solution pH 7.4

Dissolve 30.0 g of *tris(hydroxymethyl)aminomethane R*, 14.5 g of *anhydrous sodium acetate R* and 14.6 g of *sodium chloride R* in 900 mL of *water R*. Add 0.50 g of *bovine albumin R*. Adjust to pH 7.4 with *sulfuric acid R* and dilute to 1000 mL with *water R*.

Tris-sodium Acetate-Sodium Chloride Buffer Solution pH 8.0

Dissolve 30.0 g of *tris(hydroxymethyl)aminomethane R*, 14.5 g of *anhydrous sodium acetate R* and 14.6 g of *sodium chloride R* in 900 mL of *water R*. Add 0.50 g of *bovine albumin R*. Adjust to pH 8.0 with *sulfuric acid R* and dilute to 1000 mL with *water R*.

E. Reference Materials

Where the letters *BPCRS* appear after the name of a substance in a test or assay, the British Pharmacopoeia Chemical Reference Substance is to be used.
A comprehensive and up-to-date list of British Pharmacopoeia Chemical Reference Substances, together with terms of trade and supply, is available on our website at www.pharmacopoeia.com. The substances are obtainable from the BPCRS Sales Office, MHRA, 10th Floor, 10 South Colonnade, Canary Wharf, London E14 4PU, United Kingdom (telephone +44 (0)20 3080 6561, e-mail: bpcom@mhra.gov.uk, website www.pharmacopoeia.com).

In addition to *BPCRS*, monographs of the British Pharmacopoeia may also refer to reference substances available from other suppliers. These are denoted by the letters *CRS*.

Where the letters *CRS* or *EPCRS* appear, the chemical reference substance issued by the European Pharmacopoeia Commission is to be used; where the letters *BRP* or *EPBRP* appear, the Biological Reference Preparation issued by the European Pharmacopoeia Commission is to be used; where the letters *HRS* or *EPHRS* appear, the herbal reference substance issued by the European Pharmacopoeia Commission is to be used. The substances, as well as European Pharmacopoeia infrared reference spectra, are obtainable from the Council of Europe, European Directorate for the Quality of Medicines & HealthCare, CRS Sales Team, 7 allée Kastner, CS 30026, F-67081, Strasbourg Cedex, France (facsimile +33 (0)3 88 41 27 71, e-mail: crs@pheur.org, website www.edqm.eu).

Other sources of specific reference substances are shown below.

Astragaloside I CRS, Astragaloside II CRS, Astragaloside IV CRS, azadirachtin A CRS, bacopaside I CRS, bacopaside II CRS, bacoside A CRS, Paeonol CRS, Rosmarinic acid CRS, salannin CRS, Salvianolic Acid B CRS, Tanshinone IIA CRS, Withaferin A CRS, Withanolide A CRS, Withanolide B CRS, Z-Ligustilide CRS may be obtained from Chromadex Inc. through LGC Standards, Queen's Road, Teddington, TW11 0LY, United Kingdom (telephone +44 (0)20 8943 8480, facsimile +44 (0)20 8943 7554, e-mail: uksales@lgcstandards.com).

Opacity, Standard Preparation of The Standard Preparation is the 5th International Reference Preparation, established in 1975, and consists of a rod of plastic simulating the optical properties of a bacterial suspension (10 Units of opacity). It may be obtained from the National Institute for Biological Standards and Control (NIBSC), Blanche Lane, South Mimms, Potters Bar, Hertfordshire, EN6 3QG, United Kingdom (telephone +44 (0) 1707 641000, e-mail: enquiries@nibsc.org).

Piperonyl Butoxide CRS may be obtained from LGC Standards, Queen's Road, Teddington, TW11 0LY, United Kingdom (telephone +44 (0)20 8943 8480, facsimile +44 (0)20 8943 7554, e-mail: uksales@lgcstandards.com).

F. Polymorphism

(Ph. Eur. method 5.9)

Polymorphism (or crystal polymorphism) is a phenomenon related to the solid state; it is the ability of a compound in the solid state to exist in different crystalline forms having the same chemical composition. Substances that exist in a non-crystalline solid state are said to be amorphous.

When this phenomenon is observed for a chemical element (for example, sulfur), the term allotropy is used instead of polymorphism.

The term pseudopolymorphism is used to describe solvates (including hydrates), where a solvent is present in the crystal matrix in stoichiometric proportions; the term may also be extended to include compounds where the solvent is trapped in the matrix in variable proportions. However the term

pseudopolymorphism is ambiguous because of its use in different circumstances. It is therefore preferable to use only the terms "solvates" and "hydrates".

Where a monograph indicates that a substance shows polymorphism, this may be true crystal polymorphism, occurrence of solvates, allotropy or occurrence of the amorphous form.

The identity of chemical composition implies that all crystalline and amorphous forms of a given species have the same chemical behaviour in solution or as a melt; in contrast, their physico-chemical and physical characteristics (solubility, hardness, compressibility, density, melting point, etc.), and therefore their reactivity and bioavailability may be different at the solid state.

When a compound shows polymorphism, the form for which the free enthalpy is lowest at a given temperature and pressure is the most thermodynamically stable. The other forms are said to be in a metastable state. At normal temperature and pressure, a metastable form may remain unchanged or may change to a thermodynamically more stable form.

If there are several crystalline forms, one form is thermodynamically more stable at a given temperature and pressure. A given crystalline form may constitute a phase that can reach equilibrium with other solid phases and with the liquid and gas phases.

If each crystalline form is the more stable within a given temperature range, the change from one form to another is reversible and is said to be enantiotropic. The change from one phase to another is a univariate equilibrium, so that at a given pressure this state is characterised by a transition temperature. However, if only one of the forms is stable over the entire temperature range, the change is irreversible or monotropic.

Different crystalline forms or solvates may be produced by varying the crystallisation conditions (temperature, pressure, solvent, concentration, rate of crystallisation, seeding of the crystallisation medium, presence and concentration of impurities, etc.).

The following techniques may be used to study polymorphism:

- X-ray diffraction of powders (*2.9.33*),
- X-ray diffraction of single crystals,
- thermal analysis (*2.2.34*) (differential scanning calorimetry, thermogravimetry, thermomicroscopy),
- microcalorimetry,
- moisture absorption analysis,
- optical and electronic microscopy,
- solid-state nuclear magnetic resonance,
- infrared absorption spectrophotometry (*2.2.24*),
- Raman spectroscopy (*2.2.48*),
- measurement of solubility and intrinsic dissolution rate,
- density measurement.

These techniques are often complementary and it is indispensable to use several of them.

Pressure/temperature and energy/temperature diagrams based on analytical data are valuable tools for fully understanding the energetic relationship (enantiotropism, monotropism) and the thermodynamic stability of the individual modifications of a polymorphic compound.

For solvates, differential scanning calorimetry and thermogravimetry are preferable, combined with measurements of solubility, intrinsic dissolution rate and X-ray diffraction.

For hydrates, water sorption/desorption isotherms are determined to demonstrate the zones of relative stability.

In general, hydrates are less soluble in water than anhydrous forms, and likewise solvates are less soluble in their solvent than unsolvated forms.

Appendix II

A. Infrared Spectrophotometry

(Absorption Spectrophotometry, Infrared, Ph. Eur. method 2.2.24)

PRINCIPLE

Infrared absorption spectrophotometry (also known as infrared (IR) spectroscopy) is based on the interaction of infrared radiation with matter. As a result of interaction between a molecule and IR radiation, absorption of frequencies specific to that molecule can occur, and some intermolecular and intramolecular vibrations can be excited to higher vibrational levels. This results in an infrared absorption spectrum with characteristic bands that correspond to the functional groups of the molecule.

The infrared wavelength region can be further divided into 3 subregions, namely near-infrared, mid-infrared and far-infrared. These subregions have wavelength ranges that are generally accepted by convention to be 0.8-2.5 µm, 2.5-25 µm and 25-1000 µm respectively. However, in IR spectroscopy, wavenumber is more commonly used than wavelength, and can be calculated using the following equation:

$$\tilde{\nu} = \frac{1}{\lambda} \cdot 10^4$$

where ν is the wavenumber in reciprocal centimetres (cm^{-1}) and λ is the wavelength in micrometres. Thus 12 500-4000 cm^{-1} is near-infrared, 4000-400 cm^{-1} is mid-infrared and 400-10 cm^{-1} is far-infrared.

This chapter concerns only spectroscopy in the mid-infrared region, i.e. 4000-400 cm^{-1} (2.5-25 µm), which hereafter is referred to as infrared for simplicity. This region is where the fundamental molecular vibrations of functional groups appear in the spectrum as absorption bands. The region below 1500 cm^{-1} is known as the 'fingerprint region', a very complex and informative part of the spectrum which characterises the molecule being investigated.

The mid-infrared region is flanked by the near-infrared region, where overtones and combinations of fundamental vibrations, mainly C-H, N-H and O-H functional groups, are detected (*2.2.40*) and the far-infrared region, where absorption bands associated with crystal lattice modes, hydrogen bonds, angle deformation vibrations of heavy atoms and molecular rotations are observed.

APPLICATIONS

As the absorption bands in IR spectra are characteristic of the constituent functional groups of a compound, IR spectroscopy is widely used to identify substances and provide information on their structure. It can also be used for quantitative applications, which requires establishing a mathematical relationship between the intensity of the radiation absorbed by the sample and the concentration of the investigated component in the sample.

IR spectroscopy is widely used in the pharmaceutical field for chemical and physical analysis in the laboratory, and has a wide variety of applications during the manufacturing process as outlined below. IR spectroscopy thereby enables the application of Process Analytical Technology (PAT) as part of an advanced control strategy.

Chemical analysis:

- identification of active substances, excipients, dosage forms, manufacturing intermediates, chemicals and packaging materials;
- quality assessment of active substances, excipients, dosage forms, manufacturing intermediates and packaging materials, including batch-to-batch spectral comparison and supplier change assessment;
- quantification of active substances in a sample matrix, determination of water and solvent content;
- quantification of impurities, e.g. in gases, inorganic materials;
- reaction monitoring, e.g. chemical synthesis.

Physical analysis:

- determination of solid-state properties such as polymorphism.

LIMITATIONS

Notable limitations to the use of IR spectroscopy include the following:

- it may be necessary to use additional techniques to unambiguously identify a substance;
- pure enantiomers of a substance cannot be discriminated;
- it may not be a suitable method for trace analysis;
- sample preparation conditions (e.g. pressure, solvent) may change the crystalline form of a substance that exhibits polymorphism;
- for heterogeneous samples, the limited sampling volume may be problematic.

MEASUREMENT MODES

IR measurements are based on passing radiation through or into a sample and measuring the attenuation of the emerging beam at various wavelengths. This corresponds to 2 main measurement modes, i.e. transmission and attenuated total reflection (ATR). However, other modes also exist for specific applications (e.g. diffuse and specular reflection).

TRANSMISSION MODE

This mode is based on determination of the transmittance (T), namely the ability of the sample to transmit IR radiation at a given wavelength (wavenumber). It is defined by the following ratio:

$$T = \frac{I}{I_0}$$

I_0 = intensity of incident radiation;
I = intensity of transmitted radiation.

The resulting spectrum is presented in terms of transmittance (T) on the y-axis versus wavelength or wavenumber on the x-axis. It can also be presented in terms of absorbance (A) on the y-axis, which is related to transmittance (T) by the following equation:

$$A = \log_{10}\left(\frac{1}{T}\right) = \log_{10}\left(\frac{I_0}{I}\right) = a \cdot b \cdot c$$

a = molar absorption coefficient of the sample, in square centimetres per mole ($cm^2 \cdot mol^{-1}$);
b = sample thickness, in centimetres;
c = sample concentration, in moles per cubic centimetre ($mol \cdot cm^{-3}$).

ATTENUATED TOTAL REFLECTION MODE

ATR mode is based on the phenomenon of total internal reflection. The sample, with a refractive index n_2, is brought into close contact with a crystal (diamond, germanium, zinc selenide or any other suitable material), having a refractive index n_1 which is greater than n_2. A beam of IR light is then

passed through the crystal. When the angle α between the incident beam and the sample-crystal interface exceeds a critical value α_c, theoretically all of the radiation is reflected (total internal reflection). However, an evanescent wave is produced which slightly penetrates the sample and part of the energy is absorbed. The total reflection is attenuated, which makes it possible to generate an absorption spectrum.
In practice, multiple internal reflections are often used to amplify the absorption intensity, although some accessories allow absorption measurements with a single reflection.

The penetration depth d_p is usually of the order of a few micrometres and is given for a wavelength λ by the following equation:

$$d_p = \frac{\lambda/n_1}{2\pi\sqrt{sin^2\ \alpha - (n_2/n_1)2}}$$

where d_p is the penetration depth, λ is the wavelength, α is the angle of incidence and n_1, n_2 are the refractive indices of the reflection element and the sample, respectively.

Due to the relationship between these parameters, the absorption intensity in ATR is greater at higher wavelengths (i.e. smaller wavenumbers) and slight band shifts occur compared to the corresponding transmission spectrum. It is therefore not advisable to compare ATR spectra with transmission spectra when identifying compounds.

EQUIPMENT

The most commonly used IR spectrometers are Fourier-transform (FT-IR) spectrometers which typically consist of:
- a suitable polychromatic light source, e.g. a conducting ceramic rod;
- an interferometer;
- a sample presentation accessory, e.g. a sample holder;
- a detector;
- appropriate software for controlling the spectrometer, and for spectral evaluation and data processing.

Other spectrometers based on alternative principles may also be used if the requirements described under Control of equipment performance are fulfilled.

IR spectrometers can also be used in association with a microscope for the study of a small part of the sample or for chemical imaging.

IR spectroscopy can be coupled to other analytical techniques such as thermal analysis or chromatography.

CONTROL OF EQUIPMENT PERFORMANCE

Accuracy of wavenumber scale and spectral resolution are critical parameters and must be verified. The tests described below can be used for the control of instrument performance and for qualification. They can also be used as system suitability tests.

These parameters are checked using suitable reference materials which are selected and presented depending on the measurement mode (e.g. transmission or ATR).

For quantitative analysis, appropriate assessment criteria for the control of absorption intensity must also be defined.

WAVENUMBER SCALE

The wavenumber scale is typically verified using a polystyrene film that exhibits IR absorption bands at the wavenumbers shown in Table 2.2.24.-1.

Table 2.2.24.-1 - *Band positions and associated acceptable tolerances of the polystyrene film used to verify wavenumber accuracy*

Band position (cm^{-1})		Tolerance (cm^{-1})
Transmission	ATR	
906.6	906.1	± 1.0
1028.3	1027.7	± 1.0
1601.2	1601.0	± 1.0
3060.0	3059.7	± 1.0

For measurement modes other than transmission or ATR, reference materials must be defined by the user.

SPECTRAL RESOLUTION

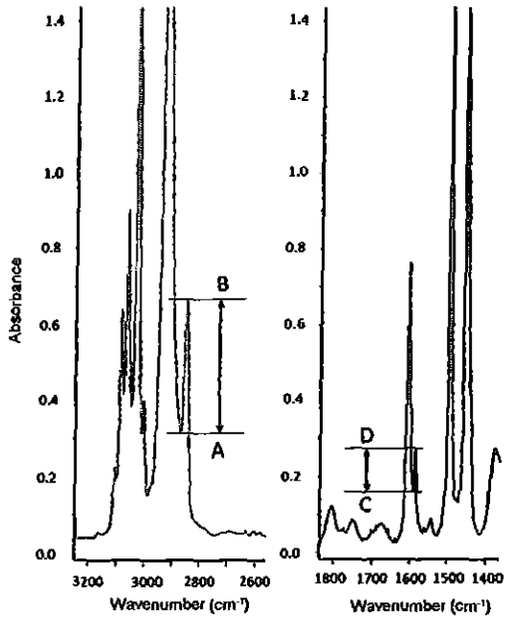


Figure 2.2.24.-1. – *Typical IR absorbance spectrum of polystyrene used to verify spectral resolution*

Spectra recorded in transmission mode

The spectral resolution is typically verified using a polystyrene film approximately 35 µm thick.

Acceptance criteria (see Figure 2.2.24.-1) The difference between the absorbance values at the absorption minimum at 2870 cm^{-1} (*A*) and the absorption maximum at 2849.5 cm^{-1} (*B*) is greater than 0.33; the difference between the absorbance values at the absorption minimum at 1589 cm^{-1} (*C*) and the absorption maximum at 1583 cm^{-1} (*D*) is greater than 0.08.

Spectra recorded in ATR mode

Appropriate assessment criteria for the control of spectral resolution according to the specifications of each instrument need to be defined.

For measurement modes other than transmission or ATR, reference materials have to be defined by the user.

PROCEDURE

SAMPLE PREPARATION AND PRESENTATION

Sample preparation and presentation vary according to the physical state of the sample and the measurement mode.

Transmission mode is applied to transparent samples, such as neat liquids, solutions, gases or suitably prepared mulls and alkali halide discs. For liquids and gases, cells with fixed or variable pathlength and IR transparent windows can be used. For alkali-halide disks, specific sample holders are used. Reflection mode, e.g. ATR, is appropriate for the measurement of a wide range of samples in the solid and liquid state.

Some preparation modes (e.g. for discs and mulls in transmission mode or for solids in ATR mode) involve grinding and/or the application of pressure, which may induce unexpected crystal modifications.

Transmission mode

Prepare the substance by one of the following methods depending on the sample state (solid, liquid or gas). Sample bands in a spectrum have a minimum transmittance not lower than 5 per cent, unless otherwise justified.

Liquids Examine liquids either in the form of a film between 2 plates transparent to infrared radiation or in a cell of suitable pathlength with windows that are transparent to infrared radiation.

Liquids or solids in solution Prepare a solution of the substance to be examined in a suitable solvent. Choose a concentration and a pathlength that give a satisfactory spectrum. Generally, good results are obtained with concentrations of 10-100 g/L for a pathlength of 0.5-0.1 mm. The absorption due to the solvent is usually compensated by successively recording the spectra of the solvent and the sample solution and subtracting the solvent absorption bands from the spectrum of the sample solution.

Solids dispersed in a solid (disc) Grind the substance to be examined taking into consideration any possible changes (e.g. crystalline form) and mix with a suitable amount of finely powdered and dried *potassium bromide R* or *potassium chloride R*, unless otherwise specified. A mixture of a few milligrams (e.g. 1-2 mg) of the substance to be examined in a few hundred milligrams (e.g. 300-400 mg) of halide is normally sufficient to give a disc of 10-15 mm diameter and a spectrum of suitable intensity. If the substance is a hydrochloride salt, it is recommended to use *potassium chloride R*. Carefully grind the mixture, spread it uniformly in a suitable die and apply a suitable pressure. A compacting force of about 800 MPa is generally sufficient to prepare a disc. For substances that are unstable under normal atmospheric conditions or are hygroscopic, the disc may be pressed under vacuum. Several factors may cause the formation of faulty discs, such as insufficient or excessive grinding, humidity or impurities in the dispersion medium. For example, any water in either the sample or the potassium bromide will cause clouding of the disc and produce a low transmission spectrum. A disc is rejected if visual examination shows a lack of uniform transparency or when, in the absence of a specific absorption band, the transmittance is less than 60 per cent or the absorbance is more than 0.22 at about 2000 cm^{-1} (5 µm) and without compensation, unless otherwise prescribed.

Solids dispersed in a liquid (mull) Triturate a small quantity of the substance to be examined with the minimum quantity of *liquid paraffin R* or other suitable liquid. A mixture of a few milligrams (e.g. 5-10 mg) of the substance to be examined in 1 drop of *liquid paraffin R* is generally sufficient to make an adequate mull. Compress the mull between 2 plates transparent to infrared radiation. A mull is rejected if a visual examination shows lack of uniform transparency or where the spectrum shows features such as:

- low transmission at 4000 cm^{-1};
- a strongly sloping baseline between 4000 and about 2500 cm^{-1};
- a ratio of relative intensities of some absorption bands that is less than expected.

Molten solids If prescribed in the monograph, make a film of a molten mass and fix it on a suitable mount.

Evaporated solution If prescribed in the monograph, dissolve the substance to be examined in a suitable solvent. Prepare a film by evaporating the solvent on a suitable carrier and fix it on a suitable mount.

Gases Use a suitable cell transparent to infrared radiation. Evacuate the air from the cell and fill to the desired pressure through a stopcock or needle valve using a suitable gas transfer line between the cell and the container of the gas to be examined. If necessary, adjust the pressure in the cell to atmospheric pressure using a gas transparent to infrared radiation (e.g. *nitrogen R* or *argon R*), or purge with carbon dioxide-free air. An appropriate measurement protocol must be followed to compensate for water, carbon dioxide or other atmospheric gases.

ATR mode

ATR is suitable for liquid and solid samples, and requires no preparation apart from simple treatments such as the grinding of large crystals and coarse material. Proceed as follows depending on the sample state (liquid or solid).

Liquids Place the sample in contact with the crystal.

Solids Ensure close and uniform contact between the substance to be examined and the whole crystal surface, either by applying pressure or by dissolving the substance in an appropriate solvent, then covering the crystal with the resulting solution and evaporating to dryness.

METHODS

Infrared spectroscopy is mostly used to identify substances, but it may also be carried out for quantitative applications. Quantitative analysis (based on the Beer-Lambert law, which relates the absorbance of a sample to its concentration) will not be described in this chapter.

The measurement is performed on an appropriately prepared sample. The data is then processed and evaluated, either to identify substances or quantify them (e.g. based on integration of IR-absorption bands).

Spectral quality may be enhanced by mathematical pretreatments. In practice, these are limited to spectral normalisation and subtraction of bands caused by carbon-dioxide and water vapour. The same pretreatments are performed on both the sample and the reference spectra.

Identification

Prepare the substance to be examined appropriately and record the spectra between 4000 and 650 cm^{-1}, unless otherwise prescribed.

Identification testing is performed by comparing the spectrum of the substance to be examined with the spectrum obtained from a Ph. Eur. chemical reference substance (CRS) or with a Ph. Eur. reference spectrum.

The spectrum of the current batch of the Ph. Eur. CRS may be recorded for immediate use or stored, for example, in a spectral library for future consultation. A stored spectrum may be used, provided traceability to the current batch of CRS is ensured.

In the case of substances that are not covered by individual monographs, a suitable reference standard may be used.

In all cases, spectra must be recorded using the same operating conditions and procedure, and especially the same measurement mode.

When comparison of the spectra recorded in the solid state show differences (see below), treat the substance to be examined and the reference substance in the same manner so that they recrystallise or are produced in the same crystalline form, or proceed as prescribed in the monograph, then record the spectra again. However, this procedure must only be done for substances where the monograph does not cover a particular form of a substance that exhibits polymorphism.

Several comparison procedures may be used, and the analyst must document and justify the method used and the specific acceptance criteria that allow a conclusion for identification. The spectra can be compared either by overlaying the spectra (in the whole spectral range or in the region of interest specified in the monograph) or by using mathematical calculations from the software. It is possible for example to perform:

- visual comparison based on band positions and relative intensities unless otherwise specified - the transmission minima (or absorption maxima) in the spectrum obtained with the substance to be examined correspond in position and relative size to those of the reference;
- calculation of the correlation coefficient between the 2 spectra - this value is calculated by the software and the identification threshold is defined by the user;
- evaluation by chemometric methods (e.g. Euclidean distance, Mahalanobis distance, classification methods); these methods involve the set-up, assessment and validation of the chemometric model by the analyst (see *5.21. Chemometric methods applied to analytical data*).

Impurities in gases

For the analysis of impurities, use a cell transparent to infrared radiation and of suitable optical pathlength (e.g. 1-20 m). Fill the cell as prescribed under Gases. For detection and quantification of the impurities, proceed as prescribed in the monograph.

Near-infrared Spectrophotometry

(Ph. Eur. method 2.2.40)

Near-infrared (NIR) spectroscopy is a technique with wide and varied applications in pharmaceutical analysis. The NIR spectral range extends from 780 nm to 2500 nm (from 12 800 cm^{-1} to 4000 cm^{-1}). NIR spectra are dominated by C-H, N-H, O-H and S-H overtones and combinations of fundamental mid-infrared (MIR) vibrations. They contain composite chemical and physical information and in most cases this information can be extracted by suitable mathematical data treatment. NIR bands are much weaker than the fundamental MIR vibrations from which they originate. Because absorptivities in the NIR range are low, radiation can penetrate up to several millimetres into materials, including solids. Furthermore, many materials such as glass are relatively transparent in this region.

Measurements can be made directly *in situ*, in addition to standard sampling and testing procedures.

NIR measurements can be performed off-line, and also at-line or in-line, and on-line for process analytical technology (PAT). Suitable chemometric methods may be required for identification. However, when the specificity criteria for a qualitative method are met, chemical identification or solid-state characterisation is possible by direct comparison of the untreated or pre-treated spectra obtained with the chemical substance being examined with a spectrum of a reference substance.

NIR spectroscopy has a wide variety of applications for chemical, physical and process analysis, for example:

Chemical analysis:

- identification of active substances, excipients, dosage forms, manufacturing intermediates, chemical materials and packaging materials;
- qualification of active substances, excipients, dosage forms, manufacturing intermediates and packaging materials, including batch-to-batch spectral comparison and supplier change assessment;
- quantification of active substances in a sample matrix, determination of chemical values such as hydroxyl value, determination of absolute water content, determination of degree of hydroxylation and control of solvent content.

Physical analysis:

- crystalline form and crystallinity, polymorphism, solvates, particle size;
- disintegration, hardness;
- film properties.

Process analysis:

- monitoring of unit operations such as synthesis, crystallisation, blending, drying, granulation and coating, for the purpose of process control;
- control and endpoint detection.

Measurements in the NIR region are influenced by many chemical and physical factors as described below; the reproducibility and relevance of results depend on control of these factors and measurements are usually valid only for a defined calibration model.

APPARATUS

All NIR measurements are based on passing light through or into a sample and measuring the attenuation of the emerging (transmitted or reflected) beam. Spectrometers for measurement in the NIR region consist of a suitable light source (such as a highly-stable quartz-tungsten lamp), a monochromator or interferometer, and a detector. Common monochromators are acousto-optic tunable filters (AOTF), gratings or prisms. Traditionally, many NIR instruments have a single-beam design, though some process instruments use internal referencing and can therefore be dual-beam (for example in diode array instruments). Silicon, lead sulfide, and indium gallium arsenide are examples of detector materials. Conventional cuvette sample holders, fibre-optic probes, transmission dip cells, neutral borosilicate vials and spinning or traversing sample holders are a few examples of sampling devices. The selection is based on the intended application, paying particular attention to the suitability of the sampling system for the type of sample to be analysed. Suitable data processing and evaluation units (e.g. software and computer) are usually part of the system.

It is common to express the wavelength (λ) in nanometres and the wavenumber (υ) in reciprocal centimetres (cm^{-1}), depending on the measurement technique and apparatus. Conversion between nm and cm^{-1} is performed according to the following expression:

$$\nu_{cm^{-1}} = 10^7 \times \frac{1}{\lambda_{nm}}$$

MEASUREMENT METHODS

Transmission mode

Transmittance (T) is a measure of the decrease in radiation intensity at given wavelengths when radiation is passed through the sample. The sample is placed in the optical beam between the source and the detector. The arrangement is analogous to that in many conventional spectrometers.

The resulting spectrum can be presented directly in terms of transmittance (T) and/or absorbance (A) (y-axis) versus the wavelength or wavenumber (x-axis).

$$T = \frac{I}{I_0}$$

I_0 = intensity of incident radiation;
I = intensity of transmitted radiation;

$$A = -\log_{10} T = \log_{10}\left(\frac{1}{T}\right) = \log_{10}\left(\frac{I_0}{I}\right)$$

Diffuse reflection mode

The diffuse reflection mode gives a measure of reflectance (R), the ratio of the intensity of light reflected from the sample (I) to that reflected from a background or reference reflective surface (I_r). Depending on the chemical composition and physical characteristics of the sample, NIR radiation can penetrate a more or less substantial distance into the sample, where it can be absorbed by the overtones and combinations of the fundamental vibrations of the analyte species present in the sample. Non-absorbed radiation is partially reflected back from the sample to the detector. NIR reflectance spectra are typically obtained by calculating and plotting $\log_{10}(1/R)$ (y-axis) versus the wavelength or wavenumber (x-axis).

$$R = \frac{I}{I_r}$$

I = intensity of light diffusively reflected from the sample;
I_r = intensity of light reflected from the background or reference reflective surface;

$$A_R = \log_{10}\left(\frac{1}{R}\right) = \log_{10}\left(\frac{I_r}{I}\right)$$

Transflection mode

This mode is a combination of transmittance and reflectance. In the measurement of transflectance (T^*), a mirror or a diffuse reflectance surface is used to reflect the transmitted radiation back through the sample, thus doubling the pathlength. Non-absorbed radiation is reflected back from the sample to the detector. The resulting spectrum can be presented directly in terms of transflectance (T^*) and/or absorbance (A^*) (y-axis) versus the wavelength or wavenumber (x-axis).

$$T^* = \frac{I}{I_T}$$

I = intensity of transflected radiation measured from the sample;
I_T = intensity of transflected radiation of the reference material as background;

$$A^* = \log_{10}\left(\frac{1}{T^*}\right) = \log_{10}\left(\frac{I_T}{I}\right)$$

SAMPLE PREPARATION/PRESENTATION

Sample preparation and presentation may vary according to the measurement mode. The following requirements are necessary for all sampling techniques:

- optimise the measuring time and number of scans to optimise the signal-to-noise ratio;
- find the best suitable measurement mode for the intended application (transmission, diffuse reflection or transflection);
- find the best orientation of the sample (e.g. to minimise the impact of debossing on tablets);
- find the best suitable accessory (e.g. transmission cell or immersion probe);
- optimise pathlength in transmission and transflection modes;
- find a suitable spectroscopic background reference material;
- show that the background reference material is reliable over time and that the measurement of the background is reproducible and stable over time;
- when measuring moving materials or samples (for process-related measurements) it is important to obtain a representative spectrum (e.g. by adjusting the measuring time, the number of scans, co-adding individual spectra, or increasing the beam size);
- ensure there is no fouling of the sensor, for example with build-up of material or contamination;
- the measuring conditions (measuring time, beam size) in relation to the minimal sample size should be justified.

In some process analysis situations it may be impossible to remove a probe for reference background data collection; various options are therefore to be considered, including internal referencing, measurement of a background reference using a 2nd detector, etc. Only spectra measured against a background possessing the same optical properties can be directly compared with one another.

Transmission mode

The measurement of transmittance (T) is dependent on a background transmittance spectrum for its calculation. Examples of background references include air, a polymeric disc, an empty cell, a solvent blank or in special cases a reference sample. The method generally applies to liquids (diluted or undiluted), dispersions, solutions and solids (including tablets and capsules). For transmittance measurements of solids, a suitable sample accessory is used. Liquid samples are examined in a cell of suitable pathlength (typically 0.5-4 mm), transparent to NIR radiation, or alternatively by immersion of a fibre-optic probe of a suitable configuration.

Diffuse reflection mode

This mode generally applies to solids. The sample is examined directly, or in a suitable device (for example a sample holder), or in direct contact with a fibre-optic probe. For process monitoring, material can be analysed through a polished window interface (e.g. sapphire), or using an in-line fibre-optic probe. Care must be taken to ensure that the measuring conditions are as reproducible as possible from one sample to another. The reflected radiation of a background reference is scanned to obtain the baseline, and then the reflectance of one or more analytical samples is measured. Common reflectance references include ceramic, thermoplastic resins and gold. Other suitable materials may be used.

Transflection mode

This mode generally applies to liquids, suspensions and clear plastic materials. A reflector is placed behind the sample so as to double the pathlength. This configuration can be adopted to share the same instrument geometry with reflectance and fibre-optic probe systems where the source and the detector are on the same side of the sample.
The sample is examined through a cell with a mirror or a suitable diffusive reflector, made either of metal or of an inert substance (for example, dried titanium dioxide) not

absorbing in the NIR region. Liquids can also be measured using in-line transflectance probes.

FACTORS AFFECTING SPECTRAL RESPONSE

Environment

The environment temperature and humidity must be taken into consideration before carrying out measurements.

Sample presentation area

The sample presentation area or probe end must be clean of residue prior to measurement. Similarly, the in-line or on-line interface to the sample should not have significant product or contamination build-up, which would interfere with the desired measurement.

Sample temperature

This parameter is important for aqueous solutions and many liquids, where a difference of a few degrees can result in measurable spectral changes which may have a significant effect on the analysis. Temperature is also an important parameter for solids and powders containing water.

Moisture and solvent residues

Moisture and solvent residues present in the samples will contribute significant absorption bands in the NIR region.

Sample thickness

Sample thickness is a known source of spectral variability and must be assessed and/or controlled, particularly for tablet and capsule analysis in transmittance mode. For the measurement of compressed powders, an infinite thickness is typically reached after 5 mm of sample depth (e.g. in a vial).

Sample optical properties

In solids, both surface and bulk scattering properties of samples must be taken into account. Spectra of physically, chemically or optically heterogeneous samples may require increasing the beam size, or examining multiple samples or spinning the sample to obtain a representative spectrum of the sample. Certain factors such as differing degree of compaction or particle size in powdered materials and surface finish can cause significant spectral differences.

Solid-state forms

Variations in solid-state forms (polymorphs, hydrates, solvates and amorphous forms) influence vibrational spectra. Hence, different crystalline forms as well as the amorphous form of a solid may be distinguished from one another on the basis of their NIR spectra. Where multiple crystalline forms are present, care must be taken to ensure that the calibration samples have a distribution of forms relevant to the intended application.

Age of samples

Samples may exhibit changes in their chemical, physical or optical properties over time. Depending on the storage conditions, solid samples may either absorb or desorb water, and portions of amorphous material may crystallise. Materials used for NIR calibration should be representative of future samples and their matrix variables.

PRE-TREATMENT OF NIR SPECTRAL DATA

In many cases, and particularly for reflection mode spectra, some form of mathematical pre-treatment of the spectrum may be useful prior to the development of a classification or calibration model. The aim can be, for example, to reduce baseline variations, to reduce the impact of known variations that are interfering in the subsequent mathematical models, or to simplify data before use. In some cases spectra may also be normalised or corrected for scatter, for example using standard normal variate (SNV) transformation. Spectral pre-treatment techniques may include, for example, windowing and noise reduction and the numerical calculation of the first- or second-order derivative of the spectrum. Higher-order derivatives are not recommended because of increased spectral noise.

CONTROL OF INSTRUMENT PERFORMANCE

Use the apparatus according to the manufacturer's instructions and carry out the prescribed verification at regular intervals, according to the use of the apparatus and the application. For in-line and on-line applications, the use of alternative means of control of instrument performance must be scientifically justified. For example, utilise the standards built into the instrument or separate channels/probes to demonstrate instrument performance (pending practicality).

System suitability tests may be required prior to sample scanning, and the instrument attributes with potential impact on suitability of the final measurement (typically photometric noise and wavelength accuracy) must be tested.

The frequency at which each performance test is carried out must be risk-assessed depending on the instrument type and its environment. For example, instruments placed in harsh environments with variations in temperature and humidity may need frequent performance testing. Cases where the measurement system cannot be removed such as an in-line probe or flow cell are also to be considered.

Some accessories are custom designed and therefore require adequate performance testing.

Verification and calibration of the wavelength or wavenumber scale (except for filter apparatus) Verify the wavelength scale employed, generally in the region between 780 nm and 2500 nm (12 800 cm^{-1} to 4000 cm^{-1}) or in the intended spectral range using one or more suitable wavelength standards which have characteristic maxima or minima within the wavelength range to be used.

For example, *methylene chloride R*, *talc R*, wavelength reference lamps or a mixture of rare-earth oxides are suitable reference materials. Other suitable standards may also be used. Obtain a spectrum and measure the position of at least 3 peaks distributed over the range used. For rare-earth oxides, the National Institute of Standards and Technology (NIST) provides suitable reference materials. Fourier transform (FT) instruments have a linear frequency range, therefore wavelength certification at a single frequency is sufficient.

Verification and calibration of photometric linearity

The photometric linearity is demonstrated with a set of transmittance or reflectance standards with known percentage values of transmittance or reflectance. For reflectance measurements, carbon-doped polymer standards are available. Ensure that the absorbance of the materials used is relevant to the intended linear working range of the method. Subsequent verifications of photometric linearity can use the initial observed absorbance values as the reference values. Non-linear calibration models and hence non-linear responses are acceptable with understanding demonstrated by the user.

Spectra obtained from reflectance and transmittance standards are subject to variability due to the differences between the experimental conditions under which they were factory-calibrated and those under which they are subsequently put to use. Hence, the percentage reflectance values supplied with a set of calibration standards may not be useful in the attempt to establish an 'absolute' calibration for a given instrument. As long as the standards do not change chemically or physically and the same reference background

is used as was used to obtain the certified values, subsequent measurements of the same standards under identical conditions, including precise sample positioning, give information on long-term stability of the photometric response. A tolerance of ± 2 per cent of the absorbance value is acceptable for long-term stability; this verification is only necessary if the spectra are used without pre-treatment.

Recommendations for the conditions used to control instrument performance for the various measurement modes are summarised in Table 2.2.40.-1.

QUALITATIVE ANALYSIS (IDENTIFICATION AND CHARACTERISATION)

Establishment of a spectral reference library

Record the spectra of a suitable number of representative samples of the substance which have known, traceable identities, and that exhibit the variation typical for the substance to be analysed (for example, solid-state form, particle size, etc.). Libraries are built using representative samples under appropriate environmental conditions. The set of spectra obtained represents the information which can be used for identification of the sample to be analysed.

The collection of spectra in the library may be represented in different ways defined by the mathematical technique used for identification. These may be:

— all individual spectra representing the substance;
— a mean spectrum of the measured batches for each chemical substance;
— if necessary, a description of the variability within the substance spectra.

The number of substances in the library depends on the specific application. All spectra in the library used have the same:

— spectral range and number of data points;
— technique of measurement;
— data pre-treatment.

If sub-groups (sub-libraries) are created, the above criteria are applied independently for each group. Sub-libraries are individually validated. Original spectral data for the preparation of the spectral library must be archived. Caution must be exercised when performing any mathematical transformation, as artefacts can be introduced or essential information (important with qualification methods) can be lost. The suitability of the algorithm used should be demonstrated by successful method validation and in all cases the rationale for the use of transform must be documented.

Direct comparison of substance and reference spectra

Direct comparison of representative spectra of the substance to be examined and of a reference substance for qualitative chemical or physical identification purposes may not require use of a reference spectral library where specificity permits.

Data evaluation

Direct comparison of the representative spectrum of the substance to be examined is made with the individual or mean reference spectra of all substances in the database on the basis of their mathematical correlation or other suitable algorithms. A set of known reference mean spectra and the variability around this mean can be used with an algorithm for classification; alternatively, this can be achieved visually by overlaying spectral data if specificity is inherent. There are different techniques available, such as principal component analysis (PCA), cluster analysis, and soft independent modelling by class analogy (SIMCA). The reliability of the technique chosen for a particular application has to be validated according to the following:

Validation of the model

Identification methods using direct spectral comparison must be validated in accordance with identification method validation procedures.

The validation parameters for qualitative methods are robustness and specificity.

LIMIT ANALYSIS

Relative comparison of spectra

A calibration is not required when comparing a set of spectra for limit analysis purposes, such as the maximum or minimum absorbance at which an analyte absorbs. Also, dryer end point control may use a qualitative approach around a specific absorbing wavelength. Appropriate spectral ranges and pre-treatments (if used) must be shown to be fit for purpose.

Specificity

The relative discriminatory power for a limit test must be demonstrated. The extent of specificity testing is dependent on the application and the risks being controlled. Variations in matrix concentrations within the operating range of the method must not affect the measurement.

TREND ANALYSIS

Relative comparison of spectra

A calibration is not necessarily required when comparing a set of spectra for trend analysis purposes, such as the moving block approach to estimate statistical parameters such as mean, median and standard deviation. For example, blend uniformity monitoring using NIR spectroscopy has adopted such data analysis approaches. Appropriate spectral ranges and algorithms must be used for trend analyses.

Specificity

The relative discriminatory power for trend analysis must be demonstrated. The extent of specificity testing is dependent on the application and the risks being controlled. Variations in matrix concentrations within the operating range of the method must not affect the trend analysis.

QUANTITATIVE ANALYSIS

Establishment of a spectral reference library for a calibration model

Calibration is the process of constructing a mathematical model to relate the response from a sample scanned using an analytical instrument to the properties of the samples.
Any calibration model that can be clearly defined in a mathematical expression and gives suitable results can be used. Record the spectra of a suitable number of representative samples with known or future-established values of the attribute of interest throughout the range to be measured (for example, content of water). The number of samples for calibration will depend on the complexity of the sample matrix and interferences (e.g. temperature, particle size, etc.). All samples must give quantitative results within a calibration interval as defined by the intended purpose of the method. Multiple linear regression (MLR), principal component regression (PCR) and partial least squares regression (PLS) are commonly used algorithms. For PLS or PCR calibrations, the regression coefficients and/or the loadings should be plotted and the regions of large coefficients or loadings compared with the spectrum of the analyte. Predicted residual error sum of squares (PRESS) plots (or similar) are useful to facilitate the optimising of the number of PCR or PLS factors.

Pre-treatment of data

Wavelength selection or excluding certain wavelength ranges may enhance the accuracy and robustness of calibration

Table 2.2.40.-1 – *Control of instrument performance*

Measurement mode	Reflection	Transflection	Transmission
Verification of wavelength scale (except for filter apparatus)	Typical tolerances for agreement with standard values are: ± 1.0 nm at 780 nm (± 16 cm^{-1} at 12 800 cm^{-1}); ± 1.0 nm at 1200 nm (± 8 cm^{-1} at 8300 cm^{-1}); ± 1.0 nm at 1600 nm (± 6 cm^{-1} at 6250 cm^{-1}); ± 1.5 nm at 2000 nm (± 4 cm^{-1} at 5000 cm^{-1}); ± 1.5 nm at 2500 nm (± 2 cm^{-1} at 4000 cm^{-1}). For the reference material used, apply the tolerance for the nearest wavelength or wavenumber for each peak used. For diode array instruments, most often the pixel resolution (wavelength between pixels) can be as large as 10 nm. The pixel resolution must be adapted to match the spectral resolution. The peak-finding algorithms are critical to wavelength accuracy. Practically, ± 2 nm is appropriate for peak wavelength accuracy using such instrumentation. Alternatively, refer to manufacturer's specifications for acceptance.		
Bench/mobile instrument	Measure *talc R* via a suitable medium or by fibre-optic probe. *Talc R* has characteristic peaks at 948 nm, 1391 nm and 2312 nm suitable for calibration. Alternatively, other suitable standards may also be used that ensure wavelength accuracy in the region of working methodology. For example, measure an internal polystyrene standard if built-in, or measure a NIST standard or other traceable material, and assess 3 peaks across the wavelength range for calibration.	A suspension of 1.2 g of dry *titanium dioxide R* in about 4 mL of *methylene chloride R* is used directly with a cell or a probe. Titanium dioxide has no absorption in the NIR range. Spectra are recorded with a maximum nominal instrument bandwidth of 10 nm at 2500 nm (16 cm^{-1} at 4000 cm^{-1}). Methylene chloride has characteristic sharp bands at 1155 nm, 1366 nm, 1417 nm, 1690 nm, 1838 nm, 1894 nm, 2068 nm and 2245 nm. Choose 3 peaks across the wavelength range for calibration. Other suitable standards may also be used, such as a liquid transflection standard mixed with titanium dioxide or some other reflective medium.	*Methylene chloride R* may be used and has characteristic sharp bands at 1155 nm, 1366 nm, 1417 nm, 1690 nm, 1838 nm, 1894 nm, 2068 nm and 2245 nm. Choose 3 peaks across the wavelength range for calibration. Other suitable standards may also be used.
Process instrument	If it is not practically possible to measure a traceable reference material at the point of sample measurement, use internal material such as polystyrene, fibreglass or solvent and/or water vapour. Alternatively, adopt a second external channel/probe. For FT instruments, the calibration of the wavenumber scale may be performed using a narrow, isolated water-vapour line, for example, the line at 7306.74 cm^{-1}, or 7299.45 cm^{-1}, or 7299.81 cm^{-1} or a narrow line from a certified reference material.		

Verification of wavelength repeatability (except for filter apparatus)	The standard deviation of the wavelength is consistent with the specifications of the instrument manufacturer, or otherwise scientifically justified.
Bench/mobile instrument	Verify the wavelength repeatability using a suitable external or internal standard.
Process instrument	Verify the wavelength repeatability using a suitable external or internal standard.

Verification of photometric linearity and response stability[1]	Measure 4 photometric standards across the working method absorbance range.		
Bench/mobile instrument	Analyse 4 reflectance standards, for example in the range of 10-99 per cent, including 10 per cent, 20 per cent, 40 per cent and 80 per cent. In some circumstances 2 per cent may be appropriate. Evaluate the observed absorbance values against the reference absorbance values, for example perform a linear regression. Acceptable tolerances are 1.00 ± 0.05 for the slope and 0.00 ± 0.05 for the intercept for the 1st verification of photometric linearity of an instrument. Subsequent verifications of photometric linearity can use the initial observed absorbance values as the reference values.	Transflection measurements can use appropriate reflectance or transmittance standards and criteria.	Analyse 4 transmittance standards to cover the absorbance values over the working absorbance range of the modelled data. Evaluate the observed absorbance values against the reference absorbance values, for example perform a linear regression. Acceptable tolerances are 1.00 ± 0.05 for the slope and 0.00 ± 0.05 for the intercept for the 1st verification of photometric linearity of an instrument. Subsequent verifications of photometric linearity can use the initial observed absorbance values as the reference values.
Process instrument	If photometric reflectance and transmittance standards cannot be measured at the point of sample measurement, use the photometric standards built into the instrument. Process instruments can use internal photometric standards for photometric linearity. Follow the manufacturer's verified tolerances in such cases.		

[1] *Verification of photometric linearity* and *Verification of photometric noise* are not required for instruments using methods to perform simple identifications which do not use the photometric absorbances as part of model strategy (for example, simple correlation with absorbing wavelengths).

Measurement mode	Reflection	Transflection	Transmission
Verification of photometric noise[1]	Determine the photometric noise at a relevant photometric region of the spectrum using a suitable reflectance standard, for example, white reflective ceramic tiles or carbon-doped polymer standards. Follow the manufacturer's methodology and specifications.		
Bench/mobile instrument	Scan the reflectance low flux standard (e.g. 5 or 10 per cent, carbon-doped polymer standard) over a suitable wavelength range in accordance with the manufacturer's recommendation and calculate the photometric noise as peak-to-peak noise.		Scan the transmittance high flux standard (e.g. 90 or 99 per cent, carbon-doped polymer standard) over a suitable wavelength/wavenumber range in accordance with the manufacturer's recommendation and calculate the photometric noise as peak-to-peak noise.
Process instrument	As above, or if not practically possible, use the standard built into the instrument for noise testing and manufacturer specifications.		As above, or if not practically possible, use the standard built into the instrument for noise testing and manufacturer specifications.

[1] *Verification of photometric linearity* and *Verification of photometric noise* are not required for instruments using methods to perform simple identifications which do not use the photometric absorbances as part of model strategy (for example, simple correlation with absorbing wavelengths).

models. Wavelength compression (wavelength averaging) techniques may be applied to the data.

Model validation parameters

Analytical performance characteristics to be considered for demonstrating the validation of NIR methods are similar to those required for any analytical procedure. Specific acceptance criteria for each validation parameter must be consistent with the intended use of the method. Validation parameters for quantitative methods are accuracy, linearity, precision (repeatability and intermediate precision), robustness and specificity.

ONGOING MODEL EVALUATION

NIR models validated for use are subjected to ongoing performance evaluation and monitoring of validation parameters.

TRANSFER OF DATABASES

When databases are transferred to another instrument, spectral range, number of data points, spectral resolution and other parameters have to be taken into consideration. Further procedures and criteria must be applied to demonstrate that the model remains valid with the new database or new instrument.

B. Ultraviolet and Visible Absorption Spectrophotometry

(Ph. Eur. method 2.2.25)

PRINCIPLE

Ultraviolet and visible (UV-Vis) spectroscopy (or spectrophotometry) is based on the ability of atoms, molecules and ions to absorb light at specific wavelengths in the ultraviolet (approximately 180-400 nm) and visible (approximately 400-800 nm) range. This absorption is associated with changes in electronic energy in the form of temporary transitions of electrons to an excited state at a higher energy orbital. As each energy level of a molecule or molecular ion also has associated vibrational and rotational sub-levels, this results in many permitted transitions, which are generally impossible to separate, thereby producing absorption bands rather than sharp lines. These bands are characteristic of the functional groups and bonds in a molecule.

UV-Vis spectroscopy measurements involve exposing a sample to light and measuring the attenuation and/or scattering of the emerging (transmitted or reflected) light either at a single wavelength or over a specified wavelength range.

APPLICATIONS

UV-Vis spectroscopy is traditionally used for the quantitative and qualitative analysis of liquid samples, but is also suitable for solid and gaseous analytes and has other applications such as the determination of physical or chemical properties.

UV-Vis spectroscopy as described in this chapter can be applied in various ways:

- when a monograph or general chapter refers to this chapter, the requirements described in the relevant paragraphs of this chapter are mandatory;
- when used as the detection method in chromatographic systems as described in general chapter *2.2.46*, the requirements listed in the relevant paragraphs of this chapter are mandatory;
- when used as a process analytical technology (PAT) tool for PAT applications similar to the applications described in this chapter, the provisions herein apply; for other PAT applications, the principles are the same, however the criteria are established bearing in mind the intended purpose of the analysis, using a risk-based approach.

EQUIPMENT

Spectrophotometers used for carrying out measurements in the UV-Vis region typically consist of:

- a suitable light source (such as a deuterium lamp for the UV region, a tungsten-halogen lamp for the visible region or a xenon lamp to cover the entire UV-Vis range); UV-Vis spectrophotometers often have 2 sources;
- a monochromator such as a grating system;
- other optical components, such as lenses or mirrors, that relay light through the instrument and that may also be used to generate more than one beam of light, i.e. in double-beam spectrophotometers, as opposed to single-beam spectrophotometers;
- a sample container, holder or sampling device; examples include conventional cuvettes, fibre-optic probes and immersed transmission cells (e.g. high-purity quartz or sapphire transparent to UV-Vis radiation); the choice depends on the intended application, paying particular attention to its suitability for the type of sample to be analysed;
- a single-channel (e.g. photomultiplier, photodiode) or multi-channel detector (e.g. photodiode array (PDA) or charge-coupled device (CCD));
- suitable computerised data processing and evaluation systems.

Control of cuvettes

For benchtop instruments, cuvettes or cells with a defined path length are used. These can be made of different materials such as quartz or glass. The tolerance for the path length of quartz and glass cuvettes is ± 0.5 per cent (e.g. ± 0.005 cm for a 1 cm cuvette). Plastic cuvettes may also be used but the tolerance interval is wider; therefore, their use must be thoroughly justified and based on a risk assessment.

The following method may be applied to check the cleanliness of optical cuvette windows and any significant differences in their thickness or parallelism: fill the cuvette with *water R* and measure its apparent absorbance against air at 240 nm for quartz cuvettes and 650 nm for glass cuvettes; rotate the cuvette 180° in its holder and measure the apparent absorbance again at the same wavelength.

When using scanning instruments, it is recommended to scan over the optical region of interest.

When using double-beam spectrophotometers, measures should be taken (e.g. matching the cuvettes) to ensure that any difference between the absorbance of the cuvettes will not have a significant impact on the analysis to be performed.

Acceptance criteria:

- the apparent absorbance is not greater than 0.093 for 1 cm quartz cuvettes (UV region) and 0.035 for 1 cm glass cuvettes (visible region);
- the absorbance measured after rotation (180°) does not differ by more than 0.005 from the value previously obtained.

MEASUREMENT

Transmission mode

Transmission mode provides a measure of the transmittance (T), at a given UV-Vis wavelength, of a sample placed between the light source and the detector. Transmittance is the ratio of the intensity of the transmitted light to the intensity of the incident light and is given by the following equation:

$$T = \frac{I}{I_0}$$

I = intensity of transmitted radiation;
I_0 = intensity of incident radiation.

A spectrum may be obtained by plotting the variation in transmittance (T) or absorbance (A) as a function of wavelength.

The absorbance is defined as the logarithm to base 10 of the reciprocal of the transmittance for monochromatic radiation. It is a dimensionless quantity expressed in absorbance units (AU), given by the following equation:

$$A = \log_{10}\left(\frac{1}{T}\right) = \log_{10}\left(\frac{I_0}{I}\right)$$

According to the Beer-Lambert law, which applies to clear diluted solutions, in the absence of interfering physico-chemical factors, the absorbance (A) is proportional to the path length (l) of the radiation through the sample, and to the concentration (c) of the substance in solution in accordance with the following equation:

$$A = \varepsilon c l$$

ε = molar absorption coefficient, in litres per mole per centimetre;
c = molar concentration of the substance in solution, in moles per litre;
l = absorption path length, in centimetres.

The specific absorbance ($A_{1\ \text{cm}}^{1\ \text{per cent}}$) of the substance is generally used in monographs and is related to absorbance (A) as follows:

$$A = A_{1\ \text{cm}}^{1\ \text{per cent}} \times c_m \times l$$

c_m = mass concentration of the substance in solution, in grams per 100 millilitres.

$A_{1\ \text{cm}}^{1\ \text{per cent}}$ represents the specific absorbance of a dissolved substance and refers to the absorbance of a 1 g/100 mL (or 1 per cent *m/V*) solution in a 1 cm cuvette or cell and is measured at a defined wavelength. The relationship between $A_{1\ \text{cm}}^{1\ \text{per cent}}$ and ε is:

$$A_{1\ \text{cm}}^{1\ \text{per cent}} = \frac{10\varepsilon}{M_r}$$

M_r = relative molecular mass.

Transmittance or absorbance measurements are generally used for liquids (dispersions and solutions), but can also be used for solids (including tablets and capsules).
For measurements of solids, a suitable sample accessory is used. Liquid samples are examined using a cell or cuvette with a suitable path length (typically 0.01-1 cm) and made of a material that is transparent to UV-Vis radiation, or by using a fibre-optic probe of a suitable configuration immersed in the liquid.

Diffuse reflection mode

Diffuse reflection mode provides a measure of reflectance (R), which is given by the following equation:

$$R = \frac{I}{I_0}$$

I = intensity of light reflected and/or scattered from the sample;
I_0 = intensity of light reflected and/or scattered from a blank or reference reflective surface.

Depending on the chemical composition and physical characteristics of the sample, the UV-Vis radiation may be absorbed as it passes through the sample. In diffuse reflection mode, it is the non-absorbed radiation which is partially reflected and/or scattered back from the sample that is measured by the detector. UV-Vis reflectance spectra are typically obtained by calculating and plotting $\log_{10}(1/R)$ as a function of the wavelength.

This measurement mode is generally selected for solids. The sample is examined either in a suitable device (e.g. a sample holder) or in direct contact with a probe. For process monitoring, the material can be analysed through a polished window interface (e.g. quartz or sapphire), or in-line using a probe. Care must be taken to ensure that the measurement conditions are as reproducible as possible from one sample to another.

Operation of the equipment

The factors below affect the spectral response and must always be taken into account.

Choose a measurement mode that is appropriate for the intended application and the sample type.

Define the measuring conditions taking into account the sample size and sample probe in such a way as to obtain a satisfactory signal-to-noise ratio (e.g. beam size, measurement time and number of measurements). For scanning spectrophotometers, also select the scan range, scan rate and slit-width that provide the necessary optical resolution for the

Table 2.2.25.-1. – *Minimum tests to be carried out for the control of equipment performance*

Purpose	Method	Wavelength accuracy	Absorbance accuracy	Photometric linearity	Stray light	Resolution/spectral bandwidth
Quantitative or limit test	Based on measurement of the absorbance at one or more identified wavelengths (e.g. assay or impurities test)	X	X	X	X	If required in the monograph
Identification test	Based on wavelength of absorption maxima and minima	X	-	-	X	-
	Based on absorption measurement and wavelength of absorption maxima	X	X	-	X	-
	Based on comparison of spectrum with that of reference substance	X	X	-	-	-

intended application without losing the required signal-to-noise ratio or the linearity of the analytical method. When using spectrophotometers with array sensors, there is no need to adjust the beam size, scan range, scan rate or slit-width since the optical resolution is typically fixed and the full spectrum is always recorded.

Before an absorbance measurement is carried out, the zero position of the absorption (baseline correction) should be set or determined for the wavelengths of interest or over the appropriate range of wavelengths.

For PAT applications, when measuring moving materials or samples, ensure that there is no fouling of the sensor (e.g. no contamination or build-up of material).

Unless otherwise prescribed in the monograph, measure the absorbance using a path length of 1 cm at the prescribed wavelength. If a single value for the position of an absorption maximum or minimum is given in a monograph, the user must determine the wavelength position. The value obtained may differ by not more than ± 2 nm, unless otherwise prescribed.

Quantitative measurements relying on absorption values above 2.0 should be avoided.

Background correction

Select a suitable spectroscopic blank (e.g. air, blank solvent, solid material). Unless otherwise prescribed, all measurements are carried out with reference to the same solvent or the same mixture of solvents (blank).

Measure the blank and the sample within a short time-frame either in parallel in double-beam spectrophotometers or sequentially in single-beam spectrophotometers.

The absorbance values of both blank and sample must be in the working range of the equipment as specified by the manufacturer.

For benchtop instruments, the absorbance of the solvent measured against air and at the prescribed wavelength must not exceed 0.4 and is preferably less than 0.2.

For chromatographic systems, the transmittance of the mobile phase may be used as the blank.

In some PAT applications, it may be impossible to remove the probe for background data collection. Various options are therefore to be considered, including the use of internal references, measurement of a blank using a second detector, etc. Only spectra measured against a blank possessing the same optical properties can be directly compared with one another.

For reflectance measurements, common reflectance blank samples include ceramics, fluoropolymers such as polytetrafluoroethylene (PTFE) and powders such as barium sulfate ($BaSO_4$) and magnesium oxide (MgO), but other suitable materials may also be used.

MATHEMATICAL TREATMENT OF SPECTRAL DATA

In the case of single wavelength analysis used to determine the concentration of an unknown sample (e.g. as prescribed in monographs), mathematical treatment consists in determining the regression of the photometric reading (absorbance) on the concentration of the standard samples.

In the case of full range spectra, data for both diffuse reflection and transmission modes may have to be treated before a classification or calibration model can be developed. The aim can be, for example, to reduce baseline drift or to correct for scatter caused by particle size changes in solid samples. For example, first-, second- or higher-order derivative spectra can typically be used to improve resolution or sensitivity. This pretreatment may be a useful means of simplifying the data and thereby reducing the variations that may cause interference in subsequently applied mathematical models.

A wide range of treatment methods, such as scaling, smoothing, normalisation and derivatisation, can be applied either singly or in combination. More information is available in general chapter *5.21. Chemometric methods applied to analytical data.*

CONTROL OF EQUIPMENT PERFORMANCE

Spectrophotometer performance is controlled (automatically or manually) at regular intervals as defined in the quality management system and dictated by the use of the equipment and the application. For example, equipment exposed to variations in temperature and humidity may need more frequent performance testing.

Requirements for control of equipment performance for the various measurement modes are summarised in Table 2.2.25.-1. Further such tests may be performed if appropriate.

Wavelength accuracy, absorbance accuracy and linearity are controlled using either certified reference materials such as

solid filters or liquid filters in appropriate sealed cells, or solutions prepared in the laboratory as described below.

Control of wavelength accuracy

Control the wavelength accuracy of an appropriate number of bands in the intended spectral range using one or more reference materials; for example, use solid or liquid filters (e.g. *holmium perchlorate solution R*) to verify the position of absorption bands, or measure the emission from a light source to check emission-line position. Table 2.2.25.-2 shows examples of wavelengths used to check wavelength accuracy. When certified reference materials are used, the reference wavelength is that stated on the corresponding certificate.

Some instruments may have an automatic or inbuilt wavelength accuracy control feature.

Table 2.2.25.-2. – *Examples of wavelengths used for the control of wavelength accuracy* *Note: the wavelength varies with the resolution of the instrument

Material		Wavelengths (nm)*
Solutions	Cerium in sulfuric acid	201.1; 211.4; 222.6; 240.4; 253.7
	Didymium in perchloric acid	511.8; 731.6; 794.2
	Holmium in perchloric acid	241.1; 287.2; 361.3; 451.4; 485.2; 536.6; 640.5
Solid filters	Didymium glass	513.5
	Holmium glass	279.3; 360.9; 453.4; 637.5
Lamps	Deuterium	486.0; 656.1
	Mercury (low pressure)	184.9; 253.7; 312.5; 365.0; 404.7; 435.8; 546.1; 577.0; 579.1
	Neon	717.4
	Xenon	541.9; 688.2; 764.2

For chromatographic systems, it is also possible to control wavelength accuracy by measuring the absorbance of a 0.05 mg/mL solution of *caffeine R* in *methanol R*; the absorption maximum is obtained at 272 nm and the minimum at 244 nm.

Acceptance criteria

It is recommended to test at least 2 wavelengths that bracket the intended spectral range.

For benchtop instruments, the tolerance for wavelength accuracy of UV-Vis spectroscopy in cuvettes is ± 1 nm at wavelengths below 400 nm and ± 3 nm at wavelengths of 400 nm and above.

For chromatographic systems, the tolerance for wavelength accuracy is ± 2 nm for the whole UV-Vis range.

For PAT applications, a tolerance of ± 2 nm for the UV-Vis range is recommended. However, wider tolerance intervals may be needed for some PAT applications, in which case the requisite wavelength accuracy must be defined by the user depending on the intended purpose, and using a risk-based approach.

The instrument parameters (especially the entrance optics such as slit-width or optical fibre diameter) influence the resolution and must be the same as those intended for the actual measurements.

Control of absorbance accuracy

Control the absorbance accuracy at an appropriate number of wavelengths in the intended spectral range, using suitable solid or liquid filters to check that the absorbance measured at the test wavelength matches the certified absorbance of the filter or the absorbance value that is calculated from a certified specific absorbance. *Nicotinic acid for equipment qualification CRS* may be used.

It is recommended to test absorbance accuracy at selected wavelengths using one or more solid or liquid filters with different absorbance levels; as a minimum, values at approximately the 2 limits of the expected absorbance range should be verified.

For chromatographic systems and PAT applications, the testing of absolute absorbance accuracy may not be necessary, providing that a standard curve is measured as required.

For measurements using *nicotinic acid for equipment qualification CRS*, the certified specific absorbance is given in the corresponding leaflet.

The solution of nicotinic acid can be prepared as follows: dissolve 57.0-63.0 mg of *nicotinic acid for equipment qualification CRS* in a 0.1 M hydrochloric acid solution prepared from *hydrochloric acid R* and dilute to 200.0 mL with the same acid solution; dilute 2.0 mL of the solution to 50.0 mL with the same acid solution to obtain a final concentration of 12 mg/L. These volumes can be adjusted to obtain nicotinic acid solutions with other concentrations (up to about 40 mg/L), for the purposes of testing different absorbance levels. The absorbance is measured at 213 nm and 261 nm.

Acceptance criteria

The difference between the measured absorbance and the absorbance of the certified material is ± 0.010 or ± 1 per cent, whichever is greater, for each combination of wavelength and absorbance assessed (applies to absorbance values not greater than 2). Tolerances for higher absorbance values should be defined on the basis of a risk assessment.

Control of photometric linearity

Control the photometric linearity in the intended spectral range. In the ultraviolet range, the filters used to control absorbance accuracy may be used, as can solutions of nicotinic acid or caffeine. In the visible range, neutral glass filters may be used. Prior to performing the test, ensure that the absorbance of the standards is compatible with the intended linear range.

Solutions with increasing concentrations (e.g. 5-40 mg/L) of *nicotinic acid for equipment qualification CRS* in a 0.1 M hydrochloric acid solution prepared from *hydrochloric acid R* may be used. The absorbance is measured at 213 nm and 261 nm.

For chromatographic systems, it is also possible to check photometric linearity using 0.5-50 mg/L solutions of *caffeine R* in *water for chromatography R*. The absorbance is measured at 273 nm.

Acceptance criterion

The coefficient of determination (R) is not less than 0.999.

Limit of stray light

Stray light is determined at an appropriate wavelength using suitable solid or liquid filters or solutions prepared in-house. The instrument parameters used for the test, such as slit-width and type of light source (e.g. deuterium or tungsten lamp), must be the same as those intended for the actual measurements.

Acceptance criterion

The acceptance criterion depends on the filters or solutions used, for example:

— the absorbance is not less than 3.0 when using a 10 g/L solution of *sodium iodide R* at 220 nm, a 10 g/L solution of *potassium iodide R* at 250 nm or a 50 g/L solution of *sodium nitrite R* at 340 nm and 370 nm;

— the absorbance is not less than 2.0 when using a 12 g/L solution of *potassium chloride R* at 198 nm.

These values apply when using a 1 cm cell and *water R* as the compensation liquid.

Control of resolution

Where prescribed in a monograph, measure the resolution of the equipment either using suitable certified reference materials, or by recording the spectrum of a 0.02 per cent *V/V* solution of *toluene R* in *hexane R* or *heptane R*, with respectively *hexane R* or *heptane R* as the compensation liquid.

Acceptance criterion

For measurements taken with a solution prepared as described above, the minimum ratio of the absorbance at the maximum (269 nm) to that at the minimum (266 nm) is stated in the monograph.

SYSTEM SUITABILITY

System suitability tests may be required prior to sample measurement to verify critical parameters that may have an impact on the result.

These tests may cover wavelength accuracy, absorbance accuracy, stray light and photometric linearity. System functionality tests, for example those performed as part of equipment autotesting, may be considered part of the system suitability tests.

In the case of UV-Vis detection for chromatographic systems, additional system suitability tests are applicable if prescribed in the monograph and/or in general chapter *2.2.46. Chromatographic separation techniques.*

C. Nuclear Magnetic Resonance Spectrometry

(Ph. Eur. method 2.2.33)

INTRODUCTION

Nuclear magnetic resonance (NMR) spectrometry is an analytical method in particular suitable for the elucidation of the chemical structure of organic molecules by means of interpretation of their NMR spectra, arising from, for example, ^{1}H or the X-nuclei ^{13}C, ^{19}F, ^{15}N, ^{31}P. The spectra can be used for qualitative and quantitative purposes.

Under suitable experimental conditions, the integrated NMR intensities of the signals are directly proportional to the number of nuclear spins of the molecular group responsible for the signal. These integrals can be used for quantitative analysis.

GENERAL PRINCIPLES

Placing an ensemble of nuclei with angular momentum and a magnetic moment in a static magnetic field (B_0) causes the nuclei to arrange themselves in different, quantum-mechanically controlled orientations in relation to the axis of the magnetic field. These orientations are different in energy. An oscillating high-frequency magnetic field (B_1), perpendicular to B_0, will cause transitions between these orientations with net energy absorption. According to the resonance condition $\omega_0 = \gamma B_0$ (γ = gyromagnetic ratio, ω_0 = Larmor frequency), either the B_0 magnetic field or the frequency (ω_1) of the B_1 field may be varied to achieve a spectrum (continuous wave (CW) method). Nowadays the B_1 irradiation is achieved by the use of a radiofrequency (RF) pulse (Fourier transform (FT) method). The coherent radiation emitted during the return to the initial state is observed in the form of a decay curve, called the free induction decay (FID). Subsequent Fourier transformation gives the spectrum in the frequency domain, providing information about the molecular structure. Additional radiofrequency fields may be applied during acquisition of the FID signal to suppress scalar (through-bond) interactions between nuclei (called 'decoupling'). One- and multi-dimensional techniques can be applied for qualitative and quantitative purposes, on samples in either the liquid or the solid state.

Important structural information is derived from the following spectroscopic features:

resonance frequency	kind of nuclei observed
number of resonance signals (singlets, multiplets)	number of chemically distinct groups of nuclei
chemical shift δ (ppm)	chemical nature and environment of the structural group observed
intensity of resonance signals	relative number of resonant nuclei per chemically distinct group
multiplicity of coupling pattern	number of nuclei that are scalar coupled to the observed nucleus
coupling constant ^{n}J (Hz)	number of bonds in the coupling pathway, and its geometry

Correlations of different spectral parameters (e.g. chemical shift and coupling constant, or chemical shifts of different nuclei within one molecular system) can be performed by homo- and hetero-nuclear two- and higher-dimensional methods. Information about the relaxation times T_1 and T_2, nuclear Overhauser effects (NOEs) and the kinetics of time-dependent processes are also accessible from appropriate experiments.

APPARATUS

A high-resolution NMR spectrometer consists of at least the following parts:

— a magnet to deliver the constant magnetic field B_0;

— a temperature-controlled probe to contain the sample, to deliver the radiofrequency pulse and to detect radiation emitted by the sample;

— an electronic console to generate high-power radiofrequency pulses and to collect and digitise the FID signal; this unit also maintains the stability of the instrument electronics;

— a data acquisition and processing unit (computer);

and may also include:

— a continuous flow cell for coupled liquid chromatographic-NMR or flow injection analysis;

— a system for pulsed field gradient NMR.

The high magnetic field is generated by a superconducting coil in a Dewar flask filled with liquid helium. The probe typically contains the sample in a 5 mm-outer-diameter sample tube or in a flow cell, and is connected to the electronics cabinet by RF cables carrying lock, ^{1}H-, and X-nucleus frequencies. Additional devices for tuning and

matching the electronic circuits are essential, and sample temperature control is often used.

The NMR spectrometer should be demonstrated to be operating correctly. Appropriate tests to demonstrate this are, typically, measurement of linewidths at half height for defined peaks under defined acquisition conditions, signal-to-noise ratios (*S/N*) for standard mixtures, pulse power (measured as a 90° pulse width), and pulse reproducibility. All instrument manufacturers publish specifications and measurement protocols for these parameters for specific instrument/probe combinations, and compliance with these specifications should be demonstrated.

FOURIER TRANSFORM NMR (FT-NMR)

Contemporary spectrometers generally operate according to the Fourier transform (FT) principle: after exciting the sample with a radiofrequency pulse of appropriate frequency (ν), amplitude (B_1) and duration (τ_p) and a succeeding short dead time (t_d) (to enable the electronics to recover), the amplified analogue FID signal is sampled during the acquisition time (t_{ac}) and digitised with an analogue-to-digital converter (ADC), and the results are stored in the spectrometer memory. The receiver output is amplified prior to digitisation to maximise sensitivity without saturating the ADC. In case of observation of X-nuclei, the standard experiment includes, if necessary, broadband ^{1}H decoupling, i.e. irradiation of all the protons during the experiment. To increase the *S/N*, multiple FID signals may be accumulated coherently and summed. Fourier transformation of this time-domain data gives the frequency-domain spectrum.

PARAMETERS

The following acquisition parameters influence the result of an FT experiment, and should be adjusted and controlled.

Pulse width (τ_p)

The excitation pulse is directed along the x-axis of the so-called rotating frame, its duration (or 'width', τ_p) determines the flip angle (θ) and thus the intensity (*I*) of the resonance signal:

$$\theta = \gamma' \times B_1 \times \tau_p \quad (1)$$

$$M_y = M_o \times \sin\theta \quad (2)$$

The observed magnetisation M_y is maximum at θ = 90°. The pulse duration should be short to guarantee that all signals in the spectral width (*SW*) are excited to a similar degree. The magnetisation decays due to relaxation processes.

Dead time (t_d)

The dead time is the time between the end of the pulse and start of the acquisition, it is necessary for technical reasons and care should be taken as it may influence signal intensities and peak phase. Rapidly decaying signals (giving rise to broad spectral lines) are reduced in intensity by more than slowly decaying signals (which give rise to narrow spectral lines).

Acquisition time (t_{ac})

The acquisition time (t_{ac}) is related to the spectral width (i.e. the whole observed region) and the number of digital data points (*DP*) collected during signal acquisition.

$$t_{ac} = \frac{DP}{2SW} \quad (3)$$

Maximal signal intensity and signal-to-noise ratio will be achieved if $t_{ac} \approx 1.2/(\pi\nu_{1/2})$, where $\nu_{1/2}$ is the full width at half-height (*fwhh*), but it should be set to greater than $5/(\pi\nu_{1/2})$ to minimise signal distortion.

Repetition time (t_r)

The spin-lattice relaxation (T_1) governs the time required for the spin system to return to equilibrium after a pulse. Relaxation can be reduced by the use of special reagents. For quantitative purposes, the repetition time used should be set relative to T_1 and θ to avoid saturation effects.

Receiver gain

The analogue signal detected by the probe is amplified prior to digitisation and storage. The amplification, or receiver gain, should be set, either automatically or manually, so that the signal does not overload the ADC, which causes signal distortion, but allows random noise generated in the probe to be digitised (i.e. is non-zero).

OPTIMISATION OF ACQUISITION AND PROCESSING PARAMETERS FOR QUANTITATIVE PURPOSES

Besides the acquisition parameters, signal intensity is also influenced by several processing parameters. After collecting a sufficient number of scans, the resulting FID is Fourier transformed. For reliable quantitative purposes the following parameters have to be optimised.

Digital resolution

The digital resolution is the frequency separation between data points. The processed signal should have at least 5 digital points above half-height of the signals to be integrated. To improve the digital resolution additional points of zero intensity may be added to the end of the experimental FID before transformation ('zero filling').

Signal-to-noise ratio (*S/N*)

This is the ratio between the intensities (as peak height) of a specified signal in the NMR spectrum and the random fluctuations in that signal, which is usually measured in a region of the spectrum that contains no signals from the analyte. A poor signal-to-noise ratio (*S/N*) limits the accuracy of peak integrations and quantitative analyses. An *S/N* equal to or greater than 150:1 allows peak integrations with a standard deviation of less than 1 per cent. Contemporary spectrometers have software algorithms to report the *S/N* of appropriate peaks. A sufficiently high *S/N* can be difficult to obtain when analysing dilute solutions, and especially when detecting nuclei other than ^{1}H. Methods to enhance the *S/N* include:

- — increasing the number of accumulated scans (*n*), as *S/N* increases with $\sqrt{n}$;
- — use of exponentional multiplication on the FID signal before Fourier transformation; the exponentional multiplication factor should be in the order of the peak full width at half-height (*fwhh*);
- — use of spectrometers with a higher magnetic field B_0, since *S/N* is proportional to $B_0^{3/2}$;
- — use of digital filtering to reduce noise;
- — use of probes that maximise the filling factor;
- — use of cryoprobes that reduce thermal noise.

Integration region

The intensity of the NMR signals is obtained by a quasi-analogue signal integration either by a stepped-line plot or, more accurately, by separate line integration and digital data presentation. In liquid state, NMR signals have Lorentzian line shape. Unless otherwise specified in the monograph or when peak overlap occurs, the same integration range,

expressed as a multiple of the peak *fwhh*, should be used for the monitored peak and the reference peak.

Dynamic range

The dynamic range of the analogue-to-digital converter (ADC) determines the minimum intensity line that can be observed or quantified when integrating 2 signals with the same linewidth in a spectrum. A 16-bit ADC allows identification of a signal of 0.003 per cent intensity relative to a strong signal completely filling the dynamic range of the ADC.

NMR OF SAMPLES IN SOLUTION

Most NMR experiments are performed on dilute solutions (about 1 per cent) of the analyte in an appropriate solvent, which can be spiked with a suitable standard for chemical shift calibration.

Solvents

The solvent should be able to dissolve the analyte without further interaction if not otherwise intended. To minimise the intense solvent signals, fully deuterated solvents (*deuterium oxide R*, *deuterated chloroform R*, *deuterated dimethyl sulfoxide R*, *deuterated acetone R*, *deuterated methanol R*, etc.) should be used. The solvent atoms give signals that are easily identified by their chemical shift and can be used to calibrate the chemical shift axis (secondary reference).

Referencing

The spectral feature most dependent on the chemical environment of the atom in the molecule is the chemical shift, designated as δ and reported in parts per million. The chemical shift between the resonance for an NMR active nucleus X ($\delta_{X,sample}$) is measured in parts per million as the difference between the resonance frequency of that nucleus ($\nu_{X,sample}$) and that of an internal shift reference standard ($\nu_{X,reference}$), both in hertz, divided by the basic spectrometer operating frequency ($\nu_{X,reference}$), in megahertz, at a given B_0:

$$\delta_{X,sample} = \frac{(\nu_{X,sample} - \nu_{X,reference})}{\nu_{X,reference}} \quad (4)$$

By convention, the standard for exact chemical shift referencing is the ^{1}H resonance of *tetramethylsilane R* (TMS), setting $\delta_{TMS} = 0$ ppm. Formally, once the ^{1}H shift scale has been referenced relative to TMS, the exact frequency of any other X resonance can be calculated and its chemical shift scale calibrated. The frequency of a (secondary) reference standard at $\delta_X = 0$ ppm ($\nu_{X,reference}$) is calculated from the ^{1}H frequency of TMS ($\nu_{H,TMS}$) and a tabulated value of the ratio ($\Xi_{X,reference}$) of the isotope-specific frequency in relation to that of ^{1}H in TMS:

$$\nu_{X,reference} = \nu_{H,TMS} \times \Xi_{X,reference} \quad (5)$$

Reference standards at $\delta_X = 0$ ppm and corresponding $\Xi_{X,reference}$ values are shown below:

Nucleus	Water[a]	$\Xi_{X,reference}$	Other solvents	$\Xi_{X,reference}$
^{1}H	DSS[b]	1.00000000	TMS	1.00000000
^{13}C	DSS[b]	0.25144953	TMS	0.25145020
^{15}N	NH_3	0.10132912	CH_3NO_2	0.10136767
^{19}F	CF_3COOH	not reported	CCl_3F	0.94094011
^{31}P	H_3PO_4 (85 per cent)	0.40480742	$(CH_3O)_3PO$	0.40480864
[a]chemical shift depends on pH				
[b]DSS = sodium 2,2-dimethyl-2-silapentane-5-sulfonate				

In practice, X chemical shifts are referenced directly using an appropriate standard. In ^{1}H and ^{13}C NMR, internal referencing is mainly used, where the reference is added directly to the system under study. In ^{15}N, ^{19}F and ^{31}P NMR, external referencing is often suitable, involving sample and reference contained separately in coaxial cylindrical sample tubes.

Lock

In order to prevent drifting of the spectrum over time, a stabilising procedure, called field-frequency locking, is performed. The ^{2}H (deuterium) signal arising from deuterated solvents is used to achieve this, unless otherwise specified in the monograph.

QUALITATIVE ANALYSIS

The principal use for qualitative NMR spectra is as an identity test, in which the ^{1}H or ^{13}C spectrum of a test sample is compared to the spectrum of a reference sample or, less commonly, with a published reference spectrum. Spectra of reference and test samples should be acquired using the same procedure and operational conditions. The peaks in the 2 spectra, or characteristic regions of the spectra, should correspond in position, intensity and multiplicity.

In appropriate cases, mathematical comparison, such as calculation of a correlation coefficient, may be appropriate.

In the absence of a reference standard, an in-house reference may be used, whose identity has been demonstrated by alternative methods, or the demonstration that the NMR spectrum is fully consistent with the reported structure for that material.

QUANTITATIVE ANALYSIS

Signal intensity in the basic NMR experiment is the integrated area under the signal curve measured. Only when 2 signals have equal *fwhh* and the same multiplicity may signal height serve as a measure of intensity. Under conditions of essentially complete relaxation between scans, the signal intensity (I_A) is a true measure of the number (N_A) of nuclei responsible for the respective signal:

$$I_A = K_S \times N_A \quad (6)$$

The constant K_S includes fundamental constants, properties of the sample and receiver parameters, and can be omitted in cases where signal intensities are compared, giving the direct relation between the numbers of nuclei in the 2 compared structure groups A and B:

$$\frac{I_A}{I_B} = \frac{N_A}{N_B} \quad (7)$$

The numbers (N_i) of nuclei belonging to different structure groups of 1 molecule are small integers. The values measured are rounded to the closest integer numbers. However, the proper operation of acquisition and processing of the spectrometer is easily checked by comparing exact intensities within a spectrum of any suitable organic compound of known structure.

In addition to the fact that the intensities of signals arising from each component in a mixture are related to each other by small integer numbers, the relative molar amounts of these components can be measured by comparison of the normalised intensities of resonances from different components. The molar ratio of 2 components of a mixture is calculated according to the following equation:

$$\frac{n_A}{n_B} = \frac{I_A}{I_B} \times \frac{N_B}{N_A} \qquad (8)$$

The determination is only valid in cases where the structure of the molecules for which I_A and I_B are determined are known (or at least the values of N for the monitored groups). Determinations are made using either an internal standard method or a peak-normalisation procedure.

Internal standard method

The mass (m_A) of an analyte (A) can be determined if a known mass (m_B) of a substance (B) with a known percentage content (P_B) is added to the solution as an intensity standard. Equation (8) can be converted to equation (9):

$$m_A = \frac{I_A}{I_B} \times \frac{N_B}{N_A} \times \frac{M_A}{M_B} \times m_B \times \frac{P_B}{100} \qquad (9)$$

Here, M_i are the molecular masses.

The intensity standard has to be carefully chosen; it should be completely soluble in the solvent used for the analyte, should produce only a small number of signals, and the 'monitor group' should have a signal in an empty spectral region. A compound of high purity and with a relatively high molecular mass is recommended for this purpose.

Normalisation procedure

The relative proportions of components in a mixture, the degree of substitution in a structurally modified polymer, or the amount of a contaminant can be determined by comparison of the relative intensities of resonances present.

The experimental method should be validated to ensure that there is no overlap of the relevant signals. When the contaminant is of poorly defined structure or molecular mass (e.g. an emulsifier), addition of known amounts of that material to the NMR tube will allow a calibration curve to be constructed.

METHOD

Sample handling

Dissolve the sample in the solvent to which the appropriate reference material may have been added to calibrate chemical shift, as prescribed in the monograph. For quantitative analysis, the solutions must be free from solid particles. Some quantitative analyses may require an internal standard to be included, so that integrations of resonances from the test sample and the reference material can be compared. Appropriate references and concentrations are indicated in the specific monographs. In other quantitative analyses, the result is obtained by comparing the relative intensities of 2 or all of the resonances that arise from the test sample. After loading the sample into a tube and capping, the sample is introduced into the NMR magnet, the experimental parameters are loaded and the experiment is executed. Key experimental parameters are indicated in the monograph.

The measurement procedure

Equilibrate the sample in the probe, and optimise the instrument to achieve best resonance conditions and to maximise the *S/N* by tuning and matching the probe, and make adjustments to maximise magnetic field homogeneity over the sample volume (called 'shimming'). Record, or save to computer, the parameter settings. An experiment may be composed of multiple pulse-acquisition-delay sequences, and the individual FIDs are summed in the computer memory, with random noise being averaged out. When an appropriate *S/N* has been achieved, the FID is stored and the frequency-domain spectrum is generated by Fourier transformation of the summed FIDs.

NMR IN THE SOLID STATE

Samples in the solid state can be analysed using NMR spectrometers specially equipped for that purpose. Certain technical procedures make observable individual lines for individual atomic sites with a valuable extension of the applicability of NMR to inorganic materials as well.

One technique is the rapid rotation (4-30 kHz) of the powdered sample in a rotor (about 4 mm outer diameter) inclined at an angle of 54.7° (the 'magic angle') to the direction of the B_0 magnetic field axis. This technique is named magic angle spinning (MAS). Another effective tool is high-power decoupling and a 3rd method is the transfer of polarisation from easily excitable nuclei towards less-polarisable nuclei, i.e. cross polarisation (CP).

The combination of these techniques makes available high-resolution spectra containing much information about chemical and structural details in solid glassy, amorphous, and crystalline materials of ceramic, polymeric or mineralogical origin.

If NMR is applied to a solid, full details of the procedure are provided in the monograph.

D. Atomic Spectrophotometry: Emission and Absorption

Atomic Emission Spectrometry

(Ph. Eur. method 2.2.22)

GENERAL PRINCIPLE

Atomic emission is a process that occurs when electromagnetic radiation is emitted by excited atoms or ions. In atomic emission spectrometry the sample is subjected to temperatures high enough to cause not only dissociation into atoms, but also to cause significant amounts of collisional excitation and ionisation of the sample atoms to take place. Once the atoms and ions are in the excited states, they can decay to lower states through thermal or radiative (emission) energy transitions and electromagnetic radiation is emitted. An emission spectrum of an element contains several more lines than the corresponding absorption spectrum.

Atomic emission spectrometry is a technique for determining the concentration of an element in a sample by measuring the intensity of one of the emission lines of the atomic vapour of the element generated from the sample. The determination is carried out at the wavelength corresponding to this emission line.

In this chapter only atomisation in flame is dealt with. The method of inductively coupled plasma-atomic emission spectrometry (ICP-AES) is described in a different general chapter.

APPARATUS

This consists essentially of:
— a sample introduction and nebulisation system;
— a flame to generate the atoms to be determined;
— a monochromator;
— a detector;
— a data-acquisition unit.

Oxygen, air and a combustible gas such as hydrogen, acetylene, propane or butane may be used in flames. The atomisation source is critical, since it must provide sufficient energy to excite and atomise the atoms. The atomic spectra emitted from flames have the advantage of being simpler than those emitted from other sources, the main limitation being that the flames are not powerful enough to cause emission for many elements allowing their determination. Acidified water is the solvent of choice for preparing test and reference solutions, although organic solvents may also be used if precautions are taken to ensure that the solvent does not interfere with the stability of the flame.

INTERFERENCES

Spectral interference is reduced or eliminated by choosing an appropriate emission line for measurement or by adjusting the slit for spectral band-width. Physical interference is corrected by diluting the sample solution, by matching the matrix or by using the method of standard additions. Chemical interference is reduced by using chemical modifiers or ionisation buffers.

MEMORY EFFECT

The memory effect caused by deposit of analyte in the apparatus may be limited by thoroughly rinsing between runs, diluting the solutions to be measured if possible and thus reducing their salt content, and by aspirating the solutions through as swiftly as possible.

METHOD

Use of plastic labware is recommended wherever possible.

Operate an atomic emission spectrometer in accordance with the manufacturer's instructions at the prescribed wavelength. Optimise the experimental conditions (flame temperature, burner adjustment, use of an ionic buffer, concentration of solutions) for the specific element to be analysed and in respect of the sample matrix. Introduce a blank solution into the atomic generator and adjust the instrument reading to zero or to its blank value. Introduce the most concentrated reference solution and adjust the sensitivity to obtain a suitable reading.

It is preferable to use concentrations which fall within the linear part of the calibration curve. If this is not possible, the calibration plots may also be curved and are then to be applied with appropriate calibration software.

Determinations are made by comparison with reference solutions with known concentrations of the element to be determined either by the method of direct calibration (Method I) or the method of standard additions (Method II).

METHOD I - DIRECT CALIBRATION

For routine measurements 3 reference solutions of the element to be determined and a blank are prepared and examined.

Prepare the solution of the substance to be examined (test solution) as prescribed in the monograph. Prepare not fewer than 3 reference solutions of the element to be determined, the concentrations of which span the expected value in the test solution. For assay purposes, optimal calibration levels are between 0.7 and 1.3 times the expected content of the element to be determined or the limit prescribed in the monograph. For purity determination, calibration levels are between the limit of detection and 1.2 times the limit specified for the element to be determined. Any reagents used in the preparation of the test solution are added to the reference solutions and to the blank solution at the same concentration.

Introduce each of the solutions into the instrument using the same number of replicates for each solution, to obtain a steady reading.

Calculation

Prepare a calibration curve from the mean of the readings obtained with the reference solutions by plotting the means as a function of concentration. Determine the concentration of the element in the test solution from the curve obtained.

METHOD II - STANDARD ADDITIONS

Add to at least 3 similar volumetric flasks equal volumes of the solution of the substance to be examined (test solution) prepared as prescribed. Add to all but 1 of the flasks progressively larger volumes of a reference solution containing a known concentration of the element to be determined to produce a series of solutions containing steadily increasing concentrations of that element known to give responses in the linear part of the curve, if at all possible. Dilute the contents of each flask to volume with solvent.

Introduce each of the solutions into the instrument using the same number of replicates for each solution, to obtain a steady reading.

Calculation

Calculate the linear equation of the graph using a least-squares fit, and derive from it the concentration of the element to be determined in the test solution.

VALIDATION OF THE METHOD

Satisfactory performance of methods prescribed in monographs is verified at suitable time intervals.

LINEARITY

Prepare and analyse not fewer than 4 reference solutions over the calibration range and a blank solution. Perform not fewer than 5 replicates.

The calibration curve is calculated by least-square regression from all measured data. The regression curve, the means, the measured data and the confidence interval of the calibration curve are plotted. The operating method is valid when:
— the correlation coefficient is at least 0.99,
— the residuals of each calibration level are randomly distributed around the calibration curve.

Calculate the mean and relative standard deviation for the lowest and highest calibration level.

When the ratio of the estimated standard deviation of the lowest and the highest calibration level is less than 0.5 or greater than 2.0, a more precise estimation of the calibration curve may be obtained using weighted linear regression. Both linear and quadratic weighting functions are applied to the data to find the most appropriate weighting function to be employed. If the means compared to the calibration curve show a deviation from linearity, two-dimensional linear regression is used.

ACCURACY

Verify the accuracy preferably by using a certified reference material (CRM). Where this is not possible, perform a test for recovery.

Recovery

For assay determinations a recovery of 90 per cent to 110 per cent is to be obtained. For other determinations, for example for trace element determination, the test is not valid if recovery is outside of the range 80 per cent to 120 per cent at the theoretical value. Recovery may be determined on a suitable reference solution (matrix solution) which is spiked with a known quantity of analyte (middle concentration of the calibration range).

REPEATABILITY

The repeatability is not greater than 3 per cent for an assay and not greater than 5 per cent for an impurity test.

LIMIT OF QUANTIFICATION

Verify that the limit of quantification (for example, determined using the 10 σ approach) is below the value to be measured.

Inductively Coupled Plasma-atomic Emission Spectrometry

(Ph. Eur. method 2.2.57)

GENERAL PRINCIPLE

Inductively coupled plasma-atomic emission spectrometry (ICP-AES) is an atomic emission spectrometry method that uses an inductively coupled plasma (ICP) as the excitation source.

An ICP is a highly ionised inert gas (usually argon) with equal numbers of electrons and ions sustained by a radio-frequency (RF) field. The high temperature reached in the plasma successively desolvates, vaporises, excites - atomic emission spectrometry (AES) detection - and ionises - mass spectrometry (MS) detection - atoms from the sample. Detection limits are, generally, in the lower nanogram (ICP-MS) to microgram (ICP-AES) per litre range.

The plasma is formed by a tangential stream of support gas through a 'torch', i.e. a system consisting of 3 concentric quartz tubes. A metal coil (the load coil) surrounds the top end of the torch and is connected to a radio-frequency (RF) generator. Power (usually 700-1500 W) is applied through the coil and an oscillating magnetic field corresponding to the frequency of the generator (in most cases 27 MHz, 40 MHz) is formed. The plasma forms when the support gas is made conductive by exposing it to an electric discharge, which produces seed electrons and ions. Inside the induced magnetic field, the charged particles (electrons and ions) are forced to flow in a closed annular path. As they meet resistance to their flow, heating takes place producing additional ionisation. The process occurs almost instantaneously, and the plasma expands to its full strength and dimensions. The radio-frequency oscillation of the power applied through the coil causes radio-frequency electric and magnetic fields to be set up in the area at the top of the torch. When a spark (produced by a Tesla tube or some other seeding device) is applied to the support gas flowing through the torch, some electrons are stripped from the support gas atoms. These electrons are then caught up in the magnetic field and accelerated. Adding energy to the electrons by the use of a coil is known as inductive coupling. These high-energy electrons in turn collide with other support-gas atoms, stripping off still more electrons. The collisional ionisation of the support gas continues in a chain reaction, breaking down the gas into a physical plasma consisting of support-gas atoms, electrons and support-gas ions. The plasma is then sustained within the torch and load coil as radio-frequency energy is continually transferred to it through the inductive coupling process.

The ICP appears as an intense, very bright, plume-shaped plasma. At the base the plasma is toroidal, and this is referred to as the induction region (IR), i.e. the region in which the inductive energy transfer from the load coil to the plasma takes place. The sample is introduced through the induction region into the centre of the plasma.

APPARATUS

The apparatus consists essentially of the following elements:

- sample-introduction system consisting of a peristaltic pump delivering the solution at constant flow rate into a nebuliser;
- radio-frequency (RF) generator;
- plasma torch;
- transfer optics focussing the image of the plasma at the entrance slit of the spectrometer; radial viewing is better for difficult matrices (alkalis, organics), whereas axial viewing gives more intensity and better detection limits in simple matrices;
- wavelength dispersive devices consisting of diffraction gratings, prisms, filters or interferometers;
- detectors converting radiant energy into electrical energy;
- data-acquisition unit.

INTERFERENCE

Interference is anything that causes the signal from an analyte in a sample to be different from the signal for the same concentration of that analyte in a calibration solution. The well-known chemical interference that is encountered in flame atomic absorption spectrometry is usually weak in ICP-AES. In rare cases where interference occurs, it may be necessary to increase the RF power or to reduce the inner support-gas flow to eliminate it. The interference in ICP-AES can be of spectral origin or even the result of high concentrations of certain elements or matrix compounds. Physical interference (due to differences in viscosity and surface tension of the sample and calibration standards) can be minimised by dilution of the sample, matrix matching, use of internal standards or through application of the method of standard additions.

Another type of interference occasionally encountered in ICP-AES is the so-called 'easily ionised elements (EIEs) effect'. The EIEs are those elements that are ionised much more easily, for example alkaline metals and alkaline earths. In samples that contain high concentrations of EIEs (more than 0.1 per cent), suppression or enhancement of emission signals is likely to occur.

Spectral interference

This may be due to other lines or shifts in background intensity. These lines may correspond to argon (observed above 300 nm), OH bands due to the decomposition of water (at about 300 nm), NO bands due to the interaction of the plasma with the ambient air (between 200 nm and 300 nm), and other elements in the sample, especially those present at high concentrations. The interference falls into 4 different categories: simple background shift, sloping background shift, direct spectral overlap, and complex background shift.

Absorption interference

This arises when part of the emission from an analyte is absorbed before it reaches the detector. This effect is observed particularly when the concentration of a strongly

emitting element is so high that the atoms or ions of that element that are in the lower energy state of transition absorb significant amounts of the radiation emitted by the relevant excited species. This effect, known as self-absorption, determines the upper end of the linear working range for a given emission line.

Multicomponent spectral fitting

Multiple emission-line determinations are commonly used to overcome problems with spectral interferences. A better, more accurate method for performing spectral interference corrections is to use the information obtained with advanced detector systems through multicomponent spectral fitting. This quantifies not only the interference, but also the background contribution from the matrix, thereby creating a correction formula. Multicomponent spectral fitting utilises a multiple linear-squares model based on the analysis of pure analyte, the matrix and the blank, creating an interference-corrected mathematical model. This permits the determination of the analyte emission in a complex matrix with improved detection limits and accuracy.

PROCEDURE

SAMPLE PREPARATION AND SAMPLE INTRODUCTION

The basic goal for the sample preparation is to ensure that the analyte concentration falls within the working range of the instrument through dilution or preconcentration, and that the sample-containing solution can be nebulised in a reproducible manner.

Several sample-introduction systems tolerate high acid concentrations, but the use of sulfuric and phosphoric acids can contribute to background emission observed in the ICP spectra. Therefore, nitric and hydrochloric acids are preferable. The availability of hydrofluoric acid-resistant (for example perfluoroalkoxy polymer) sample-introduction systems and torches also allows the use of hydrofluoric acid. In selecting a sample-introduction method, the requirements for sensitivity, stability, speed, sample size, corrosion resistance and resistance to clogging have to be considered. The use of a cross-flow nebuliser combined with a spray chamber and torch is suitable for most requirements. The peristaltic pumps used for ICP-AES usually deliver the standard and sample solutions at a rate of 1 mL/min or less.

In the case of organic solvents being used, the introduction of oxygen must be considered to avoid organic layers.

CHOICE OF OPERATING CONDITIONS

The standard operating conditions prescribed by the manufacturer are to be followed. Usually, different sets of operating conditions are used for aqueous solutions and for organic solvents. Suitable operating parameters are to be properly chosen:

- wavelength selection;
- support-gas flow rates (outer, intermediate and inner tubes of the torch);
- RF power;
- viewing position (radial or axial);
- pump speed;
- conditions for the detector (gain/voltage for photomultiplier tube detectors, others for array detectors);
- integration time (time set to measure the emission intensity at each wavelength).

CONTROL OF INSTRUMENT PERFORMANCE

System suitability

The following tests may be carried out with a multi-element control solution to ensure the adequate performance of the ICP-AES system:

- energy transfer (generator, torch, plasma); measurement of the ratio Mg II (280.270 nm)/Mg I (285.213 nm) may be used;
- sample transfer, by checking nebuliser efficiency and stability;
- resolution (optical system), by measuring peak widths at half height, for example As (189.042 nm), Mn (257.610 nm), Cu (324.754 nm) or Ba (455.403 nm);
- analytical performance, by calculating detection limits of selected elements over the wavelength range.

VALIDATION OF THE METHOD

Satisfactory performance of methods prescribed in monographs is verified at suitable time intervals.

LINEARITY

Prepare and analyse not fewer than 4 reference solutions over the calibration range plus a blank. Perform not fewer than 5 replicates.

The calibration curve is calculated by least-square regression from all measured data of the calibration test. The regression curve, the means, the measured data and the confidence interval of the calibration curve are plotted. The operating method is valid when:

- the correlation coefficient is at least 0.99;
- the residuals of each calibration level are randomly distributed around the calibration curve.

Calculate the mean and relative standard deviation for the lowest and for the highest calibration level.

When the ratio of the estimated standard deviations of the lowest and the highest calibration level is less than 0.5 or greater than 2.0, a more precise estimation of the calibration curve may be obtained using weighted linear regression. Both linear and quadratic weighting functions are applied to the data to find the most appropriate weighting function to be employed.

If the means compared to the calibration curve show a deviation from linearity, two-dimensional linear regression is used.

ACCURACY

Verify the accuracy preferably by using a certified reference material (CRM). Where this is not possible, perform a test for recovery.

Recovery

For assay determinations a recovery of 90 per cent to 110 per cent is to be obtained. The test is not valid if recovery, for example for trace-element determination, is outside of the range 80 per cent to 120 per cent of the theoretical value. Recovery may be determined on a suitable reference solution (matrix solution) spiked with a known quantity of analyte (concentration range that is relevant to the samples to be determined).

REPEATABILITY

The repeatability is not greater than 3 per cent for an assay and not greater than 5 per cent for an impurity test.

LIMIT OF QUANTIFICATION

Verify that the limit of quantification (for example, determined using the 10 σ approach) is below the value to be measured.

Atomic Absorption Spectrometry

(Ph. Eur. method 2.2.23)

GENERAL PRINCIPLE

Atomic absorption is a process that occurs when a ground state-atom absorbs electromagnetic radiation of a specific wavelength and is elevated to an excited state. The atoms in the ground state absorb energy at their resonant frequency and the electromagnetic radiation is attenuated due to resonance absorption. The energy absorption is virtually a direct function of the number of atoms present.

This chapter provides general information and defines the procedures used in element determinations by atomic absorption spectrometry, either atomisation by flame, by electrothermal vaporisation in a graphite furnace, by hydride generation or by cold vapour technique for mercury.

Atomic absorption spectrometry is a technique for determining the concentration of an element in a sample by measuring the absorption of electromagnetic radiation by the atomic vapour of the element generated from the sample. The determination is carried out at the wavelength of one of the absorption (resonance) lines of the element concerned. The amount of radiation absorbed is, according to the Lambert-Beer law, proportional to the element concentration.

APPARATUS

This consists essentially of:
— a source of radiation;
— a sample introduction device;
— a sample atomiser;
— a monochromator or polychromator;
— a detector;
— a data-acquisition unit.

The apparatus is usually equipped with a background correction system. Hollow-cathode lamps and electrodeless discharge lamps (EDL) are used as radiation source. The emission of such lamps consists of a spectrum showing very narrow lines with half-width of about 0.002 nm of the element being determined.

There are 3 types of sample atomisers:
— Flame technique

A flame atomiser is composed of a nebulisation system with a pneumatic aerosol production accessory, a gas-flow regulation and a burner. Fuel-oxidant mixtures are commonly used to produce a range of temperatures from about 2000 K to 3000 K. Fuel gases include propane, hydrogen and acetylene; air and nitrous oxide are used as oxidants. The configuration of the burner is adapted to the gases used and the gas flow is adjustable. Samples are nebulised, acidified water being the solvent of choice for preparing test and reference solutions. Organic solvents may also be used if precautions are taken to ensure that the solvent does not interfere with the stability of the flame.

— Electrothermal atomisation technique

An electrothermal atomiser is generally composed of a graphite tube furnace and an electric power source. Electrothermal atomisation in a graphite tube furnace atomises the entire sample and retains the atomic vapour in the light path for an extended period. This improves the detection limit. Samples, liquid as well as solid, are introduced directly into the graphite tube furnace, which is heated in a programmed series of steps to dry the sample and remove major matrix components by pyrolysis and to then atomise all of the analyte. The furnace is cleaned using a final temperature higher than the atomisation temperature. The flow of an inert gas during the pyrolysis step in the graphite tube furnace allows a better performance of the subsequent atomisation process.

— Cold vapour and hydride technique

The atomic vapour may also be generated outside the spectrometer. This is notably the case for the cold-vapour method for mercury or for certain hydride-forming elements such as arsenic, antimony, bismuth, selenium and tin. For mercury, atoms are generated by chemical reduction with stannous chloride or sodium borohydride and the atomic vapour is swept by a stream of an inert gas into a cold quartz cell mounted in the optical path of the instrument. Hydrides thus generated are swept by an inert gas into a heated cell in which they are dissociated into atoms.

INTERFERENCES

Chemical, physical, ionisation and spectral interferences are encountered in atomic absorption measurements. Chemical interference is compensated by addition of matrix modifiers, of releasing agents or by using high temperature produced by a nitrous oxide-acetylene flame; the use of specific ionisation buffers (for example, lanthanum and caesium) compensates for ionisation interference; by dilution of the sample, through the method of standard additions or by matrix matching, physical interference due to high salt content or viscosity is eliminated. Spectral interference results from the overlapping of resonance lines and can be avoided by using a different resonance line. The use of Zeeman background correction also compensates for spectral interference and interferences from molecular absorption, especially when using the electrothermal atomisation technique. The use of multi-element hollow-cathode lamps may also cause spectral interference. Specific or non-specific absorption is measured in a spectral range defined by the band-width selected by the monochromator (0.2-2 nm).

BACKGROUND CORRECTION

Scatter and background in the flame or the electrothermal atomisation technique increase the measured absorbance values. Background absorption covers a large range of wavelengths, whereas atomic absorption takes place in a very narrow wavelength range of about 0.005-0.02 nm. Background absorption can in principle be corrected by using a blank solution of exactly the same composition as the sample, but without the specific element to be determined, although this method is frequently impracticable. With the electrothermal atomisation technique the pyrolysis temperature is to be optimised to eliminate the matrix decomposition products causing background absorption. Background correction can also be made by using 2 different light sources, the hollow-cathode lamp that measures the total absorption (element + background) and a deuterium lamp with a continuum emission from which the background absorption is measured. Background is corrected by subtracting the deuterium lamp signal from the hollow-cathode lamp signal. This method is limited in the spectral range on account of the spectra emitted by a deuterium lamp from 190-400 nm. Background can also be measured by taking readings at a non-absorbing line near the resonance line and then subtracting the results from the measurement at the resonance line. Another method for the correction of background absorption is the Zeeman effect (based on the Zeeman splitting of the absorption line in a magnetic field). This is particularly useful when the background absorption shows fine structure. It permits an efficient background correction in the range of 185-900 nm.

CHOICE OF THE OPERATING CONDITIONS

After selecting the suitable wavelength and slit width for the specific element, the need for the following has to be ascertained:

- — correction for non-specific background absorption,
- — chemical modifiers or ionisation buffers to be added to the sample as well as to blank and reference solutions,
- — dilution of the sample to minimise, for example, physical interferences,
- — details of the temperature programme, preheating, drying, pyrolysis, atomisation, post-atomisation with ramp and hold times,
- — inert gas flow,
- — matrix modifiers for electrothermal atomisation (furnace),
- — chemical reducing reagents for measurements of mercury or other hydride-forming elements along with cold vapour cell or heating cell temperature,
- — specification of furnace design (tank, L'vov platform, etc).

METHOD

Use of plastic labware is recommended wherever possible. The preparation of the sample may require a dissolution, a digestion (mostly microwave-assisted), an ignition step or a combination thereof in order to clear up the sample matrix and/or to remove carbon-containing material. If operating in an open system, the ignition temperature should not exceed 600 °C, due to the volatility of some metals, unless otherwise stated in the monograph.

Operate an atomic absorption spectrometer in accordance with the manufacturer's instructions at the prescribed wavelength. Introduce a blank solution into the atomic generator and adjust the instrument reading so that it indicates maximum transmission. The blank value may be determined by using solvent to zero the apparatus. Introduce the most concentrated reference solution and adjust the sensitivity to obtain a maximum absorbance reading. Rinse in order to avoid contamination and memory effects. After completing the analysis, rinse with *water R* or acidified water.

If a solid sampling technique is applied, full details of the procedure are provided in the monograph.

Ensure that the concentrations to be determined fall preferably within the linear part of the calibration curve. If this is not possible, the calibration plots may also be curved and are then to be applied with appropriate calibration software.

Determinations are made by comparison with reference solutions with known concentrations of the element to be determined either by the method of direct calibration (Method I) or the method of standard additions (Method II).

METHOD I - DIRECT CALIBRATION

For routine measurements 3 reference solutions and a blank solution are prepared and examined.

Prepare the solution of the substance to be examined (test solution) as prescribed in the monograph. Prepare not fewer than 3 reference solutions of the element to be determined, the concentrations of which span the expected value in the test solution. For assay purposes, optimal calibration levels are between 0.7 and 1.3 times the expected content of the element to be determined or the limit prescribed in the monograph. For purity determination, calibration levels are the limit of detection and 1.2 times the limit specified for the element to be determined. Any reagents used in the preparation of the test solution are added to the reference and blank solutions at the same concentration.

Introduce each of the solutions into the instrument using the same number of replicates for each of the solutions to obtain a steady reading.

Calculation

Prepare a calibration curve from the mean of the readings obtained with the reference solutions by plotting the means as a function of concentration. Determine the concentration of the element in the test solution from the curve obtained.

METHOD II - STANDARD ADDITIONS

Add to at least 3 similar volumetric flasks equal volumes of the solution of the substance to be examined (test solution) prepared as prescribed. Add to all but 1 of the flasks progressively larger volumes of a reference solution containing a known concentration of the element to be determined to produce a series of solutions containing steadily increasing concentrations of that element known to give responses in the linear part of the curve, if possible. Dilute the contents of each flask to volume with solvent.

Introduce each of the solutions into the instrument, using the same number of replicates for each of the solutions, to obtain a steady reading.

Calculation

Calculate the linear equation of the graph using a least-squares fit and derive from it the concentration of the element to be determined in the test solution.

VALIDATION OF THE METHOD

Satisfactory performance of methods prescribed in monographs is verified at suitable time intervals.

LINEARITY

Prepare and analyse not fewer than 4 reference solutions over the calibration range and a blank solution. Perform not fewer than 5 replicates.

The calibration curve is calculated by least-square regression from all measured data. The regression curve, the means, the measured data and the confidence interval of the calibration curve are plotted. The operating method is valid when:

- — the correlation coefficient is at least 0.99,
- — the residuals of each calibration level are randomly distributed around the calibration curve.

Calculate the mean and relative standard deviation for the lowest and highest calibration level.

When the ratio of the estimated standard deviation of the lowest and the highest calibration level is less than 0.5 or greater than 2.0, a more precise estimation of the calibration curve may be obtained using weighted linear regression. Both linear and quadratic weighting functions are applied to the data to find the most appropriate weighting function to be employed. If the means compared to the calibration curve show a deviation from linearity, two-dimensional linear regression is used.

ACCURACY

Verify the accuracy preferably by using a certified reference material (CRM). Where this is not possible, perform a test for recovery.

Recovery

For assay determinations a recovery of 90 per cent to 110 per cent is to be obtained. For other determinations, for example, for trace element determination the test is not valid if recovery is outside of the range 80 per cent to 120 per cent at the theoretical value. Recovery may be determined on a suitable reference solution (matrix solution) which is spiked with a known quantity of analyte (middle concentration of the calibration range).

REPEATABILITY
The repeatability is not greater than 3 per cent for an assay and not greater than 5 per cent for an impurity test.

LIMIT OF QUANTIFICATION
Verify that the limit of quantification (for example, determined using the 10 σ approach) is below the value to be measured.

E. Fluorescence Spectrophotometry [Fluorimetry]

(Ph. Eur. method 2.2.21)

Fluorimetry is a procedure which uses the measurement of the intensity of the fluorescent light emitted by the substance to be examined in relation to that emitted by a given standard.

Method Dissolve the substance to be examined in the solvent or mixture of solvents prescribed in the monograph, transfer the solution to the cell or the tube of the fluorimeter and illuminate it with an excitant light beam of the wavelength prescribed in the monograph and as near as possible monochromatic.

Measure the intensity of the emitted light at an angle of 90° to the excitant beam, after passing it through a filter which transmits predominantly light of the wavelength of the fluorescence. Other types of apparatus may be used provided that the results obtained are identical.

For quantitative determinations, first introduce into the apparatus the solvent or mixture of solvents used to dissolve the substance to be examined and set the instrument to zero. Introduce the standard solution and adjust the sensitivity of the instrument so that the reading is greater than 50. If the second adjustment is made by altering the width of the slits, a new zero setting must be made and the intensity of the standard must be measured again. Finally introduce the solution of unknown concentration and read the result on the instrument. Calculate the concentration c_x of the substance in the solution to be examined, using the formula:

$$c_x = \frac{I_x c_s}{I_s}$$

c_x = concentration of the solution to be examined,
c_s = concentration of the standard solution,
I_x = intensity of the light emitted by the solution to be examined,
I_s = intensity of the light emitted by the standard solution.

If the intensity of the fluorescence is not strictly proportional to the concentration, the measurement may be effected using a calibration curve.

In some cases, measurement can be made with reference to a fixed standard (for example a fluorescent glass or a solution of another fluorescent substance). In such cases, the concentration of the substance to be examined must be determined using a previously drawn calibration curve under the same conditions.

F. X-Ray Fluorescence Spectrometry

(Ph. Eur. method Spectrometry, X.ray fluorescence (2.2.37))

PRINCIPLE OF THE TECHNIQUE

X-ray fluorescence (XRF) analysis is based on measurements of the X-rays emitted by the constituent atoms of a sample when it is excited by an external source of radiation.
If sufficiently energetic radiation impinges on an atom of the sample material, it may eject 1 of the inner-shell electrons of that atom. The vacancy created is filled by 1 of the electrons from an outer, higher-energy shell. The energy difference between the 2 electron shells involved in the process is released in the form of fluorescent X-rays. These X-rays are characteristic since their energy is specific to each element (atom). By measuring their energy and intensity, qualitative and quantitative data about the elemental composition of the test material is obtained.

XRF spectrometry is suitable for liquid, solid and powdered materials and is widely used as a means of screening pharmaceutical ingredients and products for toxic elements or elemental impurities, for quality control and in-process testing. It is also used to identify inorganic foreign elements within falsified medicinal products. As XRF can be non-invasive, it lends itself to process analytical technology (PAT) applications, such as the analysis of an unwanted trace catalyst in an active pharmaceutical ingredient.

EQUIPMENT

An XRF spectrometer (or analyser) consists of 3 essential components:
— a source of exciting radiation (e.g. an X-ray tube, an electron beam if a scanning electron microscope is used or, more rarely, a radioisotope);
— a means for reproducible sample presentation;
— a detector.

Depending on the X-ray detection method employed, either a wavelength-dispersive (WD) or an energy-dispersive (ED) XRF spectrometer is used.

In a WD-XRF spectrometer the X-rays from the sample are directed at a crystal, which diffracts them at precise angles depending on their energy. The intensity of the diffracted X-rays is measured sequentially by a detector counter.

In an ED-XRF spectrometer the X-rays from the sample are directed at a solid-state detector, which generates an electric pulse of an amplitude proportional to the energy of each X-ray photon detected. During measurement, the spectrometer acquires an X-ray spectrum of the sample that contains complete information about its composition.
An ED-XRF spectrometer can also be combined with a scanning electron microscope (SEM). Substantial advances in miniaturisation and automation have led to the development of hand-held ED-XRF spectrometers for field measurements.

Some instruments are supplied with an initial factory-set calibration that allows semi-quantitative analyses to be carried out.

MATRIX EFFECTS AND INTERFERENCE

The intensity of the characteristic X-rays of the analysed elements is not necessarily linear with concentration, owing to matrix effects. The intensity of the fluorescence measured for a given element depends not only on the concentration of that element in the sample but also on the absorption of the incident and fluorescent radiations by the matrix.
The presence and concentration of other elements (analytes) in the sample, the composition of the sample matrix, and the particle size of the sample material are known to contribute

to matrix effects. The presence of matrix effects must be taken into account in any calibration method utilised for quantitative determination.

At low concentrations the linearity is usually well preserved, which greatly facilitates calibration of the spectrometer. The intensity of the fluorescent radiation emitted by an element in a given matrix and at a given wavelength is then proportional to the concentration of that element and inversely proportional to the mass absorption coefficient of the matrix at that wavelength.

SAMPLE PREPARATION

It is essential that the sample is sufficiently thick that the intensities of characteristic X-rays measured are not affected by variations in sample thickness.

Liquid samples Samples are analysed 'as is', provided that the solution consists of a clear, single phase and has sufficiently low volatility. A special liquid-sample holder and a commercially available support window composed of a suitable polymer film (transparent to X-rays and solvent resistant) are required. Alternatively, liquid samples can be transferred onto the surface of a disc and dried before analysis.

Powdered samples Samples may be analysed 'as is' in special X-ray sample cups with bottoms made of a thin polymer film, transparent to X-rays. After transferring the powder to a sample cup, the cup is tapped gently until no further settling of the powder is observed. If necessary, more powder can be added to the cup after tapping. Another alternative for preparation of a powdered sample, which is better suited to WD-XRF analysis, is to press the sample powder into a pellet, with a binder (for example cellulose powder, wax or ethylcellulose) or without a binder. The mass of reference material and sample material must be about the same and the resulting pellets must be approximatively 5 mm thick or more.

Solid samples Samples are analysed by placing them directly on the spectrometer measuring window, making sure they cover it completely. Solid samples for WD-XRF may need to be cut into a uniform shape with a flat surface for reproducible analysis, whereas samples can be measured 'as is' when using ED-XRF, provided there is adequate sample depth.

Fusion The fusion bead method can be used to prepare solid and powdered samples (e.g. minerals or oxides) if the element of interest is not volatile. The sample is homogeneously mixed with a flux reagent (e.g. dilithium tetraborate) and transferred to a platinum vessel; a releasing agent and/or oxidising agent may be added if necessary, for example to prevent damage of the vessel. The mixture is heated at an appropriate temperature while swirling the vessel until a homogeneous melt is obtained. If necessary, the melt is then transferred to a flat-bottomed mould, kept in a horizontal position and cooled under conditions maintaining its property as a glass.

PROCEDURE

Measuring conditions

The instrument is set up and used in accordance with the manufacturer's instructions. The measurements may be carried out under vacuum, nitrogen or helium to improve the sensitivity of the method for the quantitation of light elements.

Reference standards

Standards required for the calibration, system suitability or control of equipment performance are prepared from certified reference materials (CRMs). Standards with a high carbon load may be more representative for pharmaceutical applications.

Calibration

The calibration model selected must be fit for purpose. Various calibration methods are available, including the 'fundamental parameters' approach, empirical calibration, Compton/Rayleigh normalisation and multiple linear regression (MLR).

System suitability test

This test must be carried out before the analysis to ensure that the performance of the measurement system is satisfactory. It may also be performed to verify the calibration of the system.

Acceptance criteria The measurement system is suitable if the concentration obtained for a check material containing the element(s) of interest within the used concentration range does not differ from the actual concentration by more than 5 per cent for an assay, and 20 per cent for impurity tests; when using concentrations determined from reference methods such as atomic absorption spectrometry, the accuracy of the XRF calibration should be aligned to that of the reference method; in this case an acceptance criterion of 10 per cent for the assay can be more realistic.

Analysis

The samples are measured with the same parameters as used during calibration of the instrument.

CONTROL OF EQUIPMENT PERFORMANCE

These parameters are also applicable for equipment qualification.

Specific procedures, acceptance criteria and time intervals for characterising XRF performance depend on the instrument and its intended application. Demonstrating stable instrument performance over extended periods of time provides some assurance that reliable measurements can be obtained.

The following tests may be carried out at appropriate intervals defined according to the user's quality system procedures to ensure the adequate performance of the XRF instrument.

x- and *y*-axes

It is recommended that the *x*-axis (energy or peak angle) and *y*-axis (intensity) are verified at least on installation and thereafter at appropriate intervals that are defined according to the user's quality system procedures. Consideration is to be given to peak position in ED-XRF and to peak angle in WD-XRF when verifying the *x*-axis, and to the count rate when verifying the *y*-axis.

Detector resolution

Calculate the resolution (total width at half-height) at the energy used during calibration of the instrument.

Acceptance criteria The resolution value does not deviate by more than 20 per cent for an assay, and 25 per cent for identity and elemental impurities tests, from the value determined during calibration of the instrument.

VALIDATION REQUIREMENTS

The objective of an XRF method validation is to demonstrate that the measurement procedure is fit for purpose (assay, content uniformity, limit tests and identification tests). Where sample preparation is necessary, it is essential that test materials are spiked before any preparatory steps. For example, if a test material is to be digested, the material must be spiked at the beginning of the digestion procedure.

For the determination of impurities, the validation requirements are given in general chapter *2.4.20.* For other purposes, validation is performed according to the relevant ICH guidelines.

G. Mass Spectrometry

(Ph. Eur. method 2.2.43)

Mass spectrometry is based on the direct measurement of the ratio of the mass to the number of positive or negative elementary charges of ions (*m/z*) in the gas phase obtained from the substance to be analysed. This ratio is expressed in atomic mass units (1 a.m.u. = one twelfth the mass of ^{12}C) or in daltons (1 Da = the mass of the hydrogen atom).

The ions, produced in the ion *source* of the apparatus, are accelerated and then separated by the *analyser* before reaching the *detector.* All of these operations take place in a chamber where a pumping system maintains a vacuum of 10^{-3} to 10^{-6} Pa.

The resulting spectrum shows the relative abundance of the various ionic species present as a function of *m/z.* The signal corresponding to an ion will be represented by several peaks corresponding to the statistical distribution of the various isotopes of that ion. This pattern is called the *isotopic profile* and (at least for small molecules) the peak representing the most abundant isotopes for each atom is called the *monoisotopic peak.*

Information obtained in mass spectrometry is essentially qualitative (determination of the molecular mass, information on the structure from the fragments observed) or quantitative (using internal or external standards) with limits of detection ranging from the picomole to the femtomole.

INTRODUCTION OF THE SAMPLE

The very first step of an analysis is the introduction of the sample into the apparatus without overly disturbing the vacuum. In a common method, called *direct liquid introduction,* the sample is placed on the end of a cylindrical rod (in a quartz crucible, on a filament or on a metal surface). This rod is introduced into the spectrometer after passing through a vacuum lock where a primary intermediate vacuum is maintained between atmospheric pressure and the secondary vacuum of the apparatus.

Other introduction systems allow the components of a mixture to be analysed as they are separated by an appropriate apparatus connected to the mass spectrometer.

Gas chromatography/mass spectrometry
The use of suitable columns (capillary or semi-capillary) allows the end of the column to be introduced directly into the source of the apparatus without using a separator.

Liquid chromatography/mass spectrometry
This combination is particularly useful for the analysis of polar compounds, which are insufficiently volatile or too heat-labile to be analysed by gas chromatography coupled with mass spectrometry. This method is complicated by the difficulty of obtaining ions in the gas phase from a liquid phase, which requires very special interfaces such as:

— *direct liquid introduction*: the mobile phase is nebulised, and the solvent is evaporated in front of the ion source of the apparatus,
— *particle-beam interface*: the mobile phase, which may flow at a rate of up to 0.6 mL/min, is nebulised in a desolvation chamber such that only the analytes, in neutral form, reach the ion source of the apparatus; this technique is used for compounds of relatively low polarity with molecular masses of less than 1000 Da,
— *moving-belt interface*: the mobile phase, which may flow at a rate of up to 1 mL/min, is applied to the surface of a moving belt; after the solvent evaporates, the components to be analysed are successively carried to the ion source of the apparatus where they are ionised; this technique is rather poorly suited to very polar or heat-labile compounds.

Other types of coupling (electrospray, thermospray, atmospheric-pressure chemical ionisation) are considered to be ionisation techniques in their own right and are described in the section on modes of ionisation.

Supercritical fluid chromatography/mass spectrometry
The mobile phase, usually consisting of supercritical carbon dioxide enters the gas state after passing a heated restrictor between the column and the ion source.

Capillary electrophoresis/mass spectrometry
The eluent is introduced into the ion source, in some cases after adding another solvent so that flow rates of the order of a few microlitres per minute can be attained. This technique is limited by the small quantities of sample introduced and the need to use volatile buffers.

MODES OF IONISATION

Electron impact
The sample, in the gas state, is ionised by a beam of electrons whose energy (usually 70 eV) is greater than the ionisation energy of the sample. In addition to the molecular ion M^+, fragments characteristic of the molecular structure are observed. This technique is limited mainly by the need to vaporise the sample. This makes it unsuited to polar, heat-labile or high molecular mass compounds. Electron impact is compatible with the coupling of gas chromatography to mass spectrometry and sometimes with the use of liquid chromatography.

Chemical ionisation
This type of ionisation involves a reagent gas such as methane, ammonia, nitrogen oxide, nitrogen dioxide or oxygen. The spectrum is characterised by ions of the $(M + H)^+$ or $(M - H)^-$ types, or adduct ions formed from the analyte and the gas used. Fewer fragments are produced than with electron impact. A variant of this technique is used when the substance is heat-labile: the sample, applied to a filament, is very rapidly vaporised by the Joule-Thomson effect (desorption chemical ionisation).

Fast-atom bombardment (FAB) or fast-ion bombardment ionisation (liquid secondary-ion mass spectrometry LSIMS)
The sample, dissolved in a viscous matrix such as glycerol, is applied to a metal surface and ionised by a beam of neutral atoms such as argon or xenon or high-kinetic-energy caesium ions. Ions of the $(M + H)^+$ or $(M - H)^-$ types or adduct ions formed from the matrix or the sample are produced. This type of ionisation, well suited to polar and heat-labile compounds, allows molecular masses of up to 10 000 Da to be obtained. The technique can be combined with liquid chromatography by adding 1 per cent to 2 per cent of glycerol to the mobile phase; however, the flow rates must be very low (a few microlitres per minute). These ionisation techniques also allow thin-layer chromatography plates to be analysed by applying a thin layer of matrix to the surface of these plates.

Field desorption and field ionisation
The sample is vaporised near a tungsten filament covered with microneedles (*field ionisation*) or applied to this filament (*field desorption*). A voltage of about 10 kV, applied between this filament and a counter-electrode, ionises the sample. These two techniques mainly produce molecular ions M^+, and $(M + H)^+$ ions and are used for low polarity and/or heat-labile compounds.

Matrix-assisted laser desorption ionisation (MALDI)
The sample, in a suitable matrix and deposited on a metal support, is ionised by a pulsed laser beam whose wavelength may range from UV to IR (impulses lasting from a picosecond to a few nanoseconds). This mode of ionisation plays an essential role in the analysis of very high molecular mass compounds (more than 100 000 Da) but is limited to time-of flight analysers (see below).

Electrospray
This mode of ionisation is carried out at atmospheric pressure. The samples, in solution, are introduced into the source through a capillary tube, the end of which has a potential of the order of 5 kV. A gas can be used to facilitate nebulisation. Desolvation of the resulting microdroplets produces singly or multiply charged ions in the gas phase. The flow rates vary from a few microlitres per minute to 1 mL/min. This technique is suited to polar compounds and to the investigation of biomolecules with molecular masses of up to 100 000 Da. It can be coupled to liquid chromatography or capillary electrophoresis.

Atmospheric-pressure chemical ionisation (APCI)
Ionisation is carried out at atmospheric pressure by the action of an electrode maintained at a potential of several kilovolts and placed in the path of the mobile phase, which is nebulised both by thermal effects and by the use of a stream of nitrogen. The resulting ions carry a single charge and are of the $(M + H)^+$ type in the positive mode and of the $(M - H)^-$ type in the negative mode. The high flow rates that can be used with this mode of ionisation (up to 2 mL/min) make this an ideal technique for coupling to liquid chromatography.

Thermospray
The sample, in the mobile phase consisting of water and organic modifiers and containing a volatile electrolyte (generally ammonium acetate) is introduced in nebulised form after having passed through a metal capillary tube at controlled temperature. Acceptable flow rates are of the order of 1 mL/min to 2 mL/min. The ions of the electrolyte ionise the compounds to be analysed. This ionisation process may be replaced or enhanced by an electrical discharge of about 800 volts, notably when the solvents are entirely organic. This technique is compatible with the use of liquid chromatography coupled with mass spectrometry.

ANALYSERS

Differences in the performance of analysers depend mainly on two parameters:

- the range over which *m/z* ratios can be measured, ie, the *mass range*,
- their *resolving power* characterised by the ability to separate two ions of equal intensity with *m/z* ratios differing by ΔM, and whose overlap is expressed as a given percentage of valley definition; for example, a resolving power ($M/\Delta M$) of 1000 with 10 per cent valley definition allows the separation of *m/z* ratios of 1000 and 1001 with the intensity returning to 10 per cent above baseline. However, the resolving power may in some cases (time-of-flight analysers, quadrupoles, ion-trap analysers) be defined as the ratio between the molecular mass and peak width at half height (50 per cent valley definition).

Magnetic and electrostatic analysers
The ions produced in the ion source are accelerated by a voltage V, and focused towards a magnetic analyser (magnetic field B) or an electrostatic analyser (electrostatic field E), depending on the configuration of the instrument. They follow a trajectory of radius r according to Laplace's law:

$$\frac{m}{z} = \frac{B^2 r^2}{2V}$$

Two types of scans can be used to collect and measure the various ions produced by the ion source: a scan of B holding V fixed or a scan of V with constant B. The magnetic analyser is usually followed by an electric sector that acts as a kinetic energy filter and allows the resolving power of the instrument to be increased appreciably. The maximum resolving power of such an instrument (double sector) ranges from 10 000 to 150 000 and in most cases allows the value of *m/z* ratios to be calculated accurately enough to determine the elemental composition of the corresponding ions.
For monocharged ions, the mass range is from 2000 Da to 15 000 Da. Some ions may decompose spontaneously (metastable transitions) or by colliding with a gas (collision-activated dissociation (CAD)) in field-free regions between the ion source and the detector. Examination of these decompositions is very useful for the determination of the structure as well as the characterisation of a specific compound in a mixture and involves tandem mass spectrometry. There are many such techniques depending on the region where these decompositions occur:

- *daughter-ion mode* (determination of the decomposition ions of a given parent ion): B/E = constant, *MIKES (Mass-analysed Ion Kinetic Energy Spectroscopy)*,
- *parent-ion mode* (determination of all ions which by decomposition give an ion with a specific *m/z* ratio): B^2/E = constant,
- *neutral-loss mode* (detection of all the ions that lose the same fragment):

$B/E(1 - E/E_0)^{1/2}$ = constant, where E_0 is the basic voltage of the electric sector.

Quadrupoles
The analyser consists of four parallel metal rods, which are cylindrical or hyperbolic in cross-section. They are arranged symmetrically with respect to the trajectory of the ions; the pairs diagonally opposed about the axis of symmetry of rods are connected electrically. The potentials to the two pairs of rods are opposed. They are the resultant of a constant component and an alternating component. The ions produced at the ion source are transmitted and separated by varying the voltages applied to the rods so that the ratio of continuous voltage to alternating voltage remains constant. The quadrupoles usually have a mass range of 1 a.m.u. to 2000 a.m.u., but some may range up to 4000 a.m.u. Although they have a lower resolving power than magnetic sector analysers, they nevertheless allow the monoisotopic profile of single charged ions to be obtained for the entire mass range. It is possible to obtain spectra using three quadrupoles arranged in series, Q_1, Q_2, Q_3 (Q_2 serves as a collision cell and is not really an analyser; the most commonly used collision gas is argon).
The most common types of scans are the following:

- *daughter-ion mode*: Q_1 selects an *m/z* ion whose fragments obtained by collision in Q_2 are analysed by Q_3,

— *parent-ion mode*: Q_3 filters only a specific *m/z* ratio, while Q_1 scans a given mass range. Only the ions decomposing to give the ion selected by Q_3 are detected,
— *neutral loss mode*: Q_1 and Q_3 scan a certain mass range but at an offset corresponding to the loss of a fragment characteristic of a product or family of compounds.

It is also possible to obtain spectra by combining quadrupole analysers with magnetic or electrostatic sector instruments; such instruments are called *hybrid mass spectrometers*.

Ion-trap analyser

The principle is the same as for a quadrupole, this time with the electric fields in three dimensions. This type of analyser allows product-ion spectra over several generations (MS^n) to be obtained.

Ion-cyclotron resonance analysers

Ions produced in a cell and subjected to a uniform, intense magnetic field move in circular orbits at frequencies which can be directly correlated to their *m/z* ratio by applying a Fourier transform algorithm. This phenomenon is called ion-cyclotron resonance. Analysers of this type consist of superconducting magnets and are capable of very high resolving power (up to 1000 000 and more) as well as MS^n spectra. However, very low pressures are required (of the order of 10^{-7} Pa).

Time-of-flight analysers

The ions produced at the ion source are accelerated at a voltage V of 10 kV to 20 kV. They pass through the analyser, consisting of a field-free tube, 25 cm to 1.5 m long, generally called a *flight tube*. The time (t) for an ion to travel to the detector is proportional to the square root of the *m/z* ratio. Theoretically the mass range of such an analyser is infinite. In practice, it is limited by the ionisation or desorption method. Time-of-flight analysers are mainly used for high molecular mass compounds (up to several hundred thousand daltons). This technique is very sensitive (a few picomoles of product are sufficient). The accuracy of the measurements and the resolving power of such instruments may be improved considerably by using an electrostatic mirror (reflectron).

SIGNAL ACQUISITION

There are essentially three possible modes.

Complete spectrum mode

The entire signal obtained over a chosen mass range is recorded. The spectrum represents the relative intensity of the different ionic species present as a function of *m/z*. The results are essentially qualitative. The use of spectral reference libraries for more rapid identification is possible.

Fragmentometric mode (Selected-ion monitoring)

The acquired signal is limited to one (single-ion monitoring (SIM)) or several (multiple-ion monitoring (MIM)) ions characteristic of the substance to be analysed. The limit of detection can be considerably reduced in this mode. Quantitative or semiquantitative tests can be carried out using external or internal standards (for example, deuterated standards). Such tests cannot be carried out with time-of-flight analysers.

Fragmentometric double mass spectrometry mode (multiple reaction monitoring (MRM))

The unimolecular or bimolecular decomposition of a chosen precursor ion characteristic of the substance to be analysed is followed specifically. The selectivity and the highly specific nature of this mode of acquisition provide excellent sensitivity levels and make it the most appropriate for quantitative studies using suitable internal standards (for example, deuterated standards). This type of analysis can be performed only on apparatus fitted with three quadrupoles in series, ion-trap analysers or cyclotron-resonance analysers.

CALIBRATION

Calibration allows the corresponding *m/z* value to be attributed to the detected signal. As a general rule, this is done using a reference substance. This calibration may be external (acquisition file separate from the analysis) or internal (the reference substance(s) are mixed with the substance to be examined and appear on the same acquisition file). The number of ions or points required for reliable calibration depends on the type of analyser and on the desired accuracy of the measurement, for example, in the case of a magnetic analyser where the *m/z* ratio varies exponentially with the value of the magnetic field, there should be as many points as possible.

SIGNAL DETECTION AND DATA PROCESSING

Ions separated by an analyser are converted into electric signals by a detection system such as a photomultiplier or an electron multiplier. These signals are amplified before being re-converted into digital signals for data processing, allowing various functions such as calibration, reconstruction of spectra, automatic quantification, archiving, creation or use of libraries of mass spectra. The various physical parameters required for the functioning of the apparatus as a whole are controlled by computer.

G1. Inductively Coupled Plasma-Mass Spectrometry

(Ph. Eur. method 2.2.58)

Inductively coupled plasma-mass spectrometry (ICP-MS) is a mass spectrometry method that uses an inductively coupled plasma (ICP) as the ionisation source. The basic principles of ICP formation are described in chapter *2.2.57* on inductively coupled plasma-atomic emission spectrometry (ICP-AES).

ICP-MS utilises the ability of the ICP to generate charged ions from the element species within a sample. These ions are then directed into a mass spectrometer, which separates them according to their mass-to-charge ratio (*m/z*). Most mass spectrometers have a quadrupole system or a magnetic sector. Ions are transported from the plasma through 2 cones (sampler and skimmer cones, forming the interface region) to the ion optics. The ion optics consist of an electrostatic lens, which takes ions from an area at atmospheric pressure to the mass filter at a vacuum of 10^{-8} Pa or less, maintained with a turbomolecular pump. After their filtration, ions of the selected mass/charge ratio are directed to a detector (channel electromultiplier, Faraday cup, dynodes), where ion currents are converted into electrical signals. The element is quantified according to the number of ions arriving and generating electrical pulses per unit time.

The sample-introduction system and data-handling techniques of an ICP-AES system are also used in ICP-MS.

APPARATUS

The apparatus consists essentially of the following elements:
— sample-introduction system, consisting of a peristaltic pump delivering the solution at constant flow rate into a nebuliser;
— radio-frequency (RF) generator;
— plasma torch;

— interface region including cones to transport ions to the ion optics;
— mass spectrometer;
— detector;
— data-acquisition unit.

INTERFERENCE

Mass interference is the major problem, for example by isobaric species that significantly overlap the mass signal of the ions of interest, especially in the central part of the mass range (for example 40-80 a.m.u.). The combination of atomic ions leads to polyatomic or molecular interferences (i.e. $^{40}Ar^{16}O$ with ^{56}Fe or $^{40}Ar^{40}Ar$ with ^{80}Se). Matrix interference may also occur with some analytes. Some samples have an impact on droplet formation or on the ionisation temperature in the plasma. These phenomena may lead to the suppression of analyte signals. Physical interference is to be circumvented by using the method of internal standardisation or by standard addition. The element used as internal standard depends on the element to be measured: ^{59}Co and ^{115}In, for example, can be used as internal standards.

The prime characteristic of an ICP-MS instrument is its resolution, i.e. the efficiency of separation of 2 close masses. Quadrupole instruments are, from this point of view, inferior to magnetic-sector spectrometers.

PROCEDURE

SAMPLE PREPARATIONS AND SAMPLE INTRODUCTION

The sample preparation usually involves a step of digestion of the matrix by a suitable method, for example in a microwave oven. Furthermore, it is important to ensure that the analyte concentration falls within the working range of the instrument through dilution or preconcentration, and that the sample-containing solution can be nebulised in a reproducible manner.

Several sample-introduction systems tolerate high acid concentrations, but the use of sulfuric and phosphoric acids can contribute to background emission. Therefore, nitric and hydrochloric acids are preferable. The availability of hydrofluoric acid-resistant (for example perfluoroalkoxy polymer) sample-introduction systems and torches also allows the use of hydrofluoric acid. In selecting a sample-introduction method, the requirements for sensitivity, stability, speed, sample size, corrosion resistance and resistance to clogging have to be considered. The use of a cross-flow nebuliser combined with a spray chamber and torch is suitable for most requirements. The peristaltic pumps usually deliver the standard and sample solutions at a rate of 20-1000 µL/min.

In the case of organic solvents being used, the introduction of oxygen must be considered to avoid organic layers.

CHOICE OF OPERATING CONDITIONS

The standard operating conditions prescribed by the manufacturer are to be followed. Usually, different sets of operating conditions are used for aqueous solutions and for organic solvents. Suitable operating parameters are to be properly chosen:
— selection of cones (material of sampler and skimmer);
— support-gas flow rates (outer, intermediate and inner tubes of the torch);
— RF power;
— pump speed;
— selection of one or more isotopes of the element to be measured (mass).

ISOTOPE SELECTION

Isotope selection is made using several criteria. The most abundant isotope for a given element is selected to obtain maximum sensitivity. Furthermore, an isotope with the least interference from other species in the sample matrix and from the support gas should be selected. Information about isobaric interferences and interferences from polyatomic ions of various types, for example hydrides, oxides, chlorides, etc., is usually available in the software of ICP-MS instrument manufacturers.

CONTROL OF INSTRUMENT PERFORMANCE

System suitability

— Tuning of the instrument allows to monitor and adjust the measurement before running samples. ICP-MS mass accuracy is checked with a tuning solution containing several isotopes covering the whole range of masses, for example ^{9}Be, ^{59}Co, ^{89}Y, ^{115}In, ^{140}Ce and ^{209}Bi.
— Sensitivity and short- and long-term stability are recorded. The instrument parameters (plasma condition, ion lenses and quadrupole parameter) are to be optimised to obtain the highest possible number of counts.
— Tuning for resolution and mass axis is to be done with a solution of Li, Y and Tl to ensure an acceptable response over a wide range of masses.
— Evaluation of the efficiency of the plasma to decompose oxides has to be performed in order to minimise these interferences. The ratio Ce/CeO and/or Ba/BaO is a good indicator, and a level less than about 3 per cent is required.
— Reduction of the formation of double-charged ions is made with Ba and Ce. The ratio of the signal for double-charged ions to the assigned element should be less than 2 per cent.
— Long-term stability is checked by running a standard first and at the end of the sample sequence, controlling whether salt deposits on the cones have reduced the signal throughout the run.

VALIDATION OF THE METHOD

Satisfactory performance of methods prescribed in monographs is verified at suitable time intervals.

LINEARITY

Prepare and analyse not fewer than 4 reference solutions over the calibration range plus a blank. Perform not fewer than 5 replicates.

The calibration curve is calculated by least-square regression from all measured data of the calibration test. The regression curve, the means, the measured data and the confidence interval of the calibration curve are plotted. The operating method is valid when:
— the correlation coefficient is at least 0.99;
— the residuals of each calibration level are randomly distributed around the calibration curve.

Calculate the mean and relative standard deviation for the lowest and for the highest calibration level.

When the ratio of the estimated standard deviations of the lowest and the highest calibration level is less than 0.5 or greater than 2.0, a more precise estimation of the calibration curve may be obtained using weighted linear regression. Both linear and quadratic weighting functions are applied to the data to find the most appropriate weighting function to be employed.

If the means compared to the calibration curve show a deviation from linearity, two-dimensional linear regression is used.

ACCURACY

Verify the accuracy preferably by using a certified reference material (CRM). Where this is not possible, perform a test for recovery.

Recovery

For assay determinations a recovery of 90 per cent to 110 per cent is to be obtained. The test is not valid if recovery, for example for trace-element determination, is outside the range 80 per cent to 120 per cent of the theoretical value. Recovery may be determined on a suitable reference solution (matrix solution) spiked with a known quantity of analyte (concentration range that is relevant to the samples to be determined).

REPEATABILITY

The repeatability is not greater than 3 per cent for an assay and not greater than 5 per cent for an impurity test.

LIMIT OF QUANTIFICATION

Verify that the limit of quantification (for example, determined using the 10 σ approach) is below the value to be measured.

H. Raman Spectroscopy

(Ph. Eur. method 2.2.48)

In Raman spectroscopy a sample is irradiated with an intense monochromatic light source (usually a laser). Most of the radiation is scattered from the sample at the same wavelength as that of the incident light; this process is known as Rayleigh scattering or elastic light scattering. Only 10^{-6} to 10^{-8} of the incident photons are scattered from the sample at wavelengths shifted from the original source wavelength; this scattered light is known as Raman scatter or inelastic light scatter. The differences between the incident light source wavelength and the Raman scattered wavelengths are known as the Raman shifts and are related to the molecular vibrations within the sample. The scattered light with lower energy is called Stokes scatter and with higher energy is called anti-Stokes scatter. Typically, Stokes scatter is analysed.

A Raman spectrum can be obtained from liquid, solid and even gaseous samples. Raman spectroscopy is a suitable non-invasive method for determination of solid-state properties and for chemical identification, for example, detecting changes in polymorphic form. Raman spectroscopy encompasses many methodologies, including backscattering, transmittance, resonance Raman (RR) spectroscopy, surface-enhanced Raman spectroscopy (SERS), tip-enhanced Raman spectroscopy (TERS), spatially offset Raman spectroscopy (SORS), Raman optical activity (ROA), coherent anti-Stokes Raman spectroscopy (CARS), stimulated Raman spectroscopy (SRS) and confocal Raman (CF) spectroscopy. It also lends itself to imaging techniques.

Raman spectroscopy is complementary to infrared spectroscopy. Both techniques probe the fundamental molecular vibrations in a material. However, Raman and infrared spectroscopy have different sensitivities for different functional groups within a material. Raman spectroscopy is particularly useful in examining non-polar bonds, functional groups and vibrations that are highly symmetrical (e.g. C-C single or multiple bonds); but is less sensitive to polar bonds (e.g. C=O) and vibrations that are asymmetrical.
For example, water, which strongly influences infrared spectra, exhibits weak Raman scattering and therefore has minimal interference on the resulting spectrum. Raman can therefore be used for aqueous solutions.

A major difficulty of Raman spectroscopy is that the material examined or impurities in the material may exhibit fluorescence, which can overcome the Raman signal. Fluorescence may be avoided by choosing a longer excitation wavelength, for example in the near-infrared region.

Raman spectroscopy is a rapid and non-invasive analytical method and can be performed off-line and also at-line, on-line or in-line for process analytical technology (PAT). Raman spectrometers can be situated at a great distance from the point of measurement when using long-distance optical fibres for sample interfacing. Raman spectroscopy has a wide variety of applications for chemical, physical and process analysis and can also be used for counterfeit detection and quality control, for example:

- *chemical analysis*: identification and quantification of active substances, excipients;
- *physical analysis*: identification and quantification of solid forms (e.g. polymorphs and solvates) and crystallinity;
- *process analysis*: monitoring of biological and chemical reactions, synthesis, crystallisation, granulation, mixing, drying, lyophilisation, extrusion, encapsulation and coating.

APPARATUS

There are different types of spectrometers for recording Raman spectra, the most common are benchtop dispersive instruments, including microscope-coupled Raman spectrometers, Fourier transform (FT) and hand-held Raman spectrometers.

Raman spectrometers typically consist of the following components:

- a monochromatic light source, typically a laser, with a wavelength in the ultraviolet, visible or near-infrared region; selection of laser frequency and intensity should be considered depending on the intended application and material to be examined;
- suitable optics (lens, mirrors or optical-fibre assembly) that direct the monochromatic light to and collect the Raman scattered light from the material to be examined;
- an optical device (monochromator or filter) that transmits the frequency-shifted Raman scattering and prevents the intense incident frequency of Rayleigh scattering from reaching the detector; and
- a dispersing device (grating and/or prism polychromator) combined with wavelength-selecting slits and a single or multi-channel detector (e.g. a charge-coupled device) or
- an interferometer with a detector that records the intensity of the scattered light over time, and a data-handling device (e.g. computer and appropriate software) that converts this data to the frequency or wavenumber domain by a Fourier-transform calculation.

PREPARATION OF THE SAMPLE

Raman spectra can be obtained from solids, liquids and gases either directly or in suitable containers such as glass vials, generally without prior sample preparation or dilution.

When using Raman spectroscopy, the measured sample area and volume may be small (in particular for microscope-coupled devices) and care must be taken to ensure the measurement is representative. This can be achieved by, for example, rotation of the sample, performing multiple measurements on different preparations of the sample, increasing the area of illumination by reducing the magnification (focus), by demagnification (defocusing) of the

Table 2.2.48.-1. – *Wavenumber shifts (and acceptable tolerances) of polystyrene, paracetamol and cyclohexane*

	Wavenumber shifts[A] [cm^{-1}]	Tolerances	
		Benchtop [cm^{-1}]	Hand-held [cm^{-1}]
Polystyrene[B]	620.9	± 1.5	± 2.5
	1001.4	± 1.5	± 2.0
	1031.8	± 1.5	± 2.0
	1602.3	± 1.5	± 3.0
	3054.3	± 3.0	NA[E]
Paracetamol[C]	797.2	± 1.5	± 2.5
	857.9	± 1.5	± 2.0
	1168.5	± 1.5	± 2.0
	1236.8	± 1.5	± 2.0
	1323.9	± 1.5	± 2.5
	1648.4	± 1.5	± 3.0
	2931.1	± 2.0	NA[E]
Cyclohexane[D]	801.3	± 1.5	± 2.5
	1028.3	± 1.0	± 2.0
	1266.4	± 1.0	± 2.0
	1444.4	± 1.0	± 2.5
	2852.9	± 2.0	± 3.0

[A] *Standard guide for Raman shift standards for spectrometer calibration* (American Society for Testing and Materials ASTM E 1840).
[B] Polystyrene film (e.g. 76 µm), pellets (e.g. NIST 706a) or rod.
[C] *Paracetamol for equipment qualification CRS* (which represents monoclinic form I).
[D] *Cyclohexane R.*
[E] NA: beyond detector range.

laser beam or by changing the focal length between measurements to scan at different depths.

It is not always possible to consider Raman as a non-destructive technique. The energy transmitted by the laser depends on the duration of exposure and the wavelength. It may change the physical state and may destroy the sample.

CONTROL OF INSTRUMENT PERFORMANCE

Carry out the prescribed calibrations and/or system performance tests according to the manufacturer's instructions at regular intervals, depending on the use of the apparatus. Wavenumber calibration of Fourier transform systems is maintained with an internal laser (He-Ne). For dispersive systems, emission spectra of a low pressure lamp exhibiting characteristic maxima at wavenumbers over the whole spectral range of the instrument can usually be used for calibration (e. g. neon lamps or less frequently mercury, argon, krypton or xenon lamps).

Verification of the wavenumber scale Verify the wavenumber scale of the Raman shift using a suitable standard that has characteristic maxima at the wavenumbers under investigation, for example an organic substance such as polystyrene, paracetamol or cyclohexane (see Table 2.2.48.-1).

A minimum of 3 wavenumber shifts covering the working range of the instrument intended for measurements should be chosen. For dispersive Raman spectrometers that use multiple gratings for different spectral resolutions, the wavenumber scale should be verified at the same optical resolution that will occur for sample collection. All gratings used for Raman measurements should be verified for accuracy of Raman-shift.

Verification of the response-intensity scale The absolute and relative intensities of the Raman bands are affected by variations in several factors including:

— polarisation of the irradiating light;
— polarisation of the Raman scattered light;
— intensity of the irradiating light;
— instrument response;
— focus and geometry at sample;
— packing density for solid samples;
— refractive index n or change of n (Δn) between analyte and the environment;
— the particle size and particle-size distribution;
— the scattering cross-section;
— the absorption cross-section.

Appropriate acceptance criteria will vary with the application but a day-to-day variation of ± 10 per cent or less in relative band intensities is achievable in most cases. Response calibration may involve the use of white-light standards or luminescent glass (e.g. NIST SRM 2241), usually applied by the manufacturer of Raman spectrometers.

QUALITATIVE METHODS

Since the frequency shift position is employed for identification, identical laser intensity for both the reference standard and the material to be examined may not be necessary. The material to be examined is measured in the same physical state (e.g. liquid, solid) as the reference or library material. Raman techniques offer the advantage of non-invasive measurements of the material to be examined without removal from the packaging. However, some packaging materials may alter the measurement. This is especially the case when the packaging absorbs at the laser's excitation wavelength.

Identification using a reference standard Prepare the material to be examined and the reference standard by the same procedure and record the spectra under the same instrumental conditions. The maxima in the spectrum obtained with the material to be examined correspond in position and if applicable in relative intensity to those in the spectrum obtained with the reference standard. When the spectra recorded in the solid state show differences in the positions of the maxima, treat the material to be examined and the reference standard in the same manner so that they crystallise or are produced in the same solid form, or proceed as described in the monograph, then record the spectra.

Identification using a spectral reference library
Record the spectra of a suitable number of materials which exhibit typical variation (manufacturer, batch, particle size, impurity profile, etc.) and comply with the requirements of the monograph or established specifications. The number and selection of samples in the database depends on the specific application. A suitable mathematical transformation of the Raman spectrum may facilitate qualitative comparison of spectra (e.g. baseline correction, min-max normalisation, vector normalisation, derivatives). The selectivity of the database that makes it possible to identify a given material and distinguish it adequately from other materials in the database is to be confirmed during the validation procedure.

QUANTITATIVE METHODS
Quantitative determination requires that the reference standard and the material to be examined are measured at the same laser intensity and frequency. Ensure the material to be examined is measured in the same physical state (e.g. liquid, solid) and concentration range as the reference standard used for calibration. While Beer-Lambert's law is not valid for Raman spectroscopy, Raman intensity is directly proportional to the concentration of the Raman scattering analytes, however for solid samples and suspensions the Raman intensity could be affected by the matrix (e.g. fluorescence and self-absorption). The Raman signal is influenced by the refractive index of the material, the particle size and the particle-size distribution (where the small particles give a relatively more intense Raman scattering than large particles), the packing density, the scattering cross-section, the absorption cross-section, etc. (see also under Verification of the response-intensity scale).

J. Flow Cytometry

(Ph. Eur. method 2.7.24)

Flow cytometry consists of a multiparametric analysis of optical properties of individual particles in a fluidic system.

Cells or particles in suspension are individually distributed into a linear array (stream), which flows through a detection device. Solid tissues have to be reduced to a single-cell suspension to be analysed.

The spectrum of parameters measurable by flow cytometry includes volume and morphological complexity of cells or cell-like structures, cell pigments, DNA content, RNA content, proteins, cell surface markers, intracellular markers, enzymatic activity, pH, membrane and fluidity.

It is possible to collect 2 morphological parameters plus 1 or more fluorescence signals per single cell. The multiparametric analysis allows the definition of cell populations by their phenotype.

APPARATUS
Focusing, magnifying, and choice of light source are optimised to allow the automatic detection and measurement of morphological differences and staining patterns. Flow cytofluorimetric analysis meets the following criteria:
— choice of light source depending on the parameters to be analysed;
— adjustment of instrument settings depending on the cell type to be analysed (for example, cell cultures, leucocytes, platelets, bacteria, spermatozoa, yeast) and the analysis to be performed (for example, phenotyping, cell cycle, apoptosis, cytokines, membrane fluidity, fluorescent protein).

Flow cytometry is characterised by the automated quantification of set parameters for a high number of single cells during each analysis session. For example, 100 000 particles or more (practically unlimited) are analysed one after the other, typically in about 1 min. The detection limit is as low as 100 fluorescent molecules per cell.

A flow cytometer apparatus has 5 main components:
— a fluidic system and a flow cell;
— a light source;
— a detection and Analogue to Digital Conversion (ADC) system;
— an amplification system;
— a computer provided with software for analysis of the signals.

FLUIDIC SYSTEM AND FLOW CELL
The single cell is exposed to the light source and detected in the flow cell. The fluidic system carries the suspended cells individually from the sample tube to the laser intercept point. To achieve this, the sample stream is drawn out to a very thin fluid thread by a sheath fluid in the flow cell (hydrodynamic focusing). The light beam is focused in an elliptical shape, by 2 confocal lenses, into the flow cell channel through which the cells pass. The flow rate must be constant during routine cell surface marker analysis and must ensure a suitable distance between the cells to allow counting.

LIGHT SOURCES
Commonly used light sources are:
— lamps (mercury, xenon);
— high power water-cooled lasers (argon, krypton, dye laser);
— low power air-cooled lasers (argon (488 nm), red helium-neon (633 nm), green helium-neon, helium-cadmium (UV));
— diode lasers (blue, green, red, violet).

SIGNAL DETECTION
When a particle passes across the light beam, it scatters some of the light in all directions. Fluorescent dyes, when added to the particle, give off their own light (fluorescence), which is also radiated in all directions. 2 types of signals may thereby be generated:
— scatter of light;
— fluorescence emission.

The instrument's light detectors collect some of this scattered and fluorescent light and produce electronic signals proportional to the amount of light collected.

Scatter
2 parameters of light scattering are measured:
— the amount scattered mainly forward (forward scatter (FS))
— the amount scattered at 90° from the direction of the light beam (side scatter (SS)).

Forward scatter correlates with the cell volume while side scatter is influenced by parameters such as the shape of the nucleus, the amount and type of cytoplasmic granules or the membrane roughness, and correlates with the morphological complexity of the cell, so that the higher the SS intensity, the higher the cell complexity. As a function of the morphological characteristics of cells, scatter signals will always be generated during a flow analysis; they are defined as intrinsic parameters.

Fluorescence
Depending on the type and number of light sources, when a cell passes through the sensing area, it will emit fluorescent

light. Fluorescence signals are generated from fluorescent dyes naturally present in the cells (for example, co-enzymes, chlorophyll, seaweed pigments) and/or from fluorescent probes taken up by the cells when stained for the analysis of specific characteristics (for example, fluorescent antibodies, nucleic acid dyes, pH probes, calcium probes, fluorescent proteins). Nowadays, there is a large number and a wide range of different types of fluorescent probes available. The optical filters must be adapted to the fluorochromes used and changed if necessary. Each fluorescent probe is characterised by its excitation spectrum and its emission spectrum. They are chosen depending on the nature of the excitation source and the detection system, and according to the specific purpose of the analysis.

SIGNAL MANAGEMENT AND ANALOGUE TO DIGITAL CONVERSION

Scatter and fluorescence signals emitted by cells when passing across the laser beam are sorted and addressed to their detectors using optical filters. The detectors are transducers (photomultiplier tubes (PMTs)) that convert light signals radiated from the cells into voltage pulses.

The process of counting each pulse in the appropriate channel is known as Analogue to Digital Conversion (ADC). The process is finally shown as a frequency histogram.

Amplification

Voltage pulses need to be amplified for optimal visualisation. The amplification process accentuates the differences between cell signals, and consequently increases the resolution among cell populations of different characteristics (for example, the differentiation of viable from non-viable cells, or non-specific fluorescence from antigen-specific fluorescence after staining with a fluorescent monoclonal antibody).

There are 2 methods of amplification: linear or logarithmic; the choice between the 2 types is made for every single signal according to the morphological characteristics of the cells and the staining reagents used (for example, fluorescent monoclonal antibodies, nucleic acid dyes).

Linear amplification, which enhances the differences among strong pulses, is used with those parameters that generate high intensity signals, for example:

— cell scatters;

— fluorescence from nucleic acid dyes for cell cycle studies.

Logarithmic amplification, in contrast, is for weak pulses and parameters or analysis conditions that may generate both weak and strong pulses, for example:

— cell antigens;

— scatter from platelets, bacteria, yeast;

— fluorescence from nucleic acid dyes for apoptosis studies.

Compensation of fluorescence signals

Each fluorescent dye has an absorption wavelength spectrum and a higher emission wavelength spectrum. When using 2 or more fluorescent probes simultaneously for staining cells (for example, 4-antigen immunophenotyping), the fluorochromes emission spectra may overlap. As a consequence, each fluorescence detector will sense its own specific fluorescent light and a variable quantity of light emitted by the other fluorescent probes. This results in signal over-evaluation and poor separation of the cell populations.

The solution is in the use of an electronic matrix that allows the selective subtraction of the interfering signals from each fluorescence signal after detector sensing (fluorescence compensation).

Fluorescence compensation requires the use of fluorescence calibrators, preferably positive cell samples stained with the fluorochromes of interest, combined in a manner equivalent to that for the antibody used for the analysis.

SIGNAL PLOTTING AND DISPLAY

After amplification and compensation, the signals are plotted in 2 or 3 dimensions. Histograms show the signal intensities versus the cell counts for a given parameter. Cytograms, in which each dot represents a cell, result from the combination of 2 signal intensities (dual-parameter dot plots). The type and number of plots and signal combinations are chosen on the basis of the specimens and dyes used. When analysing acquired data, the flow cytometry software can also generate other kinds of graphs (such as overlays, surface plots, tomograms, contour plots, density plots, overlay plots). Statistical data such as mean fluorescent intensities (and their shifts in time or their dependence on cell function) can also be used.

DATA ANALYSIS

Different kinds of cell populations may be present inside the cell suspensions to be analysed, some of which are unwanted (such as dead cells, debris or macro-aggregates), or simply not relevant for the analysis (for example, granulocytes when studying lymphocytes). This depends on the cell sample type (whole blood, bone marrow, cell cultures, biological fluids, cell suspensions from solid tissues) and on the handling procedures (for example, staining methods, lysis, fixation, cryopreservation, thawing, paraffin-embedded tissue preparation).

As a consequence, not all the signals generated during a flow cytometry analysis belong to the cells to be studied. 2 strategies are adopted to exclude unwanted and irrelevant cell signals.

The 1st is used during data acquisition. It is a noise threshold, applied to 1 (or more) significant parameter(s), set to acquire only the cells with signal intensities higher than the pre-defined discrimination value for that parameter. Due to its characteristics of a strong signal with a low grade of interference, forward scatter is the parameter most often used as discriminator.

The 2nd, applied during data analysis, consists of the use of gating regions to restrict the analysis only to signals from those populations that satisfy given morphological and expression profile characteristics. 2 types of logical gating are commonly used. The 1st is the morphological gate. The cell populations are identified using their morphological signals (FS and SS). A region gate is drawn around the population of interest (for example, lymphocytes, viable cells) then the fluorescence plots are gated into the selected region. The 2nd is the fluorescence-based gate. The cell population of interest is identified on the basis of the expression intensity of an antigen or a dye, then a gate region is drawn around it. Afterwards the fluorescence plots are gated into the selected region.

The analysis software allows the creation of multiple gate regions, using a sequential logic order. This feature is especially useful when studying rare cell populations or for sorting purposes.

CONTROLS

Internal control

The system's optical alignment must be validated before analysis using adapted fluorospheres and the optimum fluidic stability is checked. The data obtained are reported and allow the periodical review of control values against the mean

performance value. A positive control is highly desirable to prove that the test antibody is functional and to allow the proper setting of the flow cytometer. The positive control must include samples known to be positive for the marker of interest.

External control
To ensure reliability in the data obtained or to check inter-laboratory reproducibility, participation in a proficiency testing study is recommended.

K. Peptide Identification by Nuclear Magnetic Resonance Spectrometry

(Ph. Eur. method 2.2.64)

This general chapter is to be used in conjunction with general chapter *2.2.33. Nuclear magnetic resonance spectrometry* in the context of peptide identification. The approach to be followed is qualitative and consists of comparing the nuclear magnetic resonance (NMR) spectrum of a test sample with that of a reference sample acquired under identical conditions.

This general chapter mainly applies to the use of proton NMR (^{1}H NMR) spectrometry, to confirm the identity of small peptide products (up to approximately 15 amino acids). It is also applicable when using ^{13}C NMR spectrometry with some modifications. The scope is restricted to the use of one-dimensional NMR spectrometry.

GENERAL PRINCIPLES

Equipment
Unless otherwise specified, an apparatus with a field strength giving an operating frequency for proton NMR of at least 300 MHz.

Spectral acquisition conditions and their optimisation
After introduction into the magnet, the sample is allowed to come to thermal equilibrium, especially if analysis is carried out at a temperature significantly different from room temperature: monitoring the lock signal is often a valuable visual guide to the progress of this process.

The spectral width must encompass the complete spectrum of the peptide, with an empty spectral region at each side. Typically, a spectral width of 12 ppm or 16 ppm is appropriate.

The following parameters may be optimised to improve resolution of characteristic peaks: temperature and/or pH primarily, buffer and peptide concentrations. Control of sample temperature is recommended but is not mandatory; if not used, the effect of small temperature changes on the appearance of the spectrum is validated.

The number of data points collected is such as to define peaks adequately.

Solvent suppression is not recommended but, if used, the intensities of peaks close to the solvent resonance may be affected and this has to be validated when comparing spectra.

Chemical shift referencing
For samples in aqueous solution, sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS), sodium 3-(trimethylsilyl) propionate (TSP) or a deuterated analogue (TSP-d_4) are appropriate, and the chemical shift of the methyl signals is often set to 0 ppm. Either the reference material is added at low amounts (10-100 ppm has been found to be appropriate) to the deuterated water used to dissolve the final sample, or an easily recognised internal resonance that is consistently present (such as acetate anion) can be used as a secondary reference. In this case, a validation spectrum obtained under the same spectral conditions is used to define the chemical shift of the secondary standard.

Sample size
Usually a few milligrams are used. If sample sizes are variable, the effects of this variation on the appearance of the spectrum are validated.

Sample preparation
The test and reference samples must be comparable in terms of concentration, pH and buffer composition. Typically, samples in solution are lyophilised, and the dried samples dissolved in deuterated water or a buffer in deuterated water. It may be worthwhile to lyophilise a solution in deuterated water one or more times ('deuterium exchange') as this reduces the intensity of strong solvent signals; volatile process impurities such as ethanol will also be lost. Use of buffer for the final sample preparation can reduce aggregation and improve spectral reproducibility by reducing batch-to-batch pH variation. Some probes are intolerant to high salt concentrations, but ionic strengths up to 200 mM sodium chloride are normally tolerated. High salt concentrations tend to increase 90° pulse length.

VERIFICATION OF IDENTITY

Determination of key spectral factors
Use of a qualitative approach does not entail stringent requirements on spectral parameters (for example, fast pulse repetition rates can be used, as full relaxation is not required). The use of short pulse widths (for example, a 30° pulse) and fast repetition rates will have no significantly deleterious effect on spectra, and will allow faster acquisition of acceptable signal-to-noise ratios. Variation in the pulse width and acquisition time within wide limits will not affect the ability to compare spectra. The number of scans collected must give appropriate signal-to-noise ratios for low intensity resonances and therefore a minimum signal-to-noise ratio of 50:1 is recommended.

Identification of characteristic resonances
It is possible to compare either the complete spectrum or a portion of it. Comparison of spectra of relevant samples will highlight regions of the spectrum that are distinctive, and comparison can be constrained to these regions. It is important to define resonances from impurities, such as residual solvents, which may be essentially irrelevant to product quality and which may vary in intensity between batches.

Spectral comparison
See the provisions of general chapter *2.2.33*.

L. Chemical Imaging

(Ph. Eur. general text 5.24)

1 SCOPE

Chemical imaging (CI) combines spatially resolved sensing technologies with data analysis techniques to characterise a sample in chemical and physical terms, using information primarily obtained from its surface. Chemical imaging is particularly suited to the analysis of solid, semi-solid and liquid samples with regard to material properties, including component identity (active pharmaceutical ingredients and excipients), domain size and distribution, polymorphism, and particle morphology. Thus, imaging can be applied to assess

identity, quality and quantity of active ingredients, intermediates, and excipients in bulk or solid dosage forms, biological samples, packaging and devices. Imaging is used to explore sample homogeneity, detect physical sample defects (e.g. cracks in cores or coatings), and identify foreign particles or contaminants. It also facilitates process understanding and root cause determination. Lastly, it is a tool to evaluate falsified or counterfeit medicinal products.

This general chapter's primary focus is on chemical imaging systems (CIS) based on surface analysis performed with vibrational spectroscopy, e.g. mid-infrared spectroscopy, near-infrared spectroscopy and Raman spectroscopy. However, it also applies to other techniques that supply images.

It offers specific recommendations to assess the performance of chemical imaging systems for the qualitative and quantitative exploitation of images. Where chemical imaging systems are primarily intended for investigative purposes, the performance requirements for infrared absorption spectrophotometry (*2.2.24*), near-infrared spectroscopy (*2.2.40*) and Raman spectroscopy (*2.2.48*) need not be applied. Instead, individual criteria have to be established using a risk-based approach.

2 ASPECTS OF CHEMICAL IMAGING

2-1 DEFINITION

Chemical imaging of pharmaceutical samples is a method that consists of a collection of responses to the illumination at multiple wavelengths of positions spatially distributed across the sample surface. For a given surface location (pixel), a set of responses will be associated with the impinging wavelengths. When x and y positions are varied successively over the range of wavelengths and responses are collected at each location, an image of a wide surface of the sample is constructed.

Imaging systems resolve spatial information primarily at the sample surface. Chemical and morphological sample characteristics or features are collated into an image made up of contributions from multiple domains distributed on the surface of the sample. As a result each mapped sample point (pixel) contains a wealth of information, and the recorded signal reflects chemical and physical properties such as ingredient identity, concentration, crystallinity, orientation, domain size and particle size.

2-2 IMAGING MODES

Chemical images may be acquired in broadband, multispectral or hyperspectral modes. An example of the broadband mode is the camera, where filters are used to weight the relative importance of different wavelengths for the 3 colour variables red, green, and blue (RGB). Multispectral imaging involves sampling spectral bands that may be spread unevenly, not unlike a combination of different detectors in different frequency ranges.

Hyperspectral imaging (HS imaging, HSI) is a two-dimensional (2D) visualisation technique that records a full range spectrum for each pixel. In practice, HSI expands spectroscopic single point analysis of samples into 2D projections of a slice of finite depth for each measured area of the sample. Hyperspectral sensors take advantage of numerous contiguous channels to uncover features that usually cannot be resolved using the average spectra obtained from a single measurement in classical spectroscopy. Images may be produced with a spectral quality similar to that of conventionally obtained spectra. However, this comes at the cost of longer measurement times, larger amounts of data, and complex computation. The spectral quality is sometimes lowered to increase the image throughput. Sample domains may include complex spectral patterns embedded in the individual pixel data that cannot be identified solely by visual inspection, and appropriate image analysis is required to extract analytical information from such a multitude of measurements. Computational and numerical methods are essential for processing, extracting and analysing content in hyperspectral images.

2-3 DATACUBE

In hyperspectral imaging each pixel is associated with one spectrum. Measurements are spread over both dimensions of the sample surface. At each measurement, a column vector of dimensions equal to the number of recorded spectral data points is associated. As a result, data is packaged into a three-dimensional array called a datacube or hypercube. The datacube is a signal intensity data set made up of the pixel x-y coordinates and the corresponding series of responses (e.g. absorbance or transmittance) to the scanned spectrum (see Figure 5.24.-1).

Not unlike classical spectroscopy, which gathers chemical and physical information about the single area under examination, imaging captures spectral and spatial characteristics of surface areas. Spectral and spatial analysis of image pixels can be iterative, or combined in no particular order.

2-4 HIGHER DIMENSIONAL CHEMICAL IMAGING

Spatially resolved three-dimensional imaging can be seen as extending spatial resolution along a third direction (z) that complements x-y imaging. It is the process of stacking a collection of images covering a depth into the sample which is larger compared to the analysis depth. This can be obtained indirectly (invasive, destructive process), by sequentially collecting images of appropriately prepared solid samples, e.g. by slicing the surface stepwise with a microtome. Meanwhile there are techniques which enable direct (non-invasive, non-destructive process) 3D imaging of solids using x-y-z spatially resolved spectroscopy. These include tomography methods such as optical coherence tomography (OCT), far-infrared (FIR)/terahertz (THz) and time-domain THz spectroscopy, confocal Raman, and others, for example, X-ray tomography or nuclear magnetic resonance imaging (MRI). Ultimately 4D and higher dimensional imaging would mean simultaneous spatial (x, y, and z) and spectroscopically resolved mapping. Although higher dimensional imaging is not within the remit of the current general chapter, similar recommendations may initially apply.

2-5 APPLICATIONS

Visual display of the distribution of sample surface features complements classical analytical methods by facilitating rapid and non-destructive comparison between samples. CI can be used to analyse small to large surface areas of a sample. Imaging comes into its own as soon as constituents and morphological characteristics differ from one position to the next. Thus, the technique is particularly suited to exploring samples that are heterogeneous with regard to chemical content and physical morphology. Imaging captures the distribution of selected components and features, i.e. attributes of various parts of a sample. For example, images can display the location of features that directly impact product performance. They can show whether a component is evenly distributed on a relevant scale. Therefore, it is important to choose CI methods depending on the needs with regard to spatial resolution.

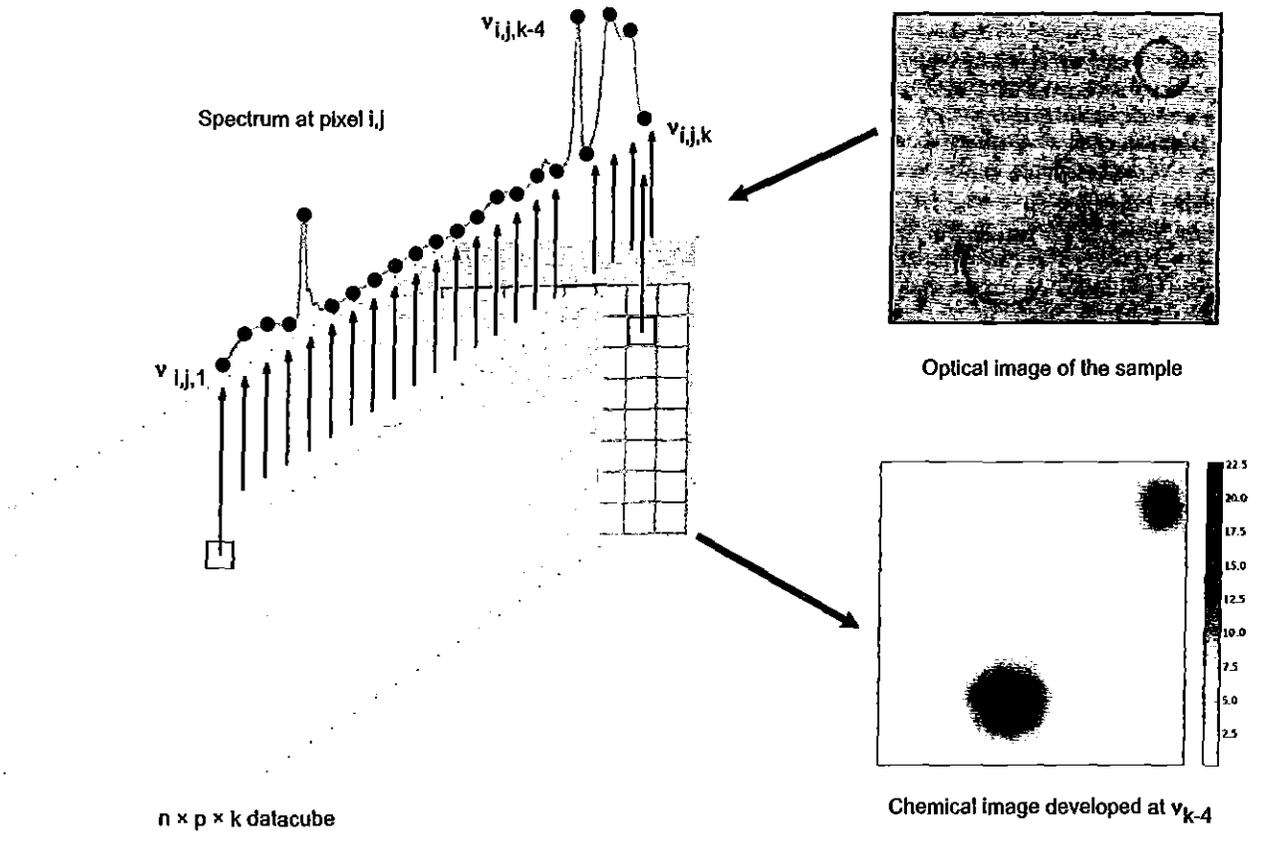


Figure 5.24.-1. – *Example of a n × p × k datacube obtained by recording spectra of k wavenumbers at n × p pixels of the sample; a grey-scale image was developed at wavenumber* v_{k-4}

The main applications of chemical imaging focus on solid-state property analysis, determination of chemical or physical features, contaminant identification, anti-counterfeit, and chemical identification. For example, the measurement of thickness and uniformity of coating on tablets, and the characterisation of surface properties, such as component mapping, determination of adhesion force and deformation depth, can be considered. Moreover, particle characterisation can be performed based on measurements of size, agglomeration and morphology, determination of roughness of the surface, and detection of broken particles and foreign particles. Analysis of particles can also be performed in liquids.

Spatial distribution of different polymorphic forms can be analysed with CI techniques and the investigation of polymorphic transitions and multiphasic materials (e.g. solid dispersions) can also be considered. The characterisation of nano- and micro-crystalline materials can be performed to monitor structural changes under stress conditions and over time, and to evaluate defects in crystals, for example, resulting from milling and micronisation of the material. The chemical characterisation of samples based on chemical images is mainly performed to identify and characterise the distribution and abundance of individual components in a mixture based on characteristic spectral features (see Figure 5.24.-2). This analysis can be performed to determine molecular but also atomic elemental species present on the surface. The kinetics and mechanism of active substance dissolution and release can be modelled, and, as another example, the drug concentration gradient between the solid/solution interface and the bulk solution can be determined.

2-6 CHEMICAL IMAGING SYSTEMS

The selection of a specific imaging instrument or technique depends on the particular analytical application intended. A chemical imaging system is characterised by setup, spatial and spectral resolutions, magnification, type and size of sample, sample preparation and presentation, moving or resting sample, acquisition time, number of measurements, data analysis algorithms, software, etc.

Numerous techniques enable the production of hyperspectral images. A brief description of techniques relying on vibrational spectroscopy is given below, along with their potential uses and limitations.

Mid-infrared (MIR)

MIR spectroscopy is based on the interaction of light with the sample, for the study of intra-molecular vibrations of a material. The electromagnetic spectrum range usually spans the region of 4000-400 cm^{-1} (2.5-25 µm). MIR imaging may be used for the characterisation of chemical species in a mixture of ingredients. Measurements are often carried out using attenuated total reflectance (ATR) microscopy where the sample is in contact with an IR-transparent crystal of higher refractive index than the sample. The measurement may be difficult for samples with excessive moisture due to interference with water bands.

Near-infrared (NIR)

NIR spectroscopy detects molecular vibrations originating from C-H, N-H, O-H and S-H overtones, and combinations of fundamental mid-infrared vibrations. The electromagnetic spectral range usually extends from 12500-4000 cm^{-1} (0.8-2.5 µm). The measurement is contactless, usually in diffuse reflectance mode, and delivers physical and chemical information.

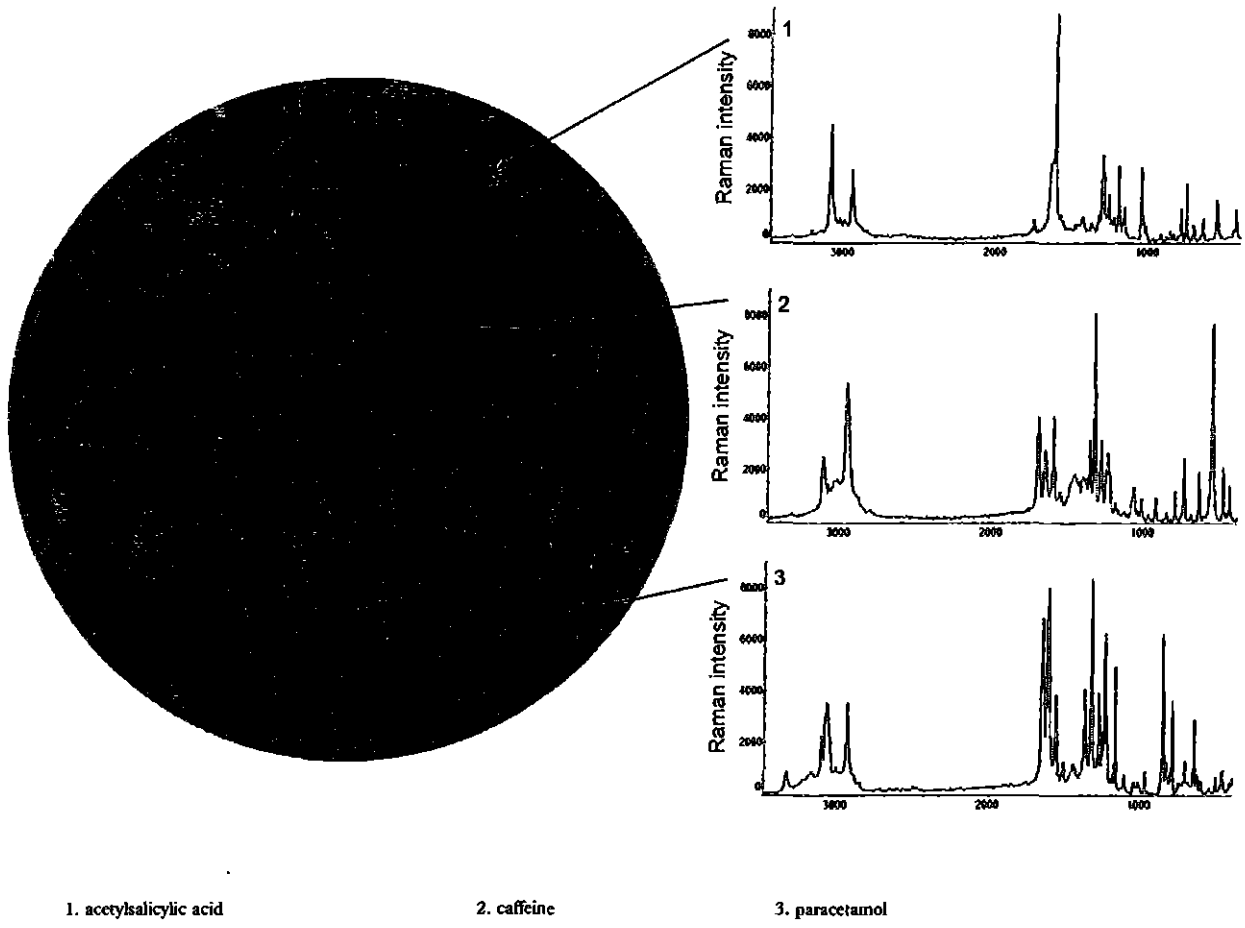


Figure 5.24.-2. – *Example of characterisation of ingredients with Raman imaging*

Far-infrared (FIR) and terahertz (THz)

In the FIR range, electromagnetic radiation typically spans from 400 to approximately 10 cm^{-1} (25-1000 µm). This allows spectra to cover inter-molecular and lattice vibration modes. The selectivity of the technique to the hydrogen bonding networks makes possible the identification and characterisation of solid state forms, e.g. polymorphic forms and degree of crystallinity.

The radiation can pass through a wide variety of non-conducting materials, but cannot pass through conducting materials such as metal and water, and penetrates deep into the sample.

Raman

Raman spectroscopy detects frequency shifts originating from the inelastic part of the light scattered by a sample previously irradiated with an intense monochromatic light source (usually a laser). Raman spectra contain numerous narrow bands which makes possible the identification of chemical substances. As a result, Raman imaging gives information on chemical species in a sample (including polymorphs).

Water, air and glass are weak Raman scatterers and therefore the analysis of aqueous samples may be performed in atmospheric conditions or in sealed vials. However, the measured signal may be disturbed by fluorescence.

2-7 ACQUISITION MODES

Either spatial sequencing or wavelength sequencing techniques are required to produce an image. The 3 modes used to record datacubes are point mapping, line mapping and global imaging. Point mapping (point scanning) is the simplest and typically follows a rectangular grid pattern. A spectrum is recorded at each point on the grid with the sample stage being moved to the next neighbouring location. Scanning is computer-controlled along both spatial axes. In line mapping (linear scanning), the sample is illuminated along a line and the image signal is dispersed along one spatial axis onto the detector. The sample is moved along the other spatial axis to capture the next neighbouring line until full mapping is eventually achieved. Here the speed and flexibility of the experimental setup enable on-line application of continuous CI. Global imaging (focal plane scanning) is performed when all image points of a sample are imaged simultaneously onto the detector array. This can be done on a per wavelength basis with filters or tuneable filters.

2-8 RESOLUTION

Pixels contain a wealth of contributions from chemical and physical features occurring at the surface and near sub-surface of the sample. The performance of an imaging system is directly related to the spatial and spectral resolutions that can be achieved. Linear line mapping systems are more sensitive to spatial interference, whereas spectral resolution is more critical with focal plane acquisition.

Depending on the imaging technique, spectral resolution may be affected for example by laser wavelength, grating, detector and spectrometer focal length. Spectral resolution has an impact on chemical feature extraction because it influences the performance of qualitative and quantitative image analysis, e.g. identity of ingredients.

Spatial resolution is the smallest distance between two consecutive points which can be distinguished. It has an impact on image processing as information may appear as unresolved. For example, the domain size of components of interest should be consistent with the spatial resolution achieved by the system. The spatial resolution obtainable is limited by the instrument characteristics, for example spot size of the laser, diffraction limits, detector size, magnification, numerical aperture, etc. The spatial resolution obtained by a specific CI system is also affected by the diffusion of radiation across the sample surface. Scattering occurring below the surface is likely to distort the image obtained and limit the spatial resolution.

2-9 REPRESENTATIVENESS OF SAMPLE SURFACE

Depending on the instrument setup, the topology of the sample surface may impact imaging performance.

The sample surface investigated has to be representative for the intended purpose of analysis. Depending on the latter, the impact of sample homogeneity has to be evaluated. For example, with a design based on reflectance, due to the limitation of penetration depth and spatial resolution, a true measure of morphology or distribution of particle shapes in the sample is only estimated. Thus, results obtained from surface image analysis may not be representative of the whole sample because it may not be homogeneous.

One approach to overcome this limitation would be to measure a number of cross-sections of the sample to improve estimations for the whole sample. Another approach would be to use an alternative optical design where penetration depth can be increased, e.g. confocal or spatially resolved offset measurements to capture information near the surface, tomography or transmission measurements to collect whole matrix information.

3 ELEMENTS OF A CHEMICAL IMAGING PROCESS

Proceeding with imaging encompasses numerous steps such as:

— sample preparation;
— control of instrument components and system performance;
— calibration of instrument and system suitability test;
— measurement, image processing, display and storage;
— image analysis and computation of numerical results.

3-1 SAMPLE

3-1-1 Sample preparation

Sample preparation has to be in accordance with the imaging technique used. *In situ* measurements with probes can be performed without sample preparation. On the other hand, setups for Raman scattering for example proceed by non-contact focusing, meaning that the sample surface may need to be prepared to obtain a reasonably flat surface. If the instrumentation is unable to compensate for topographical variation, sample surfaces may be mechanically modified, for example by flattening a concave tablet surface. Focusing adjustments by automatic refocusing during mapping compensates for slightly uneven surfaces. Sample surface preparation has also to be considered with ATR-IR imaging for which contact between optics and sample is required.

3-1-2 Sample presentation

Appropriate sample presentation is dictated by instrument setup. A particular setup will be more or less adapted to a specific sample type and analytical task. The sample is positioned so that it is optimally imaged. The setup is optimised to reduce specular reflection as much as possible. Angles and distances between probe (or beam), sample and detector match the setup requirements.

3-2 CONTROL OF INSTRUMENT PERFORMANCE

Evaluating both instrument performance and image analysis methods is essential to avoid misinterpretation or artefacts. Parameters include spectral as well as spatial components. The instrument is to be used according to the manufacturer's instructions.

Instrument performance verification consists of periodic performance qualification as well as system suitability tests. The intervals depend on the use of the instrument and its application. The system suitability tests are carried out before measurements to check if the CI system is operating properly for the intended application.

The parameters to be assessed and the acceptance criteria applied during performance qualification and system suitability tests have to be justified and depend on the CI technique and the purpose of the analysis. Parameters which might be verified are described below.

3-2-1 CIS component adjustment

In a schematic setup, a typical CIS would be made up of components including source of radiation, optical devices, sample holder, detector, and software.

The system and its individual components comply with all expected requirements.

3-2-1-1 Sources, optics and detectors

Source intensity is monitored as part of the setup verification. In particular, the source intensity should be verified before starting calibration routines or a new series of sample measurements. Optical path, confocality, wavelength accuracy and energy throughput at any *x-y* position (pixel) match the specifications.

Alignment of optics, sample and detectors comply with measurement requirements in terms of distances, angles and polarisation. This alignment may shift with the temperature. In particular, illumination of the sample or regions of interest has to be as homogeneous as possible and reproducible.

Parasitic or adverse effects, for example scattering, background, noise, bad pixels, cosmic rays and fluorescent lights in the laboratory, and side effects such as sample fluorescence have to be controlled. Stray light, ghost lines, and ghost images can be caused by reflections from imperfect surface elements. They constitute parasitic light that has to be dealt with.

3-2-1-2 Multi-wavelength and multispectral systems

Multi-wavelength systems should be tested at wavelengths spread over the wavelength scale using the peaks of the selected reference standard with good signal-to-noise properties.

Multispectral systems are verified for all signal sources involved.

3-2-1-3 Mapping

When images from more than one instrument are going to be aligned, it is important to have verified *x-y* scales.

3-2-1-4 Magnification

In the case where the CIS permits different magnifications, the optical or electronic magnification level needs to be optimised. If magnification is inadequate for resolving relevant features, the value of imaging may be diminished; whereas if magnification is set higher than required, the field of view is reduced.

3-2-2 Calibration

The purpose of calibration is to transform the recorded signal into data suitable for analysis, interpretation, or comparison with reference data.

Basic instrument calibration of imaging systems consists of *x*- and *y*-axis calibration in both wavelengths and intensity.

3-2-2-1 Spectral axis

Optimal wavenumber accuracy is critical and should be achieved at the same level as for a standard spectrometer. Spectral axis calibration assigns wavelength and intensity values to every mapped pixel of the sample surface. To determine these relationships, well-characterised light sources or reference materials can be used. Calibration should be carried out using referenced and certified/normalised internal or external standards provided either by the supplier or a third party. Technology and environmental conditions are other critical points.

3-2-2-2 Spatial axes

Spatial axis calibration has an impact on the actual field of view of the CIS and the sampled surface area, i.e. the pixel location. Calibration corrects deformations on the spatial axis such as those caused by the optics. For example, the pixel position may be accompanied by variation of the spectral resolution from the centre towards the detector boundaries. In addition, mixing of signals from adjacent pixels can occur. Both errors change the appearance of the pixel spectra and may reduce the accuracy of subsequent data analysis. If it is not possible to deduce the position of the imaged pixels on the detector surface from the pattern on the target surface, a special test target can be used or developed for the application.

Spatial resolution in both *x* and *y* directions is also limited, for example, by image size and step size of the moving table or the conveyor belt.

3-2-2-3 Method

Appropriate data processing and modelling are needed to deliver information about the property that correlates with the observed features in images. Calibration related to the analytical objective requires, for example, identifying and estimating a characteristic identity or morphological features of the sample.

3-3 IMAGE PROCESSING

Processing means that the image is treated for display and subsequent feature estimations. Imaging requires steps to improve the image for analysis, e.g. to control brightness and contrast, reduce noise, sharpen edges and borders, remove unwanted features, etc. This improvement allows features to be recognised i.e. separated and discriminated from the background.

Chemical images are generally processed by chemometric modelling for qualitative or quantitative purposes (see general chapter *5.21. Chemometric methods applied to analytical data*).

3-3-1 Spectral selection

Image contrast is built upon specific signatures of the components at the sample surface and derived from spectroscopic data such as peak intensity, peak position, baseline variations (scattering differences), or values calculated with multivariate analysis.

3-3-2 Image pre-processing

Data analysis methods for imaging data sets usually begin with the same steps as for single point spectroscopy. Pre-processing is utilised to separate chemical and physical effects, unless an analysis is based on the physical effects (e.g. scattering differences seen as baseline variations).

The collected data is typically affected by noise or instrumental distortions and pre-processing is often applied to improve image quality. There may also be a need to remove irrelevant sources of variation such as cosmic ray spikes or stripes due to detector configuration. Common pre-processing operations for spectra are: baseline correction, derivatives, smoothing, wavelet-based filtering, standard normal variate (SNV), multiplicative scatter correction (MSC), normalisation, truncation and Fourier-transform. It has to be verified that pre-processing does not cause artefacts.

3-4 IMAGE ANALYSIS

Pixel arrangements in an image of the sample represent a map of the distribution of chemical or physical properties or features. Depending on the objective of the analysis, data can be explored for spectral features, spatial features, and a combination of both. Examination of the datacube can be performed in a direct way, as for conventional spectroscopy (e.g. by analysing height of peaks or area ratios), or by techniques including image processing, multivariate data analysis, etc.

3-4-1 Visual exploration

A review by the user of an image displayed on screen may deliver a first impression before beginning enhancement, exploration and evaluation. Images can be analysed for features at the surface of the sample, such as location, distribution, size and shape of domains, constituent identification, etc. Standard image processing tools, such as morphological filtering and particle statistics, may be applied directly for samples that are significantly heterogeneous.

For a chemical image, both spectral and spatial analyses can be equally important and there are different steps of chemical image analysis. Spectral and spatial analyses are often iterative by nature. In practice, the methods used depend upon whether it is the spectral information or the spatial information that is of more interest. The goal is to reduce data dimensionality and extract as many features as possible. Unsupervised multivariate analysis such as principal component analysis (PCA) may be applied prior to feature extraction to describe the information contained in the image. A preliminary segmentation of PCA images may be used to facilitate identification of regions of interest in the image for further processing. If the ingredients of a mixture are known and available for reference measurements, the corresponding data is used to reduce the number of spectral layers in the hypercube down to a few chemically or physically meaningful layers by using supervised regression methods, e.g. multivariate curve resolution-alternating least squares (MCR-ALS), independent component analysis (ICA), and partial least squares regression (PLS).

3-4-2 Feature extraction

The extraction of chemical or morphological features of samples involves supervised techniques to perform dimensional reduction. It concentrates further image processing on characteristics that are meaningful for the analysis. For example, a selected feature could be the quantity of a given substance or a solid-state form of interest. If the sample is comprised of components whose spectral features are well separated, it is possible to proceed directly with a univariate method based on a single wavelength per feature, thus utilising only a small fraction of available data. However, in many cases spectral overlap is significant and the univariate approach is insufficient. The extraction of features can then be improved by including a greater proportion of the spectral information and applying a

multivariate approach, such as clustering, classification, PLS discriminant analysis (PLS-DA), linear discriminant analysis (LDA), etc.

3-4-3 Quantification of ingredients

There are several ways to measure ingredient variation using regression methods. A quantitative model can be computed by using average spectra or binning of regions of interest at the surface of the sample. The known bulk concentration may be used when the ingredient present varies in responses for the analytical method used. This can be performed by peak integration at a single wavelength or alternative chemometric methods.

When individual pixel spectra become increasingly mixed, chemometric tools, typically multivariate data analysis, can be deployed with similar data analytical strategies as those for single-point spectroscopy.

For example, it is possible to estimate relative component abundance by using pure component spectra as the starting point. The reference spectra used to create the library are collected from separate pure component samples. These spectra can be measured with a non-imaging spectrometer, or as separate images of pure components. The inherent variation in pure component spectra when acquired across a large number of pixels provides additional statistical robustness. The reference spectra and the unknown sample can also be measured simultaneously in the same field of view to create one single data set. If pure component reference spectra can be derived directly from an isolated area of the imaged sample, there is no need to acquire additional reference spectra for calibration. A multivariate model can be developed either based on external reference spectra or based on spectra from isolated areas of the respective image.

Both with simultaneously and separately acquired pure component spectra, it is important to verify that they are representative for the present analysis by for example regression residuals.

3-4-4 Spatial analysis of image features

Spatial analysis of the images can be performed with regard to distributions of shapes, sizes and locations of domains, etc. Analysis of shape and distribution relies on a variety of numerical methods and algorithms.

Images display the samples by setting a threshold value and converting data into a set of colours or shades according to a monochrome or pseudo-colour intensity scale to underline the presence or proportion of the analytes of interest at each pixel. Alternatively, the distribution of several analytes can be combined into colour images. Further analysis can be performed, such as counting pixels, estimating the size distributions of particles of the same chemical origin, and segmentation which is a process to divide the image between regions that correspond to surface areas of interest.

3-4-5 Measuring dimensions

Images can be used for example to determine area, dimensions, perimeter, aspect ratio and roundness.

3-4-6 Statistics

Pixel statistics can be performed to facilitate further image analysis. Application of methods such as variance analysis and box-plot for domain size, shape, distribution, and distances between domains, enables estimation of the variability within an image. These methods deliver a condensed description of relations between domains in the images with simplification of the image analysis. Property estimations can also be derived from these values.

M. Direct Amperometric and Pulsed Electrochemical Detection

(Ph. Eur. method 2.2.63)

Direct amperometric detection and pulsed electrochemical detection (PED) are used to detect electroactive compounds by oxidation (or reduction) when coupled to a separation technique such as liquid chromatography (*2.2.29*). Electrochemical detection uses the redox potential of analytes and is therefore only valid for electroactive substances.

PRINCIPLE

When a constant potential is applied between 2 electrodes, the oxidation (or reduction) of an electroactive substance generates a current proportional to the amount of analyte passing the electrode surface. This analytical technique requires a supporting electrolyte, which can be added post-column if not already present in the mobile phase. Detection usually involves oxidation of the compounds of interest.

The potential applied to the measuring electrode can serve as a selectivity parameter for the compounds of interest.

The upper limit of the electroactivity range is defined by the oxidation curve of the mobile phase, the supporting electrolyte or the measuring electrode.

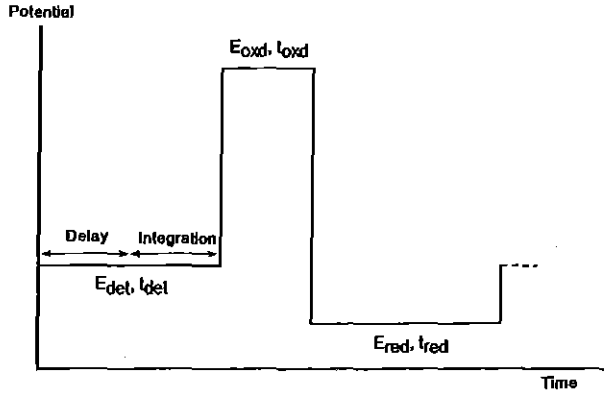
With direct amperometric detection, only a constant potential is applied. However, this method may result in the surface of the measuring electrode becoming fouled with adsorbed carbon compounds, resulting in signal variability and a decreased response. This passivation phenomenon is particularly common with sugars, thiols and phenols.

The problem of electrode fouling can be resolved by the application of a series of pulses, a process known by the generic term of pulsed electrochemical detection (PED).

The most widely used PED mode is pulsed amperometric detection (PAD), which involves a 3-step series of pulses or waveform (Figure 2.2.63.-1). After applying the detection potential (E_{det}), a high positive potential (E_{oxd}) (anodic polarisation) is applied to clean the surface of the measuring electrode, followed by a negative potential (E_{red}) (cathodic polarisation) to restore the surface of the measuring electrode. A new cycle of the waveform can then start.

Following the potential step from E_{red} to E_{det}, background signals will originate. Although these decay quickly, a brief delay must be introduced before the analyte signal can be properly measured.

Other waveforms comprising more than 3 voltage steps have been developed.



E_{det}: detection potential applied during a period t_{det}
E_{oxd}: oxidation potential applied during a period t_{oxd}
E_{red}: reduction potential applied during a period t_{red}

Figure 2.2.63.-1. – *3-Step potential-time waveform (pulse sequence)*

EQUIPMENT

An electrochemical detection system comprises 3 electrodes: a measuring electrode (gold, platinum or glassy carbon); a reference electrode (usually silver-silver chloride); and an auxiliary electrode (e.g. titanium cell body) (Figure 2.2.63.-2).

The choice of measuring electrode depends on the substance to be examined. The electrodes are coupled to a potentiostat, which controls the potentials applied to the measuring electrode without subjecting the reference electrode to a current, the auxiliary electrode functioning as a cathode whenever the measuring electrode is operating as an anode and vice versa.

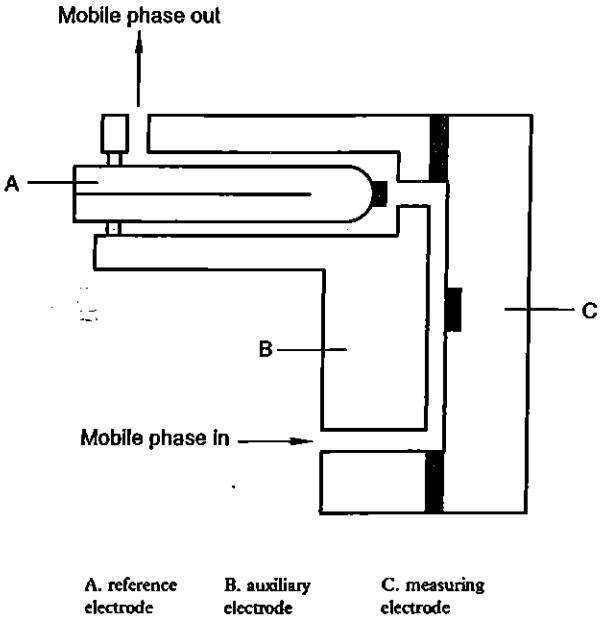


Figure 2.2.63.-2. – *Electrochemical detection system*

EQUIPMENT PERFORMANCE

The potentials to be applied depend on the analyte and the type of electrodes used, and the settings must therefore be adapted accordingly.

All electrodes should be in good condition and periodic replacement is recommended. When the detector is used for quantitative analysis, it is advisable to check the linear range, sensitivity and repeatability of the detector.

The quality of the reagents used is of utmost importance. It is advisable to employ reagents of the highest purity.

ADDITIONAL INFORMATION

Maintenance of the electrochemical cell

Although the application of an increased potential in PED cleans the surface of the measuring electrode effectively, it can be necessary to polish the measuring electrode mechanically from time to time when the background current increases and the sensitivity decreases. The cleaning of the measuring electrode must be carried out very carefully to avoid creating pits or scratches. It is also advisable to wipe the auxiliary electrode at the same time to remove deposited substances. The cell requires some time to stabilise after polishing.

Sodium hydroxide solution

Detection can be enhanced in alkaline media (at least pH 12), as is the case with aminoglycoside antibiotics. When the mobile phase is not sufficiently alkaline, this can be achieved by post-column addition of a sodium hydroxide solution to increase the pH of the column effluent. The solutions are mixed in a coil that is linked to the electrochemical cell. It is essential that the length of the mixing coil is such as to produce a homogeneous solution but with minimal peak broadening. It is important to avoid carbonates in the sodium hydroxide solution. To avoid baseline disturbances, the sodium hydroxide solution should be degassed before use, it must be added pulse-free and its flow should be constant between runs. The parts of the chromatographic system coming into contact with the sodium hydroxide-containing solutions must be alkaline resistant.

Process Analytical Technology

(Ph. Eur. method 5.25)

This general chapter focuses on the interfacing of analytical techniques with the manufacturing process as a means of enhancing process control and improving process understanding. It should be noted that it is not within the scope of the chapter to give specific instructions for all possible process analytical technology sensors; instead, it offers a general approach to the integration of analytical techniques in the process environment together with aspects to be taken into consideration for the application of process analytical technology.

1 INTRODUCTION

Process analytical technology (PAT) can be defined as a system for designing, analysing and controlling manufacturing processes through timely measurements (i.e. during processing) of critical quality attributes (CQA) of raw and in-process materials and critical performance characteristics of processes in order to ensure the quality of the final product. It is important to note that the term 'analytical' in PAT is used in a broad sense to include chemical, physical and microbiological measurements conducted in an integrated manner and combined with data analysis. The goal of PAT is control of the manufacturing process and enhanced process understanding, guided by risk management. Interfacing manufacturing processes with analytical techniques is therefore essential in PAT, as it facilitates process development in accordance with quality by design (QbD) principles, enables real-time release testing (RTRT) and supports continuous manufacturing processes.

Time delays between obtaining a sample for testing, analysis of that sample and any consequent outcomes must be taken into consideration when applying PAT. When the analytical results are used on a continuous basis to monitor and control a process, it is important to minimise such delays. This can be achieved most effectively with sensor-based continuous measurement systems directly interfacing with the process stream during a specific unit operation. The sensors interfaced with the process continuously measure the process conditions and material characteristics within the process environment (*in situ*) and send the measured data (e.g. a spectrum) to an operating system where it is recorded and analysed, and where any necessary adjustments to processing conditions can be determined on a continuous basis. These *in situ* measurements can generate very large volumes of data representative of the process.

2 INTERFACING MODES

The interfacing of analytical techniques with the manufacturing process is central to the application of PAT. Results generated by analytical equipment interfaced with a process can be used for monitoring purposes and to ensure that the process is stable, for example via automated feedback or feedforward control loops.

The terms 'off-line', 'at-line', 'on-line' and 'in-line' describe interfacing modes (see Figure 5.25.-1). Measurements using on-line and in-line systems generally support rapid and automated process adjustments since they move the analytical techniques into the process stream. In contrast, off-line and at-line measurements involve transferring the samples away from the process stream to the analytical equipment.

Off-line measurements

These correspond to conventional analytical testing in which the sample is removed from the manufacturing process environment and tested in a laboratory, typically located away from the production environment. This transfer of samples away from the process stream can result in a significant time lag and generally does not permit immediate process adjustments. However, off-line measurements can nonetheless be useful for PAT purposes if relevant analytical data can be obtained within a time frame that is compatible with process dynamics.

At-line measurements

With at-line measurements, the sample is also removed (manually or automatically) from the process stream for testing, but the testing equipment is usually located within the production environment, i.e. in close physical proximity to the process stream, and testing can therefore take place with minimal delay. This results in a shorter time frame for obtaining analytical data, and also differentiates at-line measurements from off-line measurements. It is therefore possible to make process adjustments based on the results of at-line measurements.

On-line measurements

On-line measurements typically involve sensor-based measurements made under real-time conditions by diverting a portion of the material from the process stream directly into a measuring device and the results are available after a minimal time delay.

Depending on the nature of the test, e.g. whether or not it is detrimental to the product, the diverted portion may be returned to the process stream, otherwise it is discarded.

In-line measurements

In-line measurements are taken within the process stream by placing measuring devices, typically sensors, directly in contact with or into the process stream. No portions of the material are therefore removed from the process stream.

In-line measurements must also be non-detrimental to the product.

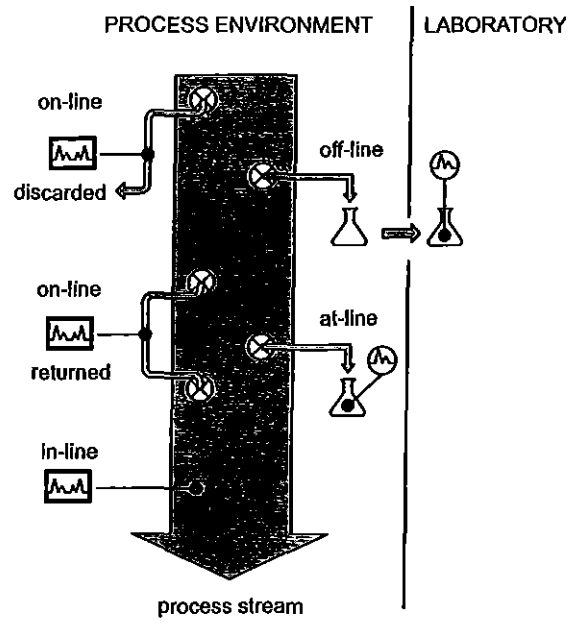


Figure 5.25.-1. – *Interfacing modes*

3 COMPARISON OF INTERFACING MODES

Both off-line and at-line measurements are based on analysis of discrete samples removed from a process stream or bulk material. These samples are considered representative of the material in the process stream at the time they are removed. Frequent at-line measurements also provide data supporting the application of PAT, as they are carried out within a short time frame and in the immediate vicinity of the process stream.

Neither in-line nor on-line measurements involve sampling in the conventional sense. The tested portion might not be separated from the process stream, and is usually smaller than a conventional sample.

Scale of scrutiny must be considered and will determine the frequency and duration of measurements. Furthermore, measurements can be influenced by physical attributes interfering with the acquisition properties of the measuring system. When measuring solids or suspensions, for example using spectroscopic methods, consideration must be given to the surface and bulk scattering properties of samples, and movement of the material. Influencing factors such as particle size, surface roughness and solid density can cause significant spectral differences, which must be taken into account when a method is designed and used in order to ensure that the process and material are described adequately.

In-line and on-line modes offer the advantage of fast data acquisition, allowing high measurement frequency, and thus enabling rapid continuous monitoring as well as immediate action and control of the process.

In some cases, PAT measurements can be used qualitatively to build models such as process trajectories or process signatures which can be used to characterise process variability and highlight unusual process behaviours. These models are not necessarily directly linked to a critical in-process quality attribute or performance characteristic. It is therefore recommended to demonstrate the causal relationship between the PAT measurements, the model and the relevant quality attributes or performance characteristics when using such models for process control purposes.

General validation principles also apply to in-line or on-line measurements although some validation procedures may be different to those for conventional quality control methods.

Analytical validation procedures for at-line and off-line measurements assume homogenous and authentic samples, which are crucial for the assessment of precision. However, this requirement is rarely fulfilled for on-line or in-line methods which are often used to measure processes in which the material rapidly changes and/or moves during the measurement, for example a fast-drying process or reaction monitoring. Comparison of results from on-line/in-line measurements with results from a reference test procedure would normally be needed.

4 STATISTICAL PROCESS CONTROL

Statistical process control (SPC) comprises a set of data analysis methods applied in order to monitor and control a process based on analysis of process variability (e.g. of CQAs). For PAT purposes, the process can be monitored using, for example, real-time data collected by process analysers.

Based on this data, SPC can be used to make process adjustments, when necessary, to maintain or attain a desired state and to ensure that the process remains under control. This includes, for example, trend analysis of quality attributes or performance characteristics. SPC can also be used to measure process variability and process capability (i.e. the ability of a process to produce a product that complies with requirements), with a view to enhancing process understanding, thereby improving lifecycle management.

Rather than using univariate SPC methods to monitor several individual variables, it is often preferable to use multivariate statistical process control (MSPC) to analyse several variables simultaneously, taking into account potential correlations (see general chapter *5.28. Multivariate statistical process control)*.

5 EUROPEAN PHARMACOPOEIA TEXTS SUPPORTING THE APPLICATION OF PAT

Typically, the analytical techniques described in the Ph. Eur. include qualification criteria designed for off-line analytical systems. These criteria are not always relevant or practical in a PAT setting, for instance when the equipment is specifically designed for on-line or in-line measurements. For this reason, certain general chapters (including *2.2.40. Near-infrared spectroscopy*, *2.2.48. Raman spectroscopy* and *2.9.47. Demonstration of uniformity of dosage units using large sample sizes*) have been revised or specifically elaborated to support and promote the use of the techniques in conjunction with PAT.

General Notices

Section 1.1, under Demonstration of compliance with the Pharmacopoeia, states that a substance can be demonstrated to be of pharmacopoeial quality on the basis of product design, together with the control strategy applied and data derived, for example, from validation studies of the manufacturing process.

The section also states that an enhanced approach to quality control could utilise PAT and/or real-time release testing strategies (including parametric release) as alternatives to end-product testing alone.

Absorption spectrophotometry, infrared

General chapter *2.2.24* has a wide variety of applications in the manufacturing process that enable PAT, such as reaction monitoring in chemical synthesis. Wavenumber shifts and acceptable tolerances for the reference material polystyrene are described for both transmission and ATR measurement modes.

Absorption spectrophotometry, ultraviolet and visible

General chapter *2.2.25* covers the use of PAT in the UV-Vis range using modern detectors such as photodiode arrays (PDA) or charge-coupled devices (CCD). Both transmission and diffuse reflection measurement modes are possible with off-line, at-line, on-line and in-line measurements. The table with reference wavelengths for the control of wavelength accuracy includes wavelengths in the UV range down to 180 nm.

X-ray fluorescence spectrometry

General chapter *2.2.37* includes references to modern equipment and the current applications of the XRF technique.

Substantial advances in miniaturisation and automation have led to the development of hand-held energy dispersive XRF (ED-XRF) spectrometers for rapid field measurements. XRF is potentially non-destructive, although it may cause sample instability. Hence, XRF lends itself to applications of PAT such as the analysis of unwanted trace catalysts in active pharmaceutical ingredients (API).

Near-infrared spectroscopy

General chapter *2.2.40* takes into account the increasing use of NIR spectroscopy for process monitoring and control, and the development of modern NIR spectrometers.
NIR measurements can be performed off-line, but the technique also lends itself to at-line, on-line and in-line testing.

The 'Sample preparation/presentation' section includes moving materials or samples, and the use of fibre-optic probe systems in different measurement modes (i.e. transmission, diffuse reflection and transflection). The use of internal referencing for process analysis purposes is permitted when it is impossible to remove a probe for reference background data collection. A detailed table for the control of instrument performance depending on measurement mode and instrument used (benchtop, mobile or process instrument) is also provided. The chapter includes a section on qualitative analysis for identification and characterisation of a substance in addition to sections on limit analysis (e.g. for dryer end-point control) and trend analysis (for monitoring blend uniformity).

Raman spectroscopy

General chapter *2.2.48* includes Raman technologies that have potential uses for the application of PAT, including hand-held instruments.

Wavenumber shifts and acceptable tolerances for the reference materials cyclohexane, paracetamol and polystyrene (values derived from an inter-laboratory study) are described for both benchtop and hand-held Raman systems.

Demonstration of uniformity of dosage units using large sample sizes

General chapter *2.9.47* allows the determination of dosage unit uniformity in a PAT environment where the sample size is markedly greater than 30 units.

Compliance with the acceptance criteria of general chapter *2.9.47* is considered as evidence that the batch would also comply with general chapter *2.9.40. Uniformity of dosage units* if tested using a small sample size (e.g. 30-unit). Therefore, general chapter *2.9.47* does not constitute stand-alone acceptance criteria.

Alternative methods for control of microbiological quality
General chapter *5.1.6* describes alternative methods that might be used for the application of PAT in order to contribute to real-time or near-real-time microbiological quality control (e.g. test for microbiological examination of non-sterile products or for sterility based on laser-induced fluorescence) of in-process samples, active substances, medicinal products or excipients (particularly water). The chapter also provides guidance on how to validate these alternative methods.

Chemometric methods applied to analytical data
Chemometrics (general chapter *5.21*) has proven to be well suited for PAT. The investigation of large data tables and treatment of intricate signals, i.e. data collections with a hidden structure, of the type accumulated during measurements in PAT setups, requires alternative analytical tools to those used in a one-variable-at-a-time approach.

Chemical imaging
Chemical imaging (CI) (general chapter *5.24*) can be used to support PAT applications. CI measures spatial distribution and contributes to the understanding of the properties of materials such as finished products, pharmaceutical intermediates, excipients, APIs and starting materials.

The uses of CI include, for example, detection of defects at the sample surface such as cracks in tablet coatings and identification of foreign particles. It is a versatile tool used in process development and improvement, root cause analysis (e.g. for out-of-specification results), and may also be used to enhance process understanding.

Multivariate statistical process control
General chapter *5.28* gives insight into how MSPC can be used to control and improve manufacturing processes, based on the collection and processing of large amounts of data on multiple variables. It describes the principle of MSPC and the development and use of multivariate control charts, and provides an overview of the theoretical background of multivariate statistical procedures.

Appendix III

Chromatographic Separation Techniques

(Ph. Eur. method 2.2.46)

Chromatographic separation techniques are multi-stage separation methods in which the components of a sample are distributed between 2 phases, one of which is stationary, while the other is mobile. The stationary phase may be a solid or a liquid supported on a solid or a gel. The stationary phase may be packed in a column, spread as a layer, or distributed as a film, etc. The mobile phase may be gaseous or liquid or supercritical fluid. The separation may be based on adsorption, mass distribution (partition), ion-exchange, etc., or may be based on differences in the physico-chemical properties of the molecules such as size, mass, volume, etc.

This chapter contains definitions and calculations of common parameters and generally applicable requirements for system suitability. Principles of separation, apparatus and methods are given in the following general methods:
— paper chromatography (*2.2.26*);
— thin-layer chromatography (*2.2.27*);
— gas chromatography (*2.2.28*);
— liquid chromatography (*2.2.29*);
— size-exclusion chromatography (*2.2.30*);
— supercritical fluid chromatography (*2.2.45*).

DEFINITIONS

The system suitability and acceptance criteria in monographs have been set using parameters as defined below. With some equipment, certain parameters, such as the signal-to-noise ratio and resolution, can be calculated using software provided by the manufacturer. It is the responsibility of the user to ensure that the calculation methods used in the software are equivalent to the requirements of the European Pharmacopoeia and to make any necessary corrections if this is not the case.

Chromatogram
A graphical or other representation of detector response, effluent concentration or other quantity used as a measure of effluent concentration, versus time or volume. Idealised

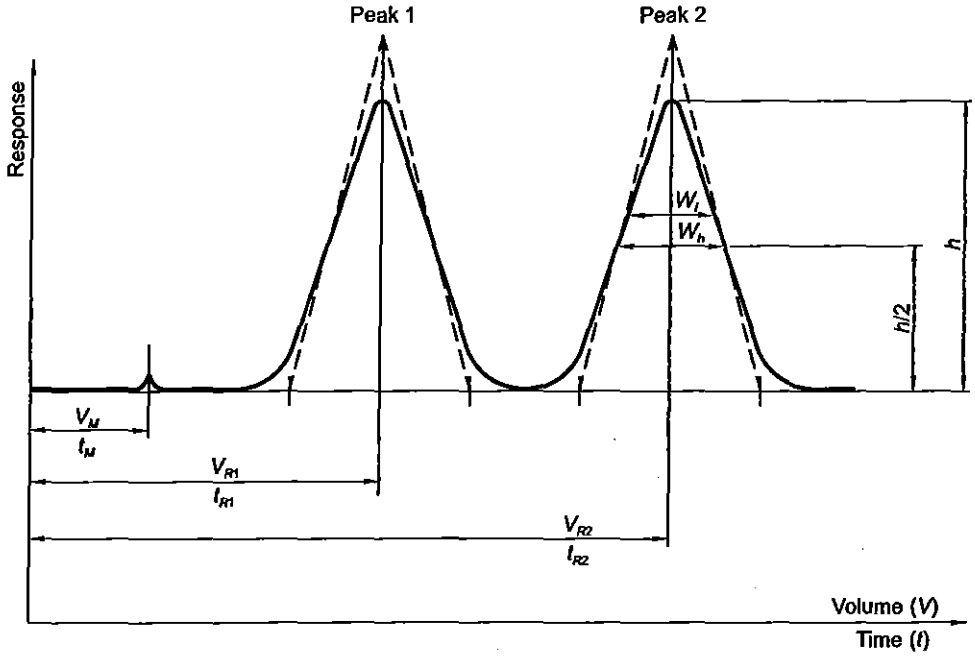


Figure 2.2.46.-1.

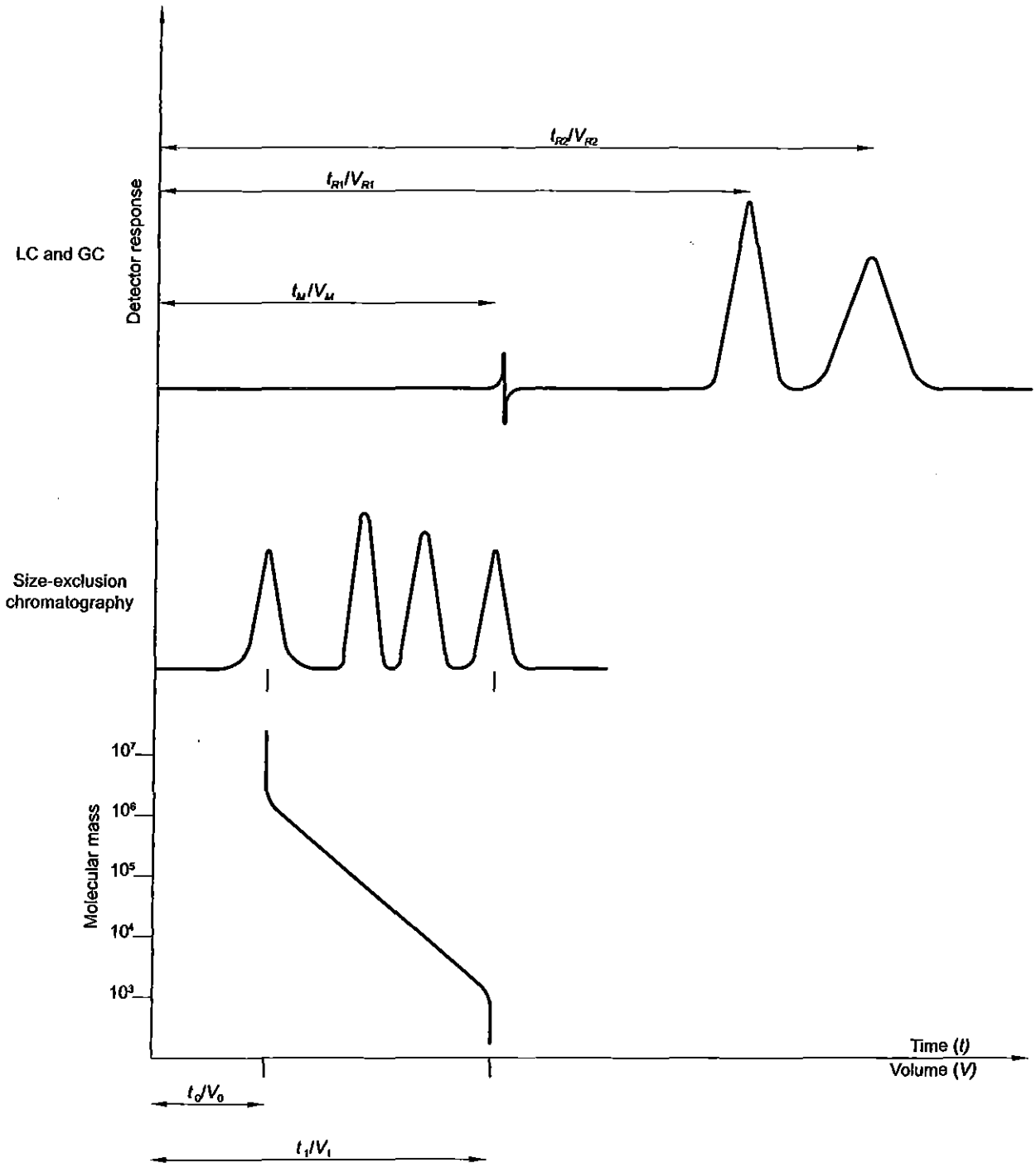


Figure 2.2.46.-2.

chromatograms are represented as a sequence of Gaussian peaks on a baseline (Figure 2.2.46.-1).

Peak

The portion of a chromatogram recording the detector response when a single component (or 2 or more unresolved components) is eluted from the column.

The peak may be defined by the peak area, or the peak height (h) and the peak width at half-height (w_h), or the peak height (h) and the peak width between the points of inflection (w_i). In Gaussian peaks (Figure 2.2.46.-1) there is the following relationship:

$$w_h = 1.18 w_i$$

Retention time (t_R)

Time required for elution of a component (Figure 2.2.46.-1, baseline scale being in minutes).

Retention volume (V_R)

Volume of the mobile phase required for elution of a component. It may be calculated from the retention time and the flow rate (F) in millilitres per minute using the following equation:

$$V_R = t_R \times F$$

Hold-up time (t_M)
Time required for elution of an unretained component (Figure 2.2.46.-1, baseline scale being in minutes). In size-exclusion chromatography, the symbol t_0 (see below) is used.

Hold-up volume (V_M)
Volume of the mobile phase required for elution of an unretained component. It may be calculated from the hold-up time and the flow rate (F) in millilitres per minute using the following equation:

$$V_M = t_M \times F$$

In size-exclusion chromatography, the symbol V_0 (see below) is used.

Retention factor (k)
The retention factor (also known as mass distribution ratio (D_m) or capacity factor (k')) is defined as:

$$k = \frac{\text{amount of component in stationary phase}}{\text{amount of component in mobile phase}} = K_C \frac{V_S}{V_M}$$

K_C = distribution constant (also known as equilibrium distribution coefficient);
V_S = volume of the stationary phase;
V_M = volume of the mobile phase.

The retention factor of a component may be determined from the chromatogram using the following equation:

$$k = \frac{t_R - t_M}{t_M}$$

Total mobile phase time (t_t)
In size-exclusion chromatography, retention time of a component whose molecules are smaller than the smallest gel pores (Figure 2.2.46.-2).

Total mobile phase volume (V_t)
In size-exclusion chromatography, retention volume of a component whose molecules are smaller than the smallest gel pores. It may be calculated from the total mobile phase time and the flow rate (F) in millilitres per minute using the following equation:

$$V_t = t_t \times F$$

Retention time of an unretained compound (t_0)
In size-exclusion chromatography, retention time of a component whose molecules are larger than the largest gel pores (Figure 2.2.46.-2).

Retention volume of an unretained compound (V_0)
In size-exclusion chromatography, retention volume of a component whose molecules are larger than the largest gel pores. It may be calculated from the retention time of an unretained compound and the flow rate (F) in millilitres per minute using the following equation:

$$V_0 = t_0 \times F$$

Distribution constant (K_0)
In size-exclusion chromatography, the elution characteristics of a component in a particular column may be given by the distribution constant (also referred to as distribution coefficient), which is calculated using the following equation:

$$K_0 = \frac{t_R - t_0}{t_t - t_0}$$

Retardation factor (R_F)
The retardation factor (also known as retention factor (R_f)), used in planar chromatography, is the ratio of the distance from the point of application to the centre of the spot and the distance travelled by the solvent front from the point of application (Figure 2.2.46.-3).

$$R_F = \frac{b}{a}$$

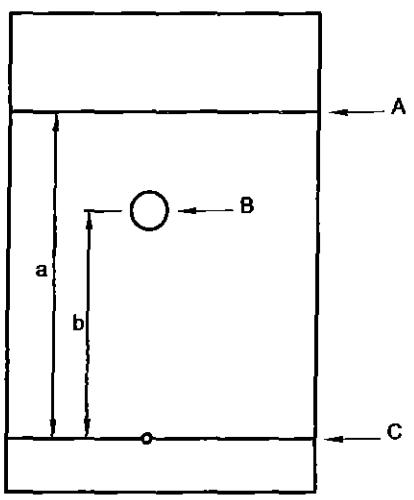
b = migration distance of the component;
a = migration distance of the solvent front.

Plate number (N)
The column performance (apparent efficiency) may be calculated from data obtained under either isothermal, isocratic or isodense conditions, depending on the technique, as the plate number (also referred to as number of theoretical plates), using the following equation, the values of t_R and w_h being expressed in the same units:

$$N = 5.54\left(\frac{t_R}{w_h}\right)^2$$

t_R = retention time of the peak corresponding to the component;
w_h = width of the peak at half-height.

The plate number varies with the component as well as with the column, the column temperature, the mobile phase and the retention time.



A. mobile phase front B. spot C. line of application

Figure 2.2.46.-3.

Dwell volume (D)
The dwell volume (also known as gradient delay volume) is the volume between the point at which the eluents meet and the top of the column. It can be determined using the following procedure.

Column Replace the chromatographic column by an appropriate capillary tubing (e.g. 1 m × 0.12 mm).

Mobile phase:
— *mobile phase A*: *water R*;
— *mobile phase B*: 0.1 per cent *V/V* solution of *acetone R*;

Time (min)	Mobile phase A (per cent *V/V*)	Mobile phase B (per cent *V/V*)
0 - 20	100 → 0	0 → 100
20 - 30	0	100

Flow rate Set to obtain sufficient back-pressure (e.g. 2 mL/min).

Detection Spectrophotometer at 265 nm.

Determine the time ($t_{0.5}$) in minutes when the absorbance has increased by 50 per cent (Figure 2.2.46.-4).

$$D = t_D \times F$$

t_D = $t_{0.5} - 0.5t_G$ (in minutes);
t_G = pre-defined gradient time (= 20 min);
F = flow rate (in millilitres per minute).

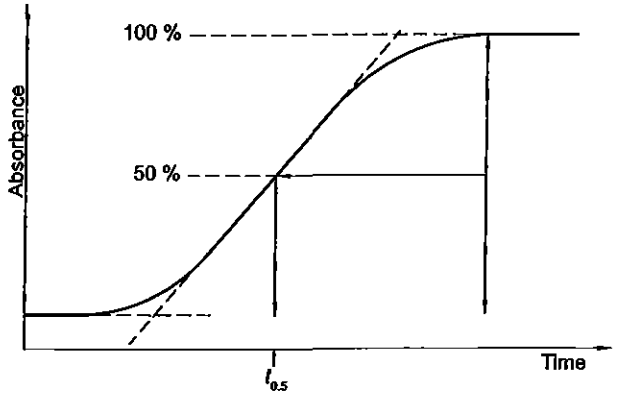


Figure 2.2.46.-4

Symmetry factor (A_s)

The symmetry factor of a peak (Figure 2.2.46.-5) is calculated using the following equation:

$$A_s = \frac{w_{0.05}}{2d}$$

$w_{0.05}$ = width of the peak at one-twentieth of the peak height;
d = distance between the perpendicular dropped from the peak maximum and the leading edge of the peak at one-twentieth of the peak height.

An A_s value of 1.0 signifies symmetry. When $A_s > 1.0$, the peak is tailing. When $A_s < 1.0$, the peak is fronting.

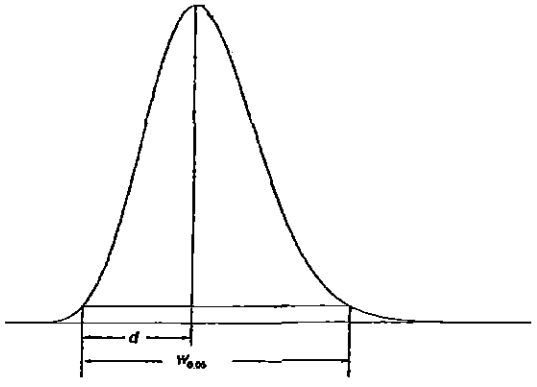


Figure 2.2.46.-5

Resolution (R_s)

The resolution between peaks of 2 components (Figure 2.2.46.-1) may be calculated using the following equation:

$$R_s = \frac{1.18(t_{R2} - t_{R1})}{w_{h1} + w_{h2}}$$

$t_{R2} > t_{R1}$

t_{R1}, t_{R2} = retention times of the peaks;
w_{h1}, w_{h2} = peak widths at half-height.

In quantitative planar chromatography, using densitometry, the migration distances are used instead of retention times and the resolution between peaks of 2 components may be calculated using the following equation:

$$R_s = \frac{1.18a(R_{F2} - R_{F1})}{w_{h1} + w_{h2}}$$

R_{F1}, R_{F2} = retardation factors of the peaks;
w_{h1}, w_{h2} = peak widths at half-height;
a = migration distance of the solvent front.

Peak-to-valley ratio (p/v)

The peak-to-valley ratio may be employed as a system suitability criterion in a test for related substances when baseline separation between 2 peaks is not achieved (Figure 2.2.46.-6).

$$p/v = \frac{H_p}{H_v}$$

H_p = height above the extrapolated baseline of the minor peak;
H_v = height above the extrapolated baseline at the lowest point of the curve separating the minor and major peaks.

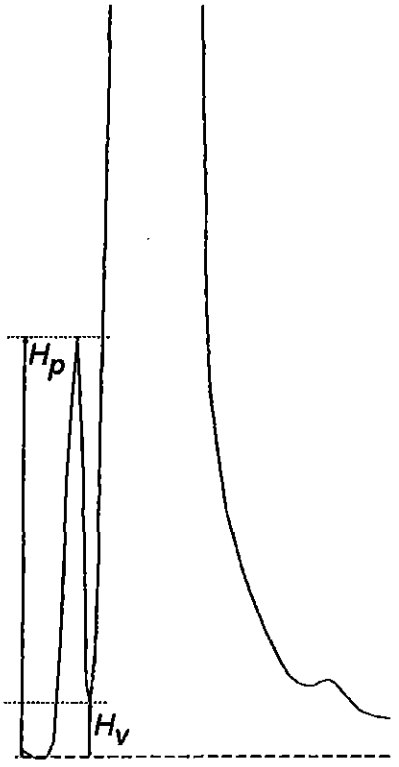


Figure 2.2.46.-6

Relative retention (r)

Relative retention is calculated as an estimate using the following equation:

$$r = \frac{t_{Ri} - t_M}{t_{Rs} - t_M}$$

t_{Ri} = retention time of the peak of interest;
t_{Rs} = retention time of the reference peak (usually the peak corresponding to the substance to be examined);
t_M = hold-up time.

The unadjusted relative retention (r_G) is calculated using the following equation:

$$r_G = \frac{t_{Ri}}{t_{Rst}}$$

Unless otherwise indicated, values for relative retention stated in monographs correspond to unadjusted relative retention.

In planar chromatography, the retardation factors R_{Fst} and R_{Fi} are used instead of t_{Rst} and t_{Ri}.

Signal-to-noise ratio (*S/N*)

The short-term noise influences the precision of quantification. The signal-to-noise ratio is calculated using the following equation:

$$S/N = \frac{2H}{h}$$

H = height of the peak (Figure 2.2.46.-7) corresponding to the component concerned, in the chromatogram obtained with the prescribed reference solution, measured from the maximum of the peak to the extrapolated baseline of the signal observed over a distance equal to at least 5 times the width at half-height;

h = range of the noise in a chromatogram obtained after injection or application of a blank, observed over a distance equal to at least 5 times the width at half-height of the peak in the chromatogram obtained with the prescribed reference solution and, if possible, situated equally around the place where this peak would be found.

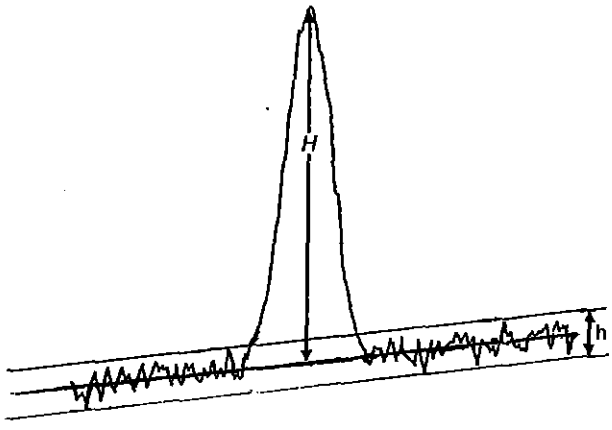


Figure 2.2.46.-7.

System repeatability

The repeatability of response is expressed as an estimated percentage relative standard deviation (s_r(%)) of a consecutive series of measurements for not fewer than 3 injections or applications of a reference solution, and is calculated using the following equation:

$$s_r(\%) = \frac{100}{\bar{y}}\sqrt{\frac{\sum(y_i - \bar{y})^2}{n-1}}$$

y_i = individual values expressed as peak area, peak height, or ratio of areas by the internal standardisation method;

$\bar{y}$ = mean of individual values;

n = number of individual values.

SYSTEM SUITABILITY

The various components of the equipment employed must be qualified and be capable of achieving the performance required to conduct the test or assay.

The system suitability tests represent an integral part of the method and are used to ensure adequate performance of the chromatographic system. Apparent efficiency, retention factor (mass distribution ratio), resolution and symmetry factor are the parameters that are usually employed in assessing the performance of the column. Factors that may affect the chromatographic behaviour include:

- the composition, ionic strength, temperature and apparent pH of the mobile phase;
- flow rate, column dimensions, column temperature and pressure;
- stationary phase characteristics including type of chromatographic support (particle-based or monolithic), particle or macropore size, porosity, specific surface area;
- reversed-phase and other surface-modification of the stationary phases, the extent of chemical modification (as expressed by end-capping, carbon loading etc.).

The following requirements and any supplementary requirements given in the individual monograph are to be fulfilled unless otherwise prescribed:

- in a related substances test or assay, for a peak in the chromatogram obtained with a reference solution used for quantification, the symmetry factor is 0.8 to 1.5, unless otherwise prescribed;
- in an assay of an active substance where the value is 100 per cent for a pure substance, the maximum permitted relative standard deviation ($s_r(\%)_{max}$) for the defined limits is calculated for a series of injections of the reference solution using the following equation:

$$s_r(\%)_{max} = \frac{KB\sqrt{n}}{t_{90\%,n-1}}$$

K = constant (0.349), obtained from the expression $K = \frac{0.6}{\sqrt{2}} \times \frac{t_{90\%,5}}{\sqrt{6}}$ in which $\frac{0.6}{\sqrt{2}}$ represents the required percentage relative standard deviation after 6 injections for $B = 1.0$;

B = upper limit given in the definition of the individual monograph minus 100 per cent;

n = number of replicate injections of the reference solution ($3 \leq n \leq 6$);

$t_{90\%,n-1}$ = Student's t at the 90 per cent probability level (double sided) with $n-1$ degrees of freedom.

Unless otherwise prescribed, the maximum permitted relative standard deviation does not exceed the appropriate value given in Table 2.2.46.-1. This requirement does not apply to tests for related substances.

Table 2.2.46.-1. – *Repeatability requirements*

	Number of individual injections			
	3	4	5	6
B (per cent)	Maximum permitted relative standard deviation			
2.0	0.41	0.59	0.73	0.85
2.5	0.52	0.74	0.92	1.06
3.0	0.62	0.89	1.10	1.27

- in a related substances test, the limit of quantification (corresponding to a signal-to-noise ratio of 10) is equal to or less than the disregard limit.

Compliance with the system suitability criteria is required throughout the chromatographic procedure. Depending on various factors, such as the frequency of use of the procedure and experience with the chromatographic system, the analyst chooses an appropriate verification scheme to monitor this.

ADJUSTMENT OF CHROMATOGRAPHIC CONDITIONS

The extent to which the various parameters of a chromatographic test may be adjusted to satisfy the system suitability criteria without fundamentally modifying the methods are listed below. Adjustment of conditions with gradient elutions is more critical than with isocratic elutions, since it may lead to shifts in peaks to a different step of the gradient, thus leading to the incorrect assignment of peaks, and to the masking of peaks or a shift such that elution occurs beyond the prescribed elution time. Changes other than those indicated require revalidation of the method. The chromatographic conditions described have been validated during the elaboration of the monograph.

The system suitability tests are included to verify that the separation required for satisfactory performance of the test or assay is achieved. Nonetheless, since the stationary phases are described in a general way and there is such a variety available commercially, with differences in chromatographic behaviour, some adjustments of the chromatographic conditions may be necessary to achieve the prescribed system suitability requirements. With reversed-phase liquid chromatographic methods in particular, adjustment of the various parameters will not always result in satisfactory chromatography. In that case, it may be necessary to replace the column with another of the same type (e.g. octadecylsilyl silica gel), which exhibits the desired chromatographic behaviour. The Knowledge database on the EDQM website usually contains information on the column(s) used during monograph elaboration.

For critical parameters the adjustments are defined clearly in the monograph to ensure the system suitability.

Thin-layer chromatography and paper chromatography

Composition of the mobile phase The amount of the minor solvent component may be adjusted by ± 30 per cent relative or ± 2 per cent absolute, whichever is the larger; for a minor component at 10 per cent of the mobile phase, a 30 per cent relative adjustment allows a range of 7-13 per cent whereas a 2 per cent absolute adjustment allows a range of 8-12 per cent, the relative value therefore being the larger; for a minor component at 5 per cent of the mobile phase, a 30 per cent relative adjustment allows a range of 3.5-6.5 per cent whereas a 2 per cent absolute adjustment allows a range of 3-7 per cent, the absolute value being the larger in this case; no other component is altered by more than 10 per cent absolute.

pH of the aqueous component of the mobile phase ± 0.2 pH, unless otherwise prescribed, or ± 1.0 pH when non-ionisable substances are to be examined.

Concentration of salts in the buffer component of a mobile phase ± 10 per cent.

Application volume 10-20 per cent of the prescribed volume if using fine particle size plates (2-10 µm).

Liquid chromatography: isocratic elution

Composition of the mobile phase The amount of the minor solvent component may be adjusted by ± 30 per cent relative or ± 2 per cent absolute, whichever is the larger (see example above); no other component is altered by more than 10 per cent absolute.

pH of the aqueous component of the mobile phase ± 0.2 pH, unless otherwise prescribed, or ± 1.0 pH when non-ionisable substances are to be examined.

Concentration of salts in the buffer component of a mobile phase ± 10 per cent.

Flow rate ± 50 per cent; a larger adjustment is acceptable when changing the column dimensions (see the formula below).

Column parameters

Stationary phase:

— no change of the identity of the substituent of the stationary phase permitted (e.g. no replacement of C18 by C8);

— *particle size*: maximum reduction of 50 per cent; no increase permitted.

Column dimensions:

— *length*: ± 70 per cent;

— *internal diameter*: ± 25 per cent.

When column dimensions are changed, the flow rate may be adjusted as necessary using the following equation:

$$F_2 = F_1 \frac{l_2 d_2^2}{l_1 d_1^2}$$

F_1 = flow rate indicated in the monograph, in millilitres per minute;
F_2 = adjusted flow rate, in millilitres per minute;
l_1 = length of the column indicated in the monograph, in millimetres;
l_2 = length of the column used, in millimetres;
d_1 = internal diameter of the column indicated in the monograph, in millimetres;
d_2 = internal diameter of the column used, in millimetres.

Temperature ± 10 °C, where the operating temperature is specified, unless otherwise prescribed.

Detector wavelength No adjustment permitted.

Injection volume May be decreased, provided detection and repeatability of the peak(s) to be determined are satisfactory; no increase permitted.

Liquid chromatography: gradient elution

Adjustment of chromatographic conditions for gradient systems requires greater caution than for isocratic systems.

Composition of the mobile phase/gradient elution Minor adjustments of the composition of the mobile phase and the gradient are acceptable provided that:

— the system suitability requirements are fulfilled;

— the principal peak(s) elute(s) within ± 15 per cent of the indicated retention time(s);

— the final composition of the mobile phase is not weaker in elution power than the prescribed composition.

Where compliance with the system suitability requirements cannot be achieved, it is often preferable to consider the dwell volume or to change the column.

Dwell volume The configuration of the equipment employed may significantly alter the resolution, retention time and relative retentions described. Should this occur, it may be due to excessive dwell volume. Monographs preferably include an isocratic step before the start of the gradient programme so that an adaptation can be made to the gradient time points to take account of differences in dwell volume between the system used for method development and that actually used. It is the user's responsibility to adapt the length of the isocratic step to the analytical equipment used. If the dwell volume used during the elaboration of the monograph is given in the monograph, the time points (t min) stated in the gradient table may be replaced by adapted time points (t_c min), calculated using the following equation:

$$t_c = t - \frac{(D - D_0)}{F}$$

D = dwell volume, in millilitres;
D_0 = dwell volume used for development of the method, in millilitres;
F = flow rate, in millilitres per minute.

The isocratic step introduced for this purpose may be omitted if validation data for application of the method without this step is available.

pH of the aqueous component of the mobile phase No adjustment permitted.

Concentration of salts in the buffer component of a mobile phase No adjustment permitted.

Flow rate Adjustment is acceptable when changing the column dimensions (see the formula below).

Column parameters

Stationary phase:
- — no change of the identity of the substituent of the stationary phase permitted (e.g. no replacement of C18 by C8);
- — *particle size*: no adjustment permitted.

Column dimensions:
- — *length*: ± 70 per cent;
- — *internal diameter*: ± 25 per cent.

When column dimensions are changed, the flow rate may be adjusted as necessary using the following equation:

$$F_2 = F_1 \frac{l_2 d_2^2}{l_1 d_1^2}$$

F_1 = flow rate indicated in the monograph, in millilitres per minute;
F_2 = adjusted flow rate, in millilitres per minute;
l_1 = length of the column indicated in the monograph, in millimetres;
l_2 = length of the column used, in millimetres;
d_1 = internal diameter of the column indicated in the monograph, in millimetres;
d_2 = internal diameter of the column used, in millimetres.

Temperature ± 5 °C, where the operating temperature is specified, unless otherwise prescribed.

Detector wavelength No adjustment permitted.

Injection volume May be decreased, provided detection and repeatability of the peak(s) to be determined are satisfactory; no increase permitted.

Gas chromatography

Column parameters

Stationary phase:
- — *particle size*: maximum reduction of 50 per cent; no increase permitted (packed columns);
- — *film thickness*: −50 per cent to + 100 per cent (capillary columns).

Column dimensions:
- — *length*: ± 70 per cent;
- — *internal diameter*: ± 50 per cent.

Flow rate ± 50 per cent.

Temperature ± 10 per cent.

Injection volume and split volume May be adjusted, provided detection and repeatability are satisfactory.

Supercritical fluid chromatography

Composition of the mobile phase For packed columns, the amount of the minor solvent component may be adjusted by ± 30 per cent relative or ± 2 per cent absolute, whichever is the larger; no adjustment is permitted for a capillary column system.

Detector wavelength No adjustment permitted.

Column parameters

Stationary phase:
- — *particle size*: maximum reduction of 50 per cent; no increase permitted (packed columns).

Column dimensions:
- — *length*: ± 70 per cent;
- — *internal diameter*:
  ± 25 per cent (packed columns);
  ± 50 per cent (capillary columns).

Flow rate ± 50 per cent.

Temperature ± 5 °C, where the operating temperature is specified.

Injection volume May be decreased, provided detection and repeatability are satisfactory; no increase permitted.

QUANTIFICATION

Peaks due to solvents and reagents or arising from the mobile phase or the sample matrix are disregarded during quantification.

- — *Detector sensitivity*. The detector sensitivity is the signal output per unit concentration or unit mass of a substance in the mobile phase entering the detector. The relative detector response factor, commonly referred to as *response factor*, expresses the sensitivity of a detector for a given substance relative to a standard substance. The *correction factor* is the reciprocal of the response factor.
- — *External standard method*. The concentration of the component(s) to be analysed is determined by comparing the response(s) (peak(s)) obtained with the test solution to the response(s) (peak(s)) obtained with a reference solution.
- — *Internal standard method*. Equal amounts of a component that will be resolved from the substance to be examined (the internal standard) are introduced into the test solution and a reference solution. The internal standard is chosen such that it does not react with the substance to be examined, is stable and does not contain impurities with the same retention time as that of the substance to be examined. The concentration of the substance to be examined is determined by comparing the ratio of the peak areas or peak heights due to the substance to be examined and the internal standard in the test solution with the ratio of the peak areas or peak heights due to the substance to be examined and the internal standard in the reference solution.
- — *Normalisation procedure*. The percentage content of a component of the substance to be examined is calculated by determining the area of the corresponding peak as a percentage of the total area of all the peaks, excluding those due to solvents or reagents or arising from the mobile phase or the sample matrix, and those at or below the disregard limit.
- — *Calibration procedure*. The relationship between the measured or evaluated signal (y) and the quantity (concentration, mass, etc.) of substance (x) is determined and the calibration function is calculated. The analytical results are calculated from the measured signal or evaluated signal of the analyte by means of the inverse function.

In tests for related substances for both the external standard method, when a dilution of the test solution is used for comparison, and the normalisation procedure, any correction factors indicated in the monograph are applied (i.e. when the response factor is outside the range 0.8-1.2).

When the related substances test prescribes the total of impurities or there is a quantitative determination of an impurity, it is important to choose an appropriate threshold setting and appropriate conditions for the integration of the peak areas. In such tests the *disregard limit*, i.e. the limit at or below which a peak is disregarded, is generally 0.05 per cent. Integration of the peak area of any impurity that is not completely separated from the principal peak is preferably performed by valley-to-valley extrapolation (tangential skim).

Additional points for monographs of the British Pharmacopoeia

System suitability

Unless otherwise stated in the monograph, the maximum permitted relative standard deviation for six replicate injections of the prescribed reference solution does not exceed 2.0%. This requirement is applicable only to assays for formulated preparations.

A. Thin-layer Chromatography

(Ph. Eur. method 2.2.27)

Thin-layer chromatography is a separation technique in which a stationary phase consisting of an appropriate material is spread in a uniform thin layer on a support (plate) of glass, metal or plastic. Solutions of analytes are deposited on the plate prior to development. The separation is based on adsorption, partition, ion-exchange or on combinations of these mechanisms and is carried out by migration (development) of solutes (solutions of analytes) in a solvent or a suitable mixture of solvents (mobile phase) through the thin-layer (stationary phase).

APPARATUS

Plates

The chromatography is carried out using pre-coated plates as described under *Reagents (4.1.1)*. The particle size of the silica gel is indicated after the name of the reagent in the tests where it is used.

Pre-treatment of the plates It may be necessary to wash the plates prior to separation. This can be done by migration of an appropriate solvent. The plates may also be impregnated by procedures such as development, immersion or spraying. At the time of use, the plates may be activated, if necessary, by heating in an oven at 120 °C for 20 min.

Chromatographic tank

With a flat bottom or twin trough, of inert, transparent material, of a size suitable for the plates used and provided with a tightly fitting lid. For horizontal development the tank is provided with a trough for the mobile phase and it additionally contains a device for directing the mobile phase to the stationary phase.

Micropipettes, microsyringes, calibrated disposable capillaries

Or other application devices suitable for the proper application of the solutions.

Fluorescence detection device

To measure direct fluorescence or the inhibition of fluorescence.

Visualisation devices and reagents

Suitable devices are used for derivatisation to transfer to the plate reagents by spraying, immersion or exposure to vapour and, where applicable, to facilitate heating for visualisation of separated components.

Documentation

A device may be used to provide documentation of the visualised chromatogram, for example a photograph or a computer file.

METHOD

Sample application

Apply the prescribed volume of the solutions at a suitable distance from the lower edge and from the sides of the plate and on a line parallel to the lower edge; allow an interval of at least 10 mm (5 mm on high-performance plates) between the centres of circular spots and 5 mm (2 mm on high-performance plates) between the edges of bands. Apply the solutions in sufficiently small portions to obtain circular spots 2-5 mm in diameter (1-2 mm on high-performance plates) or bands 10-20 mm (5-10 mm on high-performance plates) by 1-2 mm.

In a monograph, where both normal and high-performance plates may be used, the working conditions for high-performance plates are given in the brackets [ ] after those for normal plates.

Vertical development

Line the walls of the chromatographic tank with filter paper. Pour into the chromatographic tank a sufficient quantity of the mobile phase for the size of the tank to give after impregnation of the filter paper a layer of appropriate depth related to the dimension of the plate to be used.

For saturation of the chromatographic tank, replace the lid and allow to stand at 20-25 °C for 1 h. Unless otherwise indicated in the monograph, the chromatographic separation is performed in a saturated tank. Apply the prescribed volume of solutions as described above. When the solvent has evaporated from the applied solutions, place the plate in the chromatographic tank, ensuring that the plate is as vertical as possible and that the spots or bands are above the surface of the mobile phase. Close the chromatographic tank, maintain it at 20-25 °C and protect from sunlight. Remove the plate when the mobile phase has moved over the prescribed distance, measured between the points of application and the solvent front. Dry the plate and visualise the chromatograms as prescribed.

For two-dimensional chromatography, dry the plates after the first development and carry out a second development in a direction perpendicular to that of the first development.

Horizontal development

Apply the prescribed volume of the solutions as described above. When the solvent has evaporated from the applied solutions, introduce a sufficient quantity of the mobile phase into the trough of the chamber using a syringe or pipette, place the plate in the chamber after verifying that the latter is horizontal and connect the mobile phase direction device according to the manufacturer's instructions. If prescribed, develop the plate starting simultaneously at both ends. Close the chamber and maintain it at 20-25 °C. Remove the plate when the mobile phase has moved over the distance prescribed in the monograph. Dry the plate and visualise the chromatograms as prescribed.

For two-dimensional chromatography, dry the plates after the first development and carry out a second development in a direction perpendicular to that of the first development.

VISUAL EVALUATION

Identification

The principal spot in the chromatogram obtained with the test solution is visually compared to the corresponding spot in the chromatogram obtained with the reference solution by

comparing the colour, the size and the retardation factor (R_F) of both spots.

The retardation factor (R_F) is defined as the ratio of the distance from the point of application to the centre of the spot and the distance travelled by the solvent front from the point of application.

Verification of the separating power for identification Normally the performance given by the suitability test described in *Reagents (4.1.1)* is sufficient. Only in special cases an additional performance criterion is prescribed in the monograph.

Related substances test

The secondary spot(s) in the chromatogram obtained with the test solution is (are) visually compared to either the corresponding spot(s) in the chromatogram obtained with the reference solution containing the impurity(ies) or the spot in the chromatogram obtained with the reference solution prepared from a dilution of the test solution.

Verification of the separating power The requirements for the verification of the separating power are prescribed in the monographs concerned.

Verification of the detecting power The detecting power is satisfactory if a spot or band is clearly visible in the chromatogram obtained with the most dilute reference solution.

QUANTITATIVE MEASUREMENT

The requirements for resolution and separation are prescribed in the monographs concerned.

Substances separated by thin-layer chromatography and responding to UV-Vis irradiation can be determined directly on the plate, using appropriate instrumentation. While moving the plate or the measuring device, examine the plate by measuring the reflectance of the incident light. Similarly, fluorescence may be measured using an appropriate optical system. Substances containing radionuclides can be quantified in 3 ways: either directly by moving the plate alongside a suitable counter or vice versa (see *Radiopharmaceutical preparations (0125)*), by cutting the plates into strips and measuring the radioactivity on each individual strip using a suitable counter or by scraping off the stationary phase, dissolving it in a suitable scintillation cocktail and measuring the radioactivity using a liquid scintillation counter.

Apparatus

The apparatus for direct measurement on the plate consists of:

- a device for exact positioning and reproducible dispensing of the amount of substances onto the plate;
- a mechanical device to move the plate or the measuring device along the *x*-axis or the *y*-axis;
- a recorder and a suitable integrator or a computer;
- *for substances responding to UV-Vis irradiation*: a photometer with a source of light, an optical device able to generate monochromatic light and a photo cell of adequate sensitivity are used for the measurement of reflectance or transmittance; if fluorescence is measured, a suitable filter is required to prevent light used for excitation from reaching the detector while permitting emitted light or a specific portion thereof to pass;
- *for substances containing radionuclides*: a suitable counter for radioactivity. The linearity range of the counting device is to be verified.

Method

Prepare the solution of the substance to be examined (test solution) as prescribed in the monograph and, if necessary, prepare the reference solutions of the substance to be determined using the same solvent as in the test solution. Apply the same volume of each solution to the plate and develop.

Substances responding to UV-Vis irradiation Prepare and apply not fewer than 3 reference solutions of the substance to be examined, the concentrations of which span the expected value in the test solution (about 80 per cent, 100 per cent and 120 per cent). Treat with the prescribed reagent, if necessary, and record the reflectance, the transmittance or fluorescence in the chromatograms obtained with the test and reference solutions. Use the measured results for the calculation of the amount of substance in the test solution.

Substances containing radionuclides Prepare and apply a test solution containing about 100 per cent of the expected value. Determine the radioactivity as a function of the path length and report the radioactivity in each resulting peak as a percentage of the total amount of radioactivity.

Criteria for assessing the suitability of the system are described in the chapter on *Chromatographic separation techniques (2.2.46)*. The extent to which adjustments of parameters of the chromatographic system can be made to satisfy the criteria of system suitability are also given in this chapter.

Additional points for monographs of the British Pharmacopoeia

When the method prescribed in a monograph carries the instructions 'protected from light' or 'in subdued light' it is intended that the entire procedure is carried out under these conditions.

Unless otherwise indicated in the monograph, the mobile phase should be allowed to ascend 15 cm above the line of application.

The phrase *ultraviolet light (254 nm)* indicates that the plate should be examined under an ultraviolet lamp having a maximum output at 254 nm (see below); other wavelength maxima may be specified.

The term *secondary spot* means any spot other than the principal spot. Similarly, a *secondary band* is any band other than the principal band.

Where a spraying technique is prescribed it is essential that the reagent is evenly applied as a fine spray. The following method of visualisation is used when directed in the monograph.

METHOD I

Spray the dried plate with *ethanolic sulfuric acid (20%)*, heat at 105° for 30 minutes and immediately expose to nitrous fumes in a closed glass tank for 15 minutes (the nitrous fumes may be generated by adding 7M *sulfuric acid* dropwise to a solution containing 10% w/v of *sodium nitrite* and 3% w/v of *potassium iodide*). Place the plate in a current of warm air for 15 minutes and spray with a 0.5% w/v solution of *N-(1-naphthyl)ethylenediamine dihydrochloride* in *ethanol (96%)*. If necessary, allow to dry and repeat the spraying.

MATERIALS

The coating substances and precoated plates are described in Appendix I A: General Reagents. Prepare suspensions of the coating substances as recommended by the manufacturer unless otherwise prescribed. Commercial pre-coated plates

may be used for pharmacopoeial tests where a coating substances is prescribed provided that they comply with the test for chromatographic separation described for the corresponding coating substance and with any additional test for verification of separating power required in the monograph test.

Ultraviolet Ray Lamps for Analytical Purposes
(Ph. Eur. method 2.1.3)

Mercury vapour in quartz lamps is used as the source of ultraviolet light. A suitable filter may be fitted to eliminate the visible part of the spectrum emitted by the lamp. When the Pharmacopoeia prescribes in a test the use of ultraviolet light of wavelength 254 nm or 365 nm, an instrument consisting of a mercury vapour lamp and a filter which gives an emission band with maximum intensity at about 254 nm or 365 nm is used. The lamp used should be capable of revealing without doubt a standard spot of sodium salicylate with a diameter of about 5 mm on a support of *silica gel G R*, the spot being examined while in a position normal to the radiation.

For this purpose apply 5 µL of a 0.4 g/L solution of *sodium salicylate R* in *alcohol R*[1] for lamps of maximum output at 254 nm and 5 µL of a 2 g/L solution in *alcohol R*[3] for lamps of maximum output at 365 nm. The distance between the lamp and the chromatographic plate under examination used in a pharmacopoeial test should never exceed the distance used to carry out the above test.

Identification of Phenothiazines by Thin-Layer Chromatography
(Ph. Eur. method 2.3.3)

Examine by thin-layer chromatography (*2.2.27*) using *kieselguhr G R* as the coating substance. Impregnate the plate by placing it in a closed tank containing the necessary quantity of the impregnation mixture composed of a solution containing 10 per cent *V/V* of *phenoxyethanol R* and 50 g/L of *macrogol 300 R* in *acetone R* so that the plate dips about 5 mm beneath the surface of the liquid. When the impregnation mixture has risen at least 17 cm from the lower edge of the plate, remove the plate and use immediately for chromatography. Carry out the chromatography in the same direction as the impregnation.

Test solution Dissolve 20 mg of the substance to be examined in *chloroform R* and dilute to 10 mL with the same solvent.

Reference solution Dissolve 20 mg of the corresponding chemical reference substance (CRS) in *chloroform R* and dilute to 10 mL with the same solvent.

Apply separately to the plate 2 µL of each solution and develop in the dark over a path of 15 cm using a mixture of 50 mL of *light petroleum R* and 1 mL of *diethylamine R* saturated with *phenoxyethanol R* (i.e. add about 3 mL to 4 mL of *phenoxyethanol R* to the above mixture of solvents to give a persistent cloudiness on shaking, decant, and use the supernatant, even if it is cloudy). After development place the plate under ultraviolet light at 365 nm and examine after a few minutes. The spot in the chromatogram obtained with the test solution is similar in position, fluorescence and size to the spot in the chromatogram obtained with the reference solution. Spray with a 10 per cent *V/V* solution of *sulfuric acid R* in *alcohol R*. The spot in the chromatogram obtained with the test solution is of the same colour as that in the chromatogram obtained with the reference solution and has similar stability over a period of at least 20 min.

Related Substances in Phenothiazines
(No Ph. Eur. method)

Carry out the method for *thin-layer chromatography* protected from light using *silica gel* GF_{254} as the coating substance and the mobile phase prescribed in the monograph, but allowing the solvent front to ascend 12 cm above the line of application. Unless otherwise specified, apply separately to the plate 10 µL of each of two solutions of the substance being examined prepared immediately before use in a mixture of 95 volumes of *methanol* and 5 volumes of *diethylamine* containing (1) 2.0% w/v and (2) 0.010% w/v. After removal of the plate, allow it to dry in air and examine under *ultraviolet light (254 nm)*. Disregard any spot remaining on the line of application. Unless otherwise specified any *secondary spot* in the chromatogram obtained with solution (1) is not more intense than the spot in the chromatogram obtained with solution (2) (0.5%).

Mobile phases

A. A mixture of 10 volumes of *acetone*, 10 volumes of *diethylamine* and 80 volumes of *cyclohexane*.

B. A mixture of 5 volumes of *diethylamine*, 10 volumes of *acetone* and 85 volumes of *hexane*.

C. A mixture of 18 volumes of 1M *ammonia* and 90 volumes of *butan-1-ol*.

Identification of Steroids
(No Ph. Eur. method)

Carry out the method for *thin-layer chromatography* using *kieselguhr G* as the coating substance. Impregnate the dry plate by placing it in a tank containing a shallow layer of the specified impregnating solvent, allowing the solvent to ascend to the top, removing the plate from the tank and allowing the solvent to evaporate; use within 2 hours, with the flow of the mobile phase in the direction in which impregnation was carried out. Unless otherwise specified, apply separately to the plate 2 µL of each of the following three solutions in a mixture of 9 volumes of *chloroform* and 1 volume of *methanol*. Solution (1) contains 0.25% w/v of the substance being examined. Solution (2) contains 0.25% w/v of the corresponding British Pharmacopoeia Chemical Reference Substance or European Pharmacopoeia Chemical Reference Substance. Solution (3) is a mixture of equal volumes of solutions (1) and (2). Use the specified mobile phase. After removal of the plate, allow the solvent to evaporate, heat at 120° for 15 minutes and spray the hot plate with *ethanolic sulfuric acid (20%)*. Heat at 120° for a further 10 minutes, allow to cool and examine in daylight and under *ultraviolet light (365 nm)*. The principal spot in the chromatogram obtained with solution (1) is similar in position, colour in daylight, fluorescence in ultraviolet light (365 nm) and size to that in the chromatogram obtained with solution (2). The principal spot in the chromatogram obtained with solution (3) appears as a single, compact spot.

Impregnating solvents

I. A mixture of 1 volume of *formamide* and 9 volumes of *acetone*.

II. A mixture of 1 volume of *propane-1,2-diol* and 9 volumes of *acetone*.

III. A mixture of 1 volume of liquid paraffin and 9 volumes of petroleum spirit (boiling range, 40° to 60° or 50° to 70°).

[1] *The alcohol R used must be free from fluorescence.*

Mobile phases

A. *Chloroform.*

B. A mixture of 25 volumes of *chloroform* and 75 volumes of *toluene.*

C. Toluene.

D. A mixture of 20 volumes of *toluene* and 80 volumes of *cyclohexane.*

E. A mixture of equal volumes of cyclohexane and petroleum spirit (boiling range, 40° to 60° or 50° to 70°).

F. A mixture of 40 volumes of *glacial acetic acid* and 60 volumes of *water.*

G. A mixture of 20 volumes of *1,4-dioxan* and 80 volumes of *hexane.*

H. A mixture of 29 volumes of *toluene,* 56 volumes of *chloroform* and 115 volumes of *cyclohexane.*

B. Gas Chromatography

(Ph. Eur. method 2.2.28)

PRINCIPLE

Gas chromatography (GC) is a chromatographic separation technique based on the difference in the distribution of species between 2 non-miscible phases in which the mobile phase is a carrier gas moving through or passing the stationary phase contained in a column. It is applicable to substances or their derivatives which are volatilised under the temperatures employed.

GC is mainly based on mechanisms of adsorption or mass distribution.

EQUIPMENT

The equipment typically consists of:
- an injector;
- a chromatographic column contained in an oven;
- one or more detector(s);
- a data acquisition system.

The carrier gas flows through the column and then through the detector at a controlled rate or pressure.

The chromatography is carried out either at a constant temperature or according to a given temperature programme.

INJECTORS

Injection may be carried out either into a vaporisation chamber which may be equipped with a stream splitter, or directly at the head of the column using a syringe or an injection valve.

Injections of vapour phase May be effected by static or dynamic head-space injection systems.

Dynamic head-space (purge and trap) injection systems include a sparging device by which volatile substances in solution are swept into an absorbent column maintained at a low temperature. Retained substances are then desorbed into the mobile phase by rapid heating of the absorbent column.

Static head-space Injection systems include a thermostatically controlled sample heating chamber in which closed vials containing solid or liquid samples are placed for a fixed period of time to allow equilibration of the volatile components of the sample between the non-gaseous phase and the vapour phase. After equilibration, a predetermined amount of the head-space of the vial is flushed into the gas chromatograph.

STATIONARY PHASES

Stationary phases are contained in columns which may be:
- a capillary column whose stationary phase may be a solid coating the inner surface of the column (e.g. macrogol 20 000), or a liquid deposited on the inner surface (e.g. dimethylpolysiloxane); in the latter case it may be chemically bonded to the inner surface;
- a column packed with the stationary phase which may be a solid phase (e.g. alumina, silica) or an inert solid support (usually a porous polymer) impregnated or coated with a liquid.

Capillary columns, made of fused silica, are 0.1 mm to 0.53 mm in internal diameter (Ø) and at least 5 m in length. The stationary phase is a film 0.1 µm to 5.0 µm thick.

Packed columns, made of glass or metal, are usually 1 m to 3 m in length with an internal diameter (Ø) of 2 mm to 4 mm.

MOBILE PHASES

Retention time and peak efficiency depend on the carrier gas flow rate; retention time is directly proportional to column length and resolution is proportional to the square root of the column length.

The carrier gas flow rate is usually expressed in millilitres per minute at atmospheric pressure and at the stated temperature. Flow rate is measured at the detector outlet, either with a calibrated mechanical device or with a bubble tube, while the column is at operating temperature.

The linear velocity of the carrier gas through a column is inversely proportional to the square of the internal diameter of the column for a given flow volume.

Helium, nitrogen and hydrogen are commonly used carrier gases.

DETECTORS

Flame-ionisation detectors are usually employed but other detectors such as electron-capture, nitrogen-phosphorus, mass spectrometric, thermal conductivity or infrared spectrophotometric detectors may also be used.

PROCEDURE

Equilibrate the column, the injector and the detector at the temperatures and the gas flow rates/pressures specified in the monograph until a stable baseline is achieved. Prepare the test solution(s) and the reference solution(s) as prescribed. The solutions injected must be free from solid particles.

Criteria for assessing the suitability of the system are described in general chapter *2.2.46 Chromatographic separation techniques.* The extent to which adjustments of parameters of the chromatographic system can be made to satisfy the criteria of system suitability are also given in this general chapter.

STATIC HEAD-SPACE GAS CHROMATOGRAPHY

Static head-space gas chromatography is a technique particularly suitable for separating and determining volatile compounds present in solid or liquid samples. The method is based on the analysis of the vapour phase in equilibrium with the solid or liquid phase.

EQUIPMENT

The equipment consists of a gas chromatograph provided with a sample-introduction device that may be connected to a module that automatically controls the pressure and the temperature. If necessary, a device for eliminating solvents can be added.

The sample to be analysed is introduced into a container fitted with a suitable stopper and a valve-system which

permits the passage of the carrier gas. The container is placed in a thermostatically controlled chamber at a temperature set according to the substance to be examined.

The sample is held at this temperature long enough to allow equilibration between the solid or liquid phase and the vapour phase.

The carrier gas is introduced into the container and, after the prescribed time, a suitable valve is opened so that the gas expands towards the chromatographic column taking the volatilised compounds with it.

Instead of using a chromatograph specifically equipped for the introduction of samples, it is also possible to use airtight syringes and a conventional chromatograph. Equilibration is then carried out in a separate chamber and the vapour phase is carried onto the column, while necessary precautions are taken to avoid any changes in the equilibrium.

PROCEDURE

Using the reference preparations, determine suitable instrument settings to produce an adequate response.

DIRECT CALIBRATION

Introduce into separate, identical containers the preparation to be examined and each of the reference preparations, as prescribed in the monograph, avoiding contact between the sampling device and the samples.

Close the containers hermetically and place in the thermostatically controlled chamber set to the temperature and pressure prescribed in the monograph; after equilibration, carry out the chromatography under the prescribed conditions.

STANDARD ADDITIONS

Add to a set of identical suitable containers equal volumes of the preparation to be examined. Add to all but one of the containers, suitable quantities of a reference preparation containing a known concentration of the substance to be determined so as to produce a series of preparations containing steadily increasing concentrations of the substance.

Close the containers hermetically and place in the thermostatically controlled chamber set to the temperature and pressure prescribed in the monograph; after equilibration, carry out the chromatography under the prescribed conditions.

Calculate the linear equation of the graph using a least-squares fit, and derive from it the concentration of the substance to be determined in the preparation to be examined.

Alternatively, plot on a graph the mean of readings against the added quantity of the substance to be determined. Extrapolate the line joining the points on the graph until it meets the concentration axis. The distance between this point and the intersection of the axes represents the concentration of the substance to be determined in the preparation to be examined.

Additional points for monographs of the British Pharmacopoeia

APPARATUS

The design of a particular chromatograph may require modification of the conditions detailed in the monograph. In such a case, the analyst should be satisfied that the modified conditions produce comparable results. If necessary, adjust the flow rate of the carrier gas to improve the quality of the chromatogram or to modify the retention times of the peaks of interest.

METHOD

Unless otherwise stated in the monograph, use nitrogen as the carrier gas and a flame ionisation detector. Occasionally reference is made to on-column injection, in which case the sample is injected directly on to the packing material without the use of an inlet heater. When non-volatile material is to be injected on to the column, a suitable interchangeable pre-column may be used.

REAGENTS

Solvents and reagents used in the preparation of solutions for examination should be of a quality suitable for use in gas chromatography. A wide range of chemical substances is used as stationary phases, including polyethylene glycols, high-molecular weight esters and amides, hydrocarbons, silicone gums and fluids (polysiloxanes often substituted with methyl, phenyl, nitrilo, vinyl or fluoroalkyl groups or mixtures of these) and microporous cross-linked polyaromatic beads. A suitable stationary phase, its concentration and the nature and grade of a suitable solid support are stated in the monograph. The column should be conditioned in accordance with the manufacturer's instructions. In most cases reference is made to a particular commercial brand that has been found to be suitable for the purpose, but such statements do not imply that a different but equivalent commercial brand may not be used.

INTERNAL STANDARDS

Reagents used as internal standards should not contain any impurity that would produce a peak likely to interfere in the determination described in the monograph.

INJECTION VOLUME

Where no injection volume is specified in the monograph, the analyst should select an appropriate volume for their specific application. The volume chosen is dependent on the response of the analyte, the detector used, the efficiency of the column and the overall performance of the chromatographic system. Where a volume is not indicated, 1 µL is usually appropriate; however this should be checked for suitability under the local operating conditions.

SECONDARY PEAKS

Reference may be made to a secondary peak. A *secondary peak* is a peak in the chromatogram other than the principal peak and any peaks due to internal standard, solvent and derivatising agents. Peaks identified as being due to the counter-ion and/or other excipients including preservatives in the material being examined may also be excluded.

C. Size-exclusion Chromatography

(Ph. Eur. method 2.2.30)

PRINCIPLE

Size-exclusion chromatography is a liquid chromatography (*2.2.29*) technique which separates molecules in solution according to their size. With organic mobile phases, the technique is known as gel-permeation chromatography and with aqueous mobile phases it is known as gel-filtration chromatography. The sample is introduced into a column, which is filled with a gel or a porous particle packing material, and is carried by the mobile phase through the column. The size separation takes place by repeated exchange of the solute molecules between the solvent of the mobile phase and the same solvent in the stagnant liquid phase (stationary phase) within the pores of the packing

material. The pore-size range of the packing material determines the molecular-size range within which separation can occur.

Molecules small enough to penetrate all the pore spaces elute at the total mobile phase volume V_t (also known as total permeation volume). Molecules apparently larger than the maximum pore size of the packing material migrate along the column only through the spaces between the particles of the packing material without being retained and elute at the retention volume of an unretained compound V_0 (also known as exclusion volume or void volume). Separation according to molecular size occurs between the retention volume of an unretained compound and the total mobile phase volume, with useful separation usually occurring in the first two thirds of this range.

EQUIPMENT

The equipment consists essentially of a chromatographic column of varying length and internal diameter (Ø), if necessary temperature-controlled, packed with a separation material that is capable of fractionation in the appropriate range of molecular sizes. The packing material may be a soft support such as a swollen gel or a rigid support composed of a material such as glass, silica or a solvent-compatible, cross-linked organic polymer. Rigid supports usually require pressurised systems giving faster separations.

One end of the column is usually fitted with a suitable device for applying the sample such as a flow adapter, a syringe through a septum or an injection valve and may also be connected to a suitable pump for controlling the flow of the eluent. Alternatively, the sample may be applied directly to the drained bed surface or, where the sample is denser than the eluent, it may be layered beneath the eluent.

The mobile phase is chosen according to sample type, separation medium and method of detection. The eluent is passed through the column at a constant rate.

The outlet of the column is usually connected to a suitable detector fitted with an automatic recorder which enables the monitoring of the relative concentrations of separated components of the sample. Ultraviolet/visible spectrophotometers (*2.2.25*), differential refractometers (RI), luminescent detectors, multi-angle light scattering (MALS) detectors and other detectors may be used. An automatic fraction collector may be attached, if necessary.

PROCEDURE

Before carrying out the separation, the packing material is treated, and the column is packed, as described in the monograph, or according to the manufacturer's instructions.

Criteria for assessing the suitability of the system are described in general chapter *2.2.46. Chromatographic separation techniques.* The extent to which adjustments of parameters of the chromatographic system can be made to satisfy the criteria of system suitability are also given in this general chapter.

DETERMINATION OF RELATIVE COMPONENT COMPOSITION OF MIXTURES

Carry out the separation as stated in the monograph.
If possible, monitor the elution of the components continuously and measure the corresponding peak areas.
If the sample is monitored by a physico-chemical property to which all the components of interest exhibit equivalent responses (for example if they have the same specific absorbance), calculate the relative amount of each component by dividing the respective peak area by the sum of the peak areas of all the components of interest. If the responses to the property used for detection of the components of interest are not equivalent, calculate the content by means of calibration curves obtained with the calibration standards prescribed in the monograph.

DETERMINATION OF MOLECULAR MASSES

Size-exclusion chromatography may be used to determine molecular masses by comparison with appropriate calibration standards specified in the monograph. The retention volumes of the calibration standards may be plotted against the logarithm of their molecular masses. The plot usually approximates a straight line within the exclusion and total permeation limits for the separation medium used. From the calibration curve, molecular masses may be estimated.
The molecular-mass calibration is valid only for the particular macromolecular solute/solvent system used under the specified experimental conditions.

DETERMINATION OF MOLECULAR SIZE DISTRIBUTION OF POLYMERS

Size-exclusion chromatography may be used to determine the distribution of the molecular size of polymers. However, sample comparison may be valid only for results obtained under the same experimental conditions. The reference standards used for the calibration and the methods for determination of the distribution of molecular sizes of polymers are specified in the monograph.

Molecular Mass Distribution in Dextrans

(Ph. Eur. method 2.2.39)

Examine by size-exclusion chromatography (*2.2.30*).

Test solution Dissolve 0.200 g of the substance to be examined in the mobile phase and dilute to 10 mL with the mobile phase.

Marker solution Dissolve 5 mg of *glucose R* and 2 mg of *dextran V_0 CRS* in 1 mL of the mobile phase.

Calibration solutions Dissolve separately in 1 mL of the mobile phase 15 mg of *dextran 4 for calibration CRS*, 15 mg of *dextran 10 for calibration CRS*, 20 mg of *dextran 40 for calibration CRS*, 20 mg of *dextran 70 for calibration CRS* and 20 mg of *dextran 250 for calibration CRS*.

System suitability solution Dissolve either 20 mg of *dextran 40 for performance test CRS* (for dextran 40) or 20 mg of *dextran 60/70 for performance test CRS* (for dextran 60 and dextran 70) in 1 mL of the mobile phase.

The chromatographic procedure may be carried out using:
- a column 0.3 m long and 10 mm in internal diameter, packed with *cross-linked agarose for chromatography R* or a series of columns, 0.3 m long and 7.5 mm in internal diameter, packed with *polyether hydroxylated gel for chromatography R*,
- as the mobile phase, at a flow rate of 0.5-1 mL/min, kept constant to ± 1 per cent per hour, a solution containing 7 g of *anhydrous sodium sulfate R* and 1 g of *chlorobutanol R* in 1000 mL of *water for chromatography R*,
- as detector a differential refractometer,
- a 100 µL to 200 µL loop injector,

maintaining the system at a constant temperature (± 0.1 °C).

CALIBRATION OF THE CHROMATOGRAPHIC SYSTEM

Carry out replicate injections of the chosen volume of the marker solution. The chromatogram shows 2 peaks, the first of which corresponds to *dextran V_0 CRS* and the second of which corresponds to *glucose R*. From the elution volume of the peak corresponding to dextran V_0, calculate the void

volume V_0 and from the peak corresponding to dextrose, calculate the total volume V_t.

Inject the chosen volume of each of the calibration solutions. Draw carefully the baseline of each of the chromatograms. Divide each chromatogram into p (at least 60) equal vertical sections (corresponding to equal elution volumes). In each section i, corresponding to an elution volume V_i measure the height (y_i) of the chromatogram line above the baseline and calculate the coefficient of distribution K_i using the expression:

$$\frac{(V_i - V_0)}{(V_t - V_0)} \qquad (1)$$

V_0 = void volume of the column, determined using the peak corresponding to *dextran V_0 CRS* in the chromatogram obtained with the marker solution,

V_t = total volume of the column, determined using the peak corresponding to glucose in the chromatogram obtained with the marker solution,

V_i = elution volume of section i in the chromatogram obtained with each of the calibration solutions.

Carry out the calibration using either of the following methods.

Calibration by plotting of the curve

For each of the dextrans for calibration calculate the coefficient of distribution K_{max} corresponding to the maximum height of the chromatographic line, using expression (1). Plot on semilogarithmic paper the values of K_{max} (on the x-axis) against the declared molecular mass at the maximum height of the chromatographic line (M_{max}) of each of the dextrans for calibration and glucose. Draw a first calibration curve through the points obtained, extrapolating it from the point K_{max} obtained with *dextran 250 for calibration CRS* to the lowest K value obtained for this CRS (Figure 2.2.39.-1). Using this first calibration curve, transform, for each chromatogram, all K_i values into the corresponding molecular mass M_i, thus obtaining the molecular mass distribution. Calculate for each dextran for calibration the average molecular mass M_w using equation (3) below. If the calculated values for M_w do not differ by more than 5 per cent from those declared for each of the dextrans for calibration and the mean difference is within ± 3 per cent, the calibration curve is approved. If not, move the calibration curve along the y-axis and repeat the procedure above until the calculated and the declared values for M_w do not differ by more than 5 per cent.

Calibration by calculation of the curve

Calculate from equations (2) and (3) below, using a suitable method[1], values for b_1, b_2, b_3, b_4 and b_5 that give values of M_w within 5 per cent of the declared values of each of the dextrans for calibration and 180 ± 2 for glucose:

$$M_i = b_5 + e^{\left(b_4 + b_1 K_i + b_2 K_i^2 + b_3 K_i^3\right)} \qquad (2)$$

$$\overline{M}_w = \frac{\sum_{i=1}^{p}(y_i M_i)}{\sum_{i=1}^{p} y_i} \qquad (3)$$

p = number of sections dividing the chromatograms,

y_i = height of the chromatographic line above the baseline in section i,

M_i = molecular mass in section i.

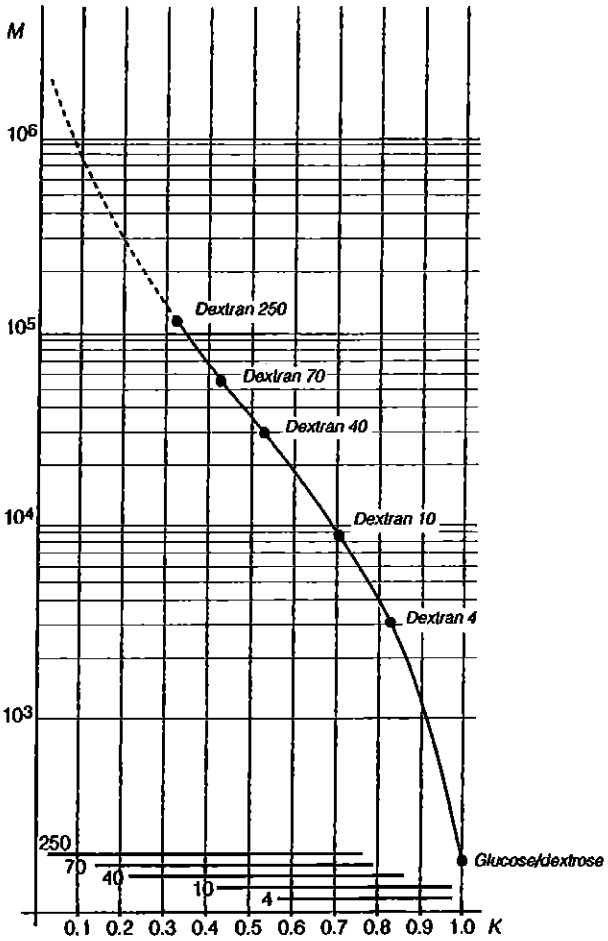


Figure 2.2.39.-1. – *Example of a calibration curve.*

The dotted line corresponds to the part of the curve that is extrapolated. Horizontal lines at the bottom of the figure represent the width and the position of the chromatographic line obtained with each of the dextrans for calibration.

SYSTEM SUITABILITY

Inject the chosen volume of the appropriate system suitability solution.

Average molecular mass of dextran for performance test CRS

Calculate the average molecular mass M_w as indicated under Calibration of the chromatographic system, using either the plotted calibration curve or the values obtained above for b_1, b_2, b_3, b_4 and b_5. The test is not valid unless M_w is:

— 41 000 to 47 000 (*dextran 40 for performance test CRS*),

— 67 000 to 75 000 (*dextran 60/70 for performance test CRS*).

Average molecular mass of the 10 per cent high-fraction dextran

Calculate M_w for the 10 per cent high-fraction dextran eluted through section n using the equation:

$$M_w = \frac{\sum_{i=1}^{n}(y_i M_i)}{\sum_{i=1}^{n} y_i} \qquad (4)$$

[1] *An iterative method such as the Gauss-Newton method modified by Hartley is suitable (see O. Hartley, Tecnometrics, 3 (1961) and G. Nilsson and K. Nilsson, J. Chromat. 101, 137 (1974)). A curve-fitting programme for microcomputers, capable of non-linear regression, may be used.*

in which n is defined by the expressions:

$$\sum_{i=1}^{n} y_i \leq 0.1\left(\sum_{i=1}^{p} y_i\right) \quad (5)$$

$$\sum_{i=1}^{n+1} y_i > 0.1\left(\sum_{i=1}^{p} y_i\right) \quad (6)$$

p = number of sections dividing the chromatograms,
y_i = height of the chromatographic line above the baseline in section i,
M_i = molecular mass in section i.

The test is not valid unless M_w of the 10 per cent high fraction dextran is:
— 110 000 to 130 000 (*dextran 40 for performance test CRS*),
— 190 000 to 230 000 (*dextran 60/70 for performance test CRS*).

Average molecular mass of the 10 per cent low-fraction dextran

Calculate M_w for the 10 per cent low-fraction dextran eluted in and after section m using the expression:

$$M_w = \frac{\sum_{i=m}^{p}(y_i M_i)}{\sum_{i=m}^{p} y_i} \quad (7)$$

in which m is defined by the expressions:

$$\sum_{i=m}^{p} y_i \leq 0.1\left(\sum_{i=1}^{p} y_i\right) \quad (8)$$

$$\sum_{i=m-1}^{p} y_i > 0.1\left(\sum_{i=1}^{p} y_i\right) \quad (9)$$

p = number of sections dividing the chromatograms,
y_i = height of the chromatographic line above the baseline in section i,
M_i = molecular mass in section i.

The test is not valid unless M_w of the 10 per cent low-fraction dextran is:
— 6000 to 8500 (*dextran 40 for performance test CRS*),
— 7000 to 11 000 (*dextran 60/70 for performance test CRS*).

MOLECULAR MASS DISTRIBUTION OF THE DEXTRAN TO BE ANALYSED

Inject the chosen volume of the test solution and calculate M_w of the total molecular mass distribution, M_w of the 10 per cent high-fraction dextran and M_w of the 10 per cent low-fraction dextran as indicated under System suitability.

D. Liquid Chromatography

(Ph. Eur. method 2.2.29)

PRINCIPLE

Liquid chromatography (LC) is a method of chromatographic separation based on the difference in the distribution of species between 2 non-miscible phases, in which the mobile phase is a liquid which percolates through a stationary phase contained in a column.

LC is mainly based on mechanisms of adsorption, mass distribution, ion exchange, size exclusion or stereochemical interaction.

Unless otherwise specified, all the information below is valid for both standard LC and LC using reduced particle-size columns (e.g. sub-2.0 µm).

The latter requires instrumentation that is able to withstand higher pressures (typically up to 100 MPa, i.e. about 15 000 psi), generates lower extra-column band broadening, provides improved gradient mixing and allows a higher sampling rate in the detection system.

EQUIPMENT

The equipment typically consists of:
— a pumping system;
— an injector;
— a chromatographic column (a column temperature controller may be used);
— 1 or more detectors;
— a data acquisition system.

The mobile phase is supplied from 1 or more reservoirs and is pumped to the injector, then through the column, usually at a constant rate, and then through the detector(s).

PUMPING SYSTEMS

LC pumping systems deliver the mobile phase at a controlled flow rate. Pressure fluctuations are to be minimised, for example by passing the pressurised solvent through a pulse-dampening device. Tubing and connections are capable of withstanding the pressures developed by the pumping system. LC pumps may be fitted with a facility for 'bleeding' the system of entrapped air bubbles.

Microprocessor-controlled pumping systems are capable of accurately delivering a mobile phase of either constant (isocratic elution) or varying composition (gradient elution), according to a defined programme. In the case of gradient elution, pumping systems which deliver solvent(s) from several reservoirs are available and solvent mixing can be achieved on either the low or high-pressure side of the pump(s).

INJECTORS

The sample solution is introduced into the flowing mobile phase at or near the head of the column using an injection system which can operate at high pressure. Fixed-loop and variable volume devices operated manually or by an autosampler are used. Partial filling of loops during manual injection may adversely affect injection volume precision.

STATIONARY PHASES

There are many types of stationary phases employed in LC, including:
— silica or alumina, commonly used in normal-phase LC (polar stationary phase and non-polar mobile phase), where the separation is based on differences in adsorption on the stationary phase and/or mass distribution between the mobile phase and the stationary phase (partition chromatography);
— a variety of chemically modified supports prepared from polymers, silica or porous graphite, used in normal-phase and reversed-phase LC (non-polar stationary phase and polar mobile phase), where the separation is based principally on partition of the molecules;
— resins or polymers with acidic or basic groups, used in ion-exchange chromatography, where separation is based

on competition between the ions to be separated and those in the mobile phase;
- porous silica or polymers, used in size-exclusion chromatography (*2.2.30*), where separation is based on differences between the volumes of the molecules, corresponding to steric exclusion;
- specially modified stationary phases, e.g. cellulose or amylose derivatives, proteins or peptides, cyclodextrins etc., for the separation of enantiomers (chiral chromatography).

Most separations are based on reversed-phase LC utilising chemically modified silica as the stationary phase.
The surface of the support, i.e. the silanol groups of silica, is reacted with various silane reagents to produce covalently bound silyl derivatives covering a varying number of active sites on the surface of the support. The nature of the bonded phase is an important parameter for determining the separation properties of the chromatographic system.

Unless otherwise stated by the manufacturer, silica-based reversed-phase columns are considered to be stable in mobile phases having an apparent pH in the range 2.0 to 8.0. Columns containing porous graphite or particles of polymeric materials such as styrene-divinylbenzene copolymer are stable over a wider pH range.

Analysis using normal-phase LC with unmodified silica or polar chemically modified silica (e.g. cyanopropyl or diol) as the stationary phase, with a non-polar mobile phase is applicable in certain cases.

For analytical separations, the particle size of the most commonly used stationary phases varies between 2 and 10 µm. The particles may be spherical or irregular, and of varying porosity and specific surface area. These properties contribute to the chromatographic behaviour of a particular stationary phase. In the case of reversed phases, the nature of the stationary phase, the extent of bonding, e.g. expressed as the carbon loading, and whether the stationary phase is end-capped (i.e. part of the residual silanol groups are silylated) are additional determining factors. Tailing of peaks, particularly of basic substances, can occur when residual silanol groups are present.

In addition to porous particles, superficially porous or monolithic materials may be used.

Unless otherwise prescribed in the monograph, columns made of stainless steel of varying length and internal diameter (Ø) are used for analytical chromatography. Columns with internal diameters of less than 2 mm are often referred to as microbore columns.

The temperature of the mobile phase and the column must be kept constant during the analysis. A column temperature may be specified in the monograph for optimal performance but most separations are performed at 20-25 °C.

MOBILE PHASES

For normal-phase LC, low-polarity organic solvents are generally employed. The residual water content of the solvents used in the mobile phase is to be strictly controlled to obtain reproducible results.

In reversed-phase LC, aqueous mobile phases, usually with organic solvents and/or modifiers, are employed.

The components of the mobile phase are usually filtered to remove particles greater than 0.45 µm in size (or greater than 0.2 µm when the stationary phase is made of sub-2.0 µm particles, and when special detectors, e.g. light scattering detectors, are used). Multicomponent mobile phases are prepared by measuring the required volumes (unless masses are specified) of the individual components, followed by mixing. Alternatively, the solvents may be delivered by individual pumps controlled by proportioning valves, by which mixing is performed according to the desired proportion. Solvents are normally degassed before pumping by sparging with helium, sonication and/or using in-line membrane/vacuum modules to avoid the creation of gas bubbles in the detector cell.

Solvents for the preparation of the mobile phase are normally free of stabilisers and, if an ultraviolet detector is employed, are transparent at the wavelength of detection. Solvents and other components employed are to be of appropriate quality. In particular, *water for chromatography R* is used for the preparation of mobile phases when water, or an aqueous solution, is 1 of the components. Any necessary adjustments of the pH are made to the aqueous component of the mobile phase and not the mixture. If buffer solutions or saline solutions are used, adequate rinsing of the system is carried out with a mixture of water and a small proportion of the organic part of the mobile phase (5 per cent *V/V*) to prevent crystallisation of salts after completion of the analysis.

Mobile phases may contain other components, for example a counter-ion for ion-pair chromatography or a chiral selector for chiral chromatography using an achiral stationary phase.

DETECTORS

Ultraviolet/visible (UV/Vis) spectrophotometers (including diode array detectors) (*2.2.25*), are the most commonly employed detectors. Fluorescence spectrophotometers, differential refractometers (RI), electrochemical detectors (ECD), light scattering detectors, charged aerosol detectors (CAD), mass spectrometers (MS) (*2.2.43*), radioactivity detectors, multi-angle light scattering (MALS) detectors or other detectors may be used.

PROCEDURE

Equilibrate the column with the prescribed mobile phase and flow rate, at 20-25 °C or at the temperature specified in the monograph, until a stable baseline is achieved. Prepare the solution(s) of the substance to be examined and the reference solution(s) required. The solutions must be free from solid particles.

Criteria for assessing the suitability of the system are described in general chapter *2.2.46. Chromatographic separation techniques*. The extent to which adjustments of parameters of the chromatographic system can be made to satisfy the criteria of system suitability are also given in this chapter.

Additional points for monographs of the British Pharmacopoeia

The composition and flow rate of the mobile phase are stated in the monograph. It is advisable to use as the mobile phase solvent mixtures that have been de-aerated using a vacuum pump or other suitable means of de-aeration that has no effect on the composition of the mixture.

In quantitative work, particularly where the use of an internal standard is not specified in the monograph, the use of a fixed-volume loop injector is recommended. In certain exceptional cases the use of peak heights alone is prescribed in the monograph; where this is the case peak heights should be used irrespective of the symmetry factor.

The column is usually made of stainless steel and its dimensions are stated in the monograph. The dimensions are stated as (length × internal diameter). When the monograph prescribes the use of a stationary phase designated by a letter, the relevant stationary phase defined below is intended.

The nominal diameter of the particles of the stationary phase is stated in parentheses immediately following the designating letter. In most cases reference is made to a particular commercial brand that has been found to be suitable for the purpose, but such statements do not imply that a different but equivalent commercial brand may not be used.
The separation should be carried out at a constant ambient temperature unless otherwise specified in the monograph.
When using mobile phases of high pH with a silica-based column, it is advisable to use a pre-column before the analytical column.

Unless otherwise specified in the monograph the detector consists of a photometric detector fitted with a low-volume flow cell (about 10 µL is suitable); the wavelength setting is specified in the monograph.

The design of a particular chromatograph may require modification of the conditions detailed in the monograph. In such a case the analyst should be satisfied that the modified conditions produce comparable results.

INJECTION VOLUME

Where no injection volume is specified in the monograph, the analyst should select an appropriate volume for their specific application. The volume chosen is dependent on the response of the analyte, the detector used, the efficiency of the column and the overall performance of the chromatographic system. Where a volume is not indicated, 20 µL is usually appropriate; however this should be checked for suitability under the local operating conditions.

RESOLUTION FACTOR

Unless otherwise stated in the monograph, for monographs with a stated *resolution factor* read Resolution (*Rs*) as referred to in Appendix III Chromatographic Separation Techniques.

RUN TIME

Where no run time is specified in the monograph, the analyst should select an appropriate run time for their specific application. The run time chosen is dependent on the type of test. For example, where a run time is not indicated in a Related substances test the analyst should ensure that the run time is greater than all known or likely *secondary peaks;* similarly in an Assay the run time should be chosen to allow the baseline to stabilise following the elution of the peak of interest.

SECONDARY PEAKS

Reference may be made to secondary peaks. A *secondary peak* is a peak in the chromatogram other than the principal peak and any peak due to internal standard, solvent or derivatising agents. Peaks identified as being due to the counter-ion and/or other excipients including preservatives in the material being examined may also be excluded.

MATERIALS

Solvents and reagents used in the preparation of solutions for examination should be of a quality suitable for use in liquid chromatography.

E. Paper Chromatography

(Ph. Eur. method 2.2.26)

ASCENDING PAPER CHROMATOGRAPHY

Apparatus The apparatus consists of a glass tank of suitable size for the chromatographic paper used, ground at the top to take a closely fitting lid. In the top of the tank is a device which suspends the chromatographic paper and is capable of being lowered without opening the chamber.
In the bottom of the tank is a dish to contain the mobile phase into which the paper may be lowered.
The chromatographic paper consists of suitable filter paper, cut into strips of sufficient length and not less than 2.5 cm wide; the paper is cut so that the mobile phase runs in the direction of the grain of the paper.

Method Place in the dish a layer 2.5 cm deep of the mobile phase prescribed in the monograph. If prescribed in the monograph, pour the stationary phase between the walls of the tank and the dish. Close the tank and allow to stand for 24 h at 20 °C to 25 °C. Maintain the tank at this temperature throughout the subsequent procedure. Draw a fine pencil line horizontally across the paper 3 cm from one end. Using a micro pipette, apply to a spot on the pencil line the volume of the solution prescribed in the monograph.
If the total volume to be applied would produce a spot more than 10 mm in diameter, apply the solution in portions allowing each to dry before the next application. When more than one chromatogram is to be run on the same strip of paper, space the solutions along the pencil line at points not less than 3 cm apart. Insert the paper into the tank, close the lid and allow to stand for 1 h 30 min. Lower the paper into the mobile phase and allow elution to proceed for the prescribed distance or time. Remove the paper from the tank and allow to dry in air. Protect the paper from bright light during the elution process.

DESCENDING PAPER CHROMATOGRAPHY

Apparatus The apparatus consists of a glass tank of suitable size for the chromatographic paper used, ground at the top to take a closely fitting glass lid. The lid has a central hole about 1.5 cm in diameter closed by a heavy glass plate or a stopper. In the upper part of the tank is suspended a solvent trough with a device for holding the chromatographic paper. On each side of the trough, parallel to and slightly above its upper edges, are two glass guide rods to support the paper in such a manner that no part of it is in contact with the walls of the tank. The chromatographic paper consists of suitable filter paper, cut into strips of sufficient length, and of any convenient width between 2.5 cm and the length of the trough; the paper is cut so that the mobile phase runs in the direction of the grain of the paper.

Method Place in the bottom of the tank a layer 2.5 cm deep of the solvent prescribed in the monograph, close the tank and allow to stand for 24 h at 20 °C to 25 °C. Maintain the tank at this temperature throughout the subsequent procedure. Draw a fine pencil line horizontally across the paper at such a distance from one end that when this end is secured in the solvent trough and the remainder of the paper is hanging freely over the guide rod, the line is a few centimetres below the guide rod and parallel with it. Using a micro-pipette, apply on the pencil line the volume of the solution prescribed in the monograph. If the total volume to be applied would produce a spot more than 10 mm in diameter, apply the solution in portions, allowing each to dry before the next application. When more than one chromatogram is to be run on the same strip of paper, space the solutions along the pencil line at points not less than 3 cm apart. Insert the paper in the tank, close the lid, and allow to stand for 1 h 30 min. Introduce into the solvent trough, through the hole in the lid, a sufficient quantity of the mobile phase, close the tank and allow elution to proceed for the prescribed distance or time. Remove the paper from the tank and allow to dry in air. The paper should be protected from bright light during the elution process.

F. Electrophoresis[1]

(Ph. Eur. method 2.2.31)

♦1 GENERAL PRINCIPLE

Under the influence of an electrical field, charged particles dissolved or dispersed in an electrolyte solution migrate in the direction of the electrode bearing the opposite polarity. In gel electrophoresis, the movements of the particles are retarded by interactions with the surrounding gel matrix, which acts as a molecular sieve. The opposing interactions of the electrical force and molecular sieving result in differential migration rates according to sizes, shapes and charges of particles. Because of their different physico-chemical properties, different macromolecules of a mixture will migrate at different speeds during electrophoresis and will thus be separated into discrete fractions. Electrophoretic separations can be conducted in systems without support phases (e.g. free solution separation in capillary electrophoresis) and in stabilising media such as thin-layer plates, films or gels.

2 FREE OR MOVING BOUNDARY ELECTROPHORESIS

This method is mainly used for the determination of mobility, the experimental characteristics being directly measurable and reproducible. It is chiefly employed with substances of high relative molecular mass and low diffusibility. The boundaries are initially located by a physical process such as refractometry or conductimetry. After applying a given electric field for an accurately measured time, the new boundaries and their respective positions are observed. The operating conditions must be such as to make it possible to determine as many boundaries as there are components.

3 ZONE ELECTROPHORESIS USING A SUPPORTING MEDIUM

This method requires the use of small samples only.

The nature of the support, such as paper, agar gel, cellulose acetate, starch, agarose, methacrylamide, mixed gel, introduces a number of additional factors modifying the mobility:

a) owing to channelling in the supporting medium, the apparent distance covered is less than the real distance,

b) some supporting media are not electrically neutral. As the medium is a stationary phase it may sometimes give rise to a considerable electro-endosmotic flow,

c) any heating due to the joule effect may cause some evaporation of the liquid from the supporting medium which, by capillarity, causes the solution to move from the ends towards the centre. The ionic strength therefore tends to increase gradually.

The rate of migration then depends on four main factors: the mobility of the charged particle, the electro-endosmotic flow, the evaporation flow, and the field strength. Hence it is necessary to operate under clearly defined experimental conditions and to use, wherever possible, reference substances.

An *apparatus* for electrophoresis consists of:

— a *generator supplying direct current* whose voltage can be controlled and, preferably, stabilised,

— an *electrophoresis chamber*. This is usually rectangular and made of glass or rigid plastic, with 2 separate compartments, the anodic and the cathodic, containing the electrolyte solution. In each compartment is immersed an electrode, for example of platinum or graphite. These are connected by means of an appropriately isolated circuit to the corresponding terminal of the power supply to form the anode and the cathode. The level of the liquid in the 2 compartments is kept equal to prevent siphoning.

The electrophoresis chamber is fitted with an airtight lid which maintains a moisture-saturated atmosphere during operation and reduces evaporation of the solvent. A safety device may be used to cut off the power when the lid is removed. If the electrical power measured across the strip exceeds 10 W, it is preferable to cool the support.

— a *support-carrying device*:

Strip electrophoresis The supporting strip, previously wetted with the same conducting solution and dipped at each end into an electrode compartment is appropriately tightened and fixed on to a suitable carrier designed to prevent diffusion of the conducting electrolyte, such as a horizontal frame, inverted-V stand or a uniform surface with contact points at suitable intervals.

Gel electrophoresis The device consists essentially of a glass plate (for example, a microscope slide) over the whole surface of which is deposited a firmly adhering layer of gel of uniform thickness. The connection between the gel and the conducting solution is effected in various ways according to the type of apparatus used. Precautions must be taken to avoid condensation of moisture or drying of the solid layer.

— measuring *device or means of detection*.

Method Introduce the electrolyte solution into the electrode compartments. Place the support suitably impregnated with electrolyte solution in the chamber under the conditions prescribed for the type of apparatus used. Locate the starting line and apply the sample. Apply the electric current for the prescribed time. After the current has been switched off, remove the support from the chamber, dry and visualise.

4 POLYACRYLAMIDE ROD GEL ELECTROPHORESIS

In polyacrylamide rod gel electrophoresis, the stationary phase is a gel which is prepared from a mixture of acrylamide and *N,N'*-methylenebisacrylamide. Rod gels are prepared in tubes 7.5 cm long and 0.5 cm in internal diameter, one solution being applied to each rod.

Apparatus This consists of 2 buffer solution reservoirs made of suitable material such as poly(methyl methacrylate) and mounted vertically one above the other. Each reservoir is fitted with a platinum electrode. The electrodes are connected to a power supply allowing operation either at constant current or at constant voltage. The apparatus has in the base of the upper reservoir a number of holders equidistant from the electrode.

Method The solutions should usually be degassed before polymerisation and the gels used immediately after preparation. Prepare the gel mixture as prescribed and pour into suitable glass tubes, stoppered at the bottom, to an equal height in each tube and to about 1 cm from the top, taking care to ensure that no air bubbles are trapped in the tubes. Cover the gel mixture with a layer of *water R* to exclude air and allow to set. Gel formation usually takes about 30 min and is complete when a sharp interface appears between the gel and the water layer. Remove the water layer. Fill the lower reservoir with the prescribed buffer solution and remove the stoppers from the tubes. Fit the tubes into the holders of the upper reservoir and adjust so that the bottom

[1] *This chapter has undergone pharmacopoeial harmonisation. See chapter 5.8 Pharmacopoeial harmonisation.*

of the tubes are immersed in the buffer solution in the lower reservoir. Carefully fill the tubes with the prescribed buffer solution. Prepare the test and reference solutions containing the prescribed marker dye and make them dense by dissolving in them *sucrose R*, for example. Apply the solutions to the surface of a gel using a different tube for each solution. Add the same buffer to the upper reservoir. Connect the electrodes to the power supply and allow electrophoresis to proceed at the prescribed temperature and using the prescribed constant voltage or current. Switch off the power supply when the marker dye has migrated almost into the lower reservoir. Immediately remove each tube from the apparatus and extrude the gel. Locate the position of the bands in the electropherogram as prescribed.♦

5 SODIUM DODECYL SULFATE POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

5-1 SDS-PAGE - UNIFORM PERCENTAGE GELS

Scope

Polyacrylamide gel electrophoresis is used for the qualitative characterisation of proteins in biological preparations, for control of purity and for quantitative determinations.

Purpose

Analytical gel electrophoresis is an appropriate method with which to identify and to assess the homogeneity of proteins in pharmaceutical preparations. The method is routinely used for the estimation of protein subunit molecular masses and for determination of the subunit compositions of purified proteins.

Ready-to-use gels and reagents are commercially available and can be used instead of those described in this text, provided that they give equivalent results and that they meet the validity requirements given below under Validation of the test.

5-1-1 Characteristics of polyacrylamide gels

The sieving properties of polyacrylamide gels are established by the three-dimensional network of fibres and pores which is formed as the bifunctional bisacrylamide cross-links adjacent polyacrylamide chains. Polymerisation is usually catalysed by a free radical-generating system composed of ammonium persulfate and *N,N,N′,N′*-tetramethylethylenediamine (TEMED).

As the acrylamide concentration of a gel increases, its effective pore size decreases. The effective pore size of a gel is operationally defined by its sieving properties, i.e. by the resistance it imparts to the migration of macromolecules. There are limits on the acrylamide concentrations that can be used. At high acrylamide concentrations, gels break much more easily and are difficult to handle. As the pore size of a gel decreases, the migration rate of a protein through the gel decreases. By adjusting the pore size of a gel, through manipulating the acrylamide concentration, the resolution of the method can be optimised for a given protein product. Thus, a given gel is physically characterised by its respective composition of acrylamide and bisacrylamide.

In addition to the composition of the gel, the state of the protein is an important component to the electrophoretic mobility. In the case of proteins, the electrophoretic mobility is dependent on the pK value of the charged groups and the size of the molecule. It is influenced by the type, the concentration and the pH of the buffer, by the temperature and the field strength, and by the nature of the support material.

5-1-2 Denaturing polyacrylamide gel electrophoresis

The method cited as an example is limited to the analysis of monomeric polypeptides with a mass range of 14 000 to 100 000 daltons. It is possible to extend this mass range using various techniques (e.g. gradient gels, particular buffer system). For instance, tricine-SDS gels, with tricine as the trailing ion in the electrophoresis running-buffer (instead of glycine as in the method described here), can separate very small proteins and peptides under 10 000 to 15 000 daltons.

Denaturing polyacrylamide gel electrophoresis using glycine-SDS is the most common mode of electrophoresis used in assessing the pharmaceutical quality of protein products and will be the focus of the example method. Typically, analytical electrophoresis of proteins is carried out in polyacrylamide gels under conditions that ensure dissociation of the proteins into their individual polypeptide subunits and that minimise aggregation. Most commonly, the strongly anionic detergent SDS is used in combination with heat to dissociate the proteins before they are loaded on the gel. The denatured polypeptides bind to SDS, become negatively charged and exhibit a consistent charge-to-mass ratio regardless of protein type. Because the amount of SDS bound is almost always proportional to the molecular mass of the polypeptide and is independent of its sequence, SDS-polypeptide complexes migrate through polyacrylamide gels with mobilities dependent on the size of the polypeptide.

The electrophoretic mobilities of the resultant detergent-polypeptide complexes all assume the same functional relationship to their molecular masses. SDS complexes will migrate toward the anode in a predictable manner, with low-molecular-mass complexes migrating faster than larger ones. The molecular mass of a protein can therefore be estimated from its relative mobility in calibrated SDS-PAGE, and the intensity of a single band relative to other undesired bands in such a gel can be a measure of purity.

Modifications to the polypeptide backbone, such as *N*- or *O*-linked glycosylation, can change the apparent molecular mass of a protein since SDS does not bind to a carbohydrate moiety in a manner similar to a polypeptide; therefore, a consistent charge-to-mass ratio is not maintained.

Depending on the extent of glycosylation and other post-translational modifications, the apparent molecular mass of proteins may not be a true reflection of the mass of the polypeptide chain.

Reducing conditions

Polypeptide subunits and three-dimensional structure are often maintained in proteins by the presence of disulfide bonds. A goal of SDS-PAGE analysis under reducing conditions is to disrupt this structure by reducing disulfide bonds. Complete denaturation and dissociation of proteins by treatment with 2-mercaptoethanol (2-ME) or dithiothreitol (DTT) will result in unfolding of the polypeptide backbone and subsequent complexation with SDS. Using these conditions, the molecular mass of the polypeptide subunits can reasonably be calculated by linear regression (or, more accurately, by non-linear regression) with the aid of suitable molecular-mass standards.

Non-reducing conditions

For some analyses, complete dissociation of the protein into subunit peptides is not desirable. In the absence of treatment with reducing agents such as 2-ME or DTT, disulfide covalent bonds remain intact, preserving the oligomeric form of the protein. Oligomeric SDS-protein complexes migrate more slowly than their SDS-polypeptide subunits.
In addition, non-reduced proteins may not be completely

saturated with SDS and, hence, may not bind the detergent in a constant mass ratio. Moreover, intra-chain disulfide bonds constrain the molecular shape, usually in such a way as to reduce the Stokes radius of the molecule, thereby reducing the apparent molecular mass M_r. This makes molecular-mass determinations of these molecules by SDS-PAGE less straightforward than analyses of fully denatured polypeptides, since it is necessary that both standards and unknown proteins be in similar configurations for valid comparisons.

5-1-3 Characteristics of discontinuous buffer system gel electrophoresis

The most popular electrophoretic method for the characterisation of complex mixtures of proteins uses a discontinuous buffer system involving 2 contiguous, but distinct gels: a resolving or separating (lower) gel and a stacking (upper) gel. The 2 gels are cast with different porosities, pH, and ionic strengths. In addition, different mobile ions are used in the gel and electrode buffers.

The buffer discontinuity acts to concentrate large volume samples in the stacking gel, resulting in improved resolution. When power is applied, a voltage drop develops across the sample solution which drives the proteins into the stacking gel. Glycinate ions from the electrode buffer follow the proteins into the stacking gel. A moving boundary region is rapidly formed with the highly mobile chloride ions in the front and the relatively slow glycinate ions in the rear.

A localised high-voltage gradient forms between the leading and trailing ion fronts, causing the SDS-protein complexes to form into a thin zone (stack) and migrate between the chloride and glycinate phases. Within broad limits, regardless of the height of the applied sample, all SDS-proteins condense into a very narrow region and enter the resolving gel as a well-defined, thin zone of high protein density.

The large-pore stacking gel does not retard the migration of most proteins and serves mainly as an anti-convective medium. At the interface of the stacking and resolving gels, the proteins undergo a sharp increase in retardation due to the restrictive pore size of the resolving gel and the buffer discontinuity, which also contributes to the focusing of the proteins. Once in the resolving gel, proteins continue to be slowed by the sieving of the matrix. The glycinate ions overtake the proteins, which then move in a space of uniform pH formed by the tris(hydroxymethyl)aminomethane and glycine. Molecular sieving causes the SDS-polypeptide complexes to separate on the basis of their molecular masses.

5-1-4 Preparing vertical discontinuous buffer SDS polyacrylamide gels

This section describes the preparation of gels using particular instrumentation. This does not apply to pre-cast gels.

For pre-cast gels or any other commercially available equipment, the manufacturer's instructions must be used for guidance.

The use of commercial reagents that have been purified in solution is recommended. When this is not the case and where the purity of the reagents used is not sufficient, a pre-treatment is applied. For instance, any solution sufficiently impure to require filtration must also be deionised with a mixed-bed (anion/cation exchange) resin to remove acrylic acid and other charged degradation products. When stored according to recommendations, acrylamide/bisacrylamide solutions and solid persulfate are stable for long periods.

Assembling the gel moulding cassette

Clean the 2 glass plates (e.g. 10 cm by 8 cm in size), the polytetrafluoroethylene comb, the 2 spacers and the silicone rubber tubing (e.g. 0.6 mm in diameter by 35 cm) with mild detergent and rinse extensively with water, followed by anhydrous ethanol, and allow the plates to dry at room temperature. Lubricate the spacers and the tubing with non-silicone grease. Apply the spacers along each of the 2 short sides of the glass plate 2 mm away from the edges and 2 mm away from the long side corresponding to the bottom of the gel. Begin to lay the tubing on the glass plate by using one spacer as a guide. Carefully twist the tubing at the bottom of the spacer and follow the long side of the glass plate. While holding the tubing with 1 finger along the long side, twist again the tubing and lay it on the second short side of the glass plate, using the spacer as a guide. Place the second glass plate in perfect alignment and hold the mould together by hand pressure. Apply 2 clamps on each of the 2 short sides of the mould. Carefully apply four clamps on the longer side of the gel mould thus forming the bottom of the gel mould. Verify that the tubing runs along the edge of the glass plates and has not been extruded while placing the clamps. The gel mould is now ready for pouring the gel.

Preparation of the gel

In a discontinuous buffer SDS polyacrylamide gel, it is recommended to pour the resolving gel, let the gel set, and then pour the stacking gel since the composition of the 2 gels in acrylamide-bisacrylamide, buffer and pH are different.

Preparation of the resolving gel In a conical flask, prepare the appropriate volume of solution containing the desired concentration of acrylamide for the resolving gel, using the values given in Table 2.2.31.-1. Mix the components in the order shown. Where appropriate, before adding the ammonium persulfate solution and the TEMED, filter the solution if necessary under vacuum through a cellulose acetate membrane (pore diameter 0.45 µm). Keep the solution under vacuum, while swirling the filtration unit, until no more bubbles are formed in the solution.

Add appropriate amounts of ammonium persulfate solution and TEMED as indicated in Table 2.2.31.-1, swirl and pour immediately into the gap between the 2 glass plates of the mould. Leave sufficient space for the stacking gel (the length of the teeth of the comb plus 1 cm). Using a tapered glass pipette, carefully overlay the solution with water-saturated isobutanol. Leave the gel in a vertical position at room temperature to allow polymerisation.

Preparation of the stacking gel After polymerisation is complete (about 30 min), pour off the isobutanol and wash the top of the gel several times with water to remove the isobutanol overlay and any unpolymerised acrylamide. Drain as much fluid as possible from the top of the gel, and then remove any remaining water with the edge of a paper towel.

In a conical flask, prepare the appropriate volume of solution containing the desired concentration of acrylamide, using the values given in Table 2.2.31.-2. Mix the components in the order shown. Where appropriate, before adding the ammonium persulfate solution and the TEMED, filter the solution if necessary under vacuum through a cellulose acetate membrane (pore diameter 0.45 µm). Keep the solution under vacuum, while swirling the filtration unit, until no more bubbles are formed in the solution.

Add appropriate amounts of ammonium persulfate solution and TEMED as indicated in Table 2.2.31.-2. Swirl and pour immediately into the gap between the 2 glass plates of the mould directly onto the surface of the polymerised resolving gel. Immediately insert a clean polytetrafluoroethylene comb into the stacking gel solution, being careful to avoid trapping air bubbles. Add more stacking gel solution to fill the spaces

Table 2.2.31.-1. – *Preparation of resolving gel*

Solution components	Component volumes (mL) per gel mould volume of							
	5 mL	10 mL	15 mL	20 mL	25 mL	30 mL	40 mL	50 mL
6 per cent acrylamide								
Water R	2.6	5.3	7.9	10.6	13.2	15.9	21.2	26.5
Acrylamide solution(1)	1.0	2.0	3.0	4.0	5.0	6.0	8.0	10.0
1.5 M Tris (pH 8.8)(2)	1.3	2.5	3.8	5.0	6.3	7.5	10.0	12.5
100 g/L SDS(3)	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
100 g/L APS(4)	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
TEMED(5)	0.004	0.008	0.012	0.016	0.02	0.024	0.032	0.04
8 per cent acrylamide								
Water R	2.3	4.6	6.9	9.3	11.5	13.9	18.5	23.2
Acrylamide solution(1)	1.3	2.7	4.0	5.3	6.7	8.0	10.7	13.3
1.5 M Tris (pH 8.8)(2)	1.3	2.5	3.8	5.0	6.3	7.5	10.0	12.5
100 g/L SDS(3)	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
100 g/L APS(4)	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
TEMED(5)	0.003	0.006	0.009	0.012	0.015	0.018	0.024	0.03
10 per cent acrylamide								
Water R	1.9	4.0	5.9	7.9	9.9	11.9	15.9	19.8
Acrylamide solution(1)	1.7	3.3	5.0	6.7	8.3	10.0	13.3	16.7
1.5 M Tris (pH 8.8)(2)	1.3	2.5	3.8	5.0	6.3	7.5	10.0	12.5
100 g/L SDS(3)	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
100 g/L APS(4)	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
TEMED(5)	0.002	0.004	0.006	0.008	0.01	0.012	0.016	0.02
12 per cent acrylamide								
Water R	1.6	3.3	4.9	6.6	8.2	9.9	13.2	16.5
Acrylamide solution(1)	2.0	4.0	6.0	8.0	10.0	12.0	16.0	20.0
1.5 M Tris (pH 8.8)(2)	1.3	2.5	3.8	5.0	6.3	7.5	10.0	12.5
100 g/L SDS(3)	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
100 g/L APS(4)	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
TEMED(5)	0.002	0.004	0.006	0.008	0.01	0.012	0.016	0.02
14 per cent acrylamide								
Water R	1.4	2.7	3.9	5.3	6.6	8.0	10.6	13.8
Acrylamide solution(1)	2.3	4.6	7.0	9.3	11.6	13.9	18.6	23.2
1.5 M Tris (pH 8.8)(2)	1.2	2.5	3.6	5.0	6.3	7.5	10.0	12.5
100 g/L SDS(3)	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
100 g/L APS(4)	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
TEMED(5)	0.002	0.004	0.006	0.008	0.01	0.012	0.016	0.02
15 per cent acrylamide								
Water R	1.1	2.3	3.4	4.6	5.7	6.9	9.2	11.5
Acrylamide solution(1)	2.5	5.0	7.5	10.0	12.5	15.0	20.0	25.0
1.5 M Tris (pH 8.8)(2)	1.3	2.5	3.8	5.0	6.3	7.5	10.0	12.5
100 g/L SDS(3)	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
100 g/L APS(4)	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
TEMED(5)	0.002	0.004	0.006	0.008	0.01	0.012	0.016	0.02

(1) Acrylamide solution: *30 per cent acrylamide/bisacrylamide (29:1) solution R.*
(2) 1.5 M Tris (pH 8.8): *1.5 M tris-hydrochloride buffer solution pH 8.8 R.*
(3) 100 g/L SDS: a 100 g/L solution of *sodium dodecyl sulfate R.*
(4) 100 g/L APS: a 100 g/L solution of *ammonium persulfate R.* Ammonium persulfate provides the free radicals that drive polymerisation of acrylamide and bisacrylamide. Since ammonium persulfate solution decomposes rapidly, fresh solutions must be prepared daily.
(5) TEMED: *tetramethylethylenediamine R.*

Table 2.2.31.-2. – *Preparation of stacking gel*

Solution components	Component volumes (mL) per gel mould volume of							
	1 mL	2 mL	3 mL	4 mL	5 mL	6 mL	8 mL	10 mL
Water R	0.68	1.4	2.1	2.7	3.4	4.1	5.5	6.8
Acrylamide solution(1)	0.17	0.33	0.5	0.67	0.83	1.0	1.3	1.7
1.0 M Tris (pH 6.8)(2)	0.13	0.25	0.38	0.5	0.63	0.75	1.0	1.25
100 g/L SDS(3)	0.01	0.02	0.03	0.04	0.05	0.06	0.08	0.1
100 g/L APS(4)	0.01	0.02	0.03	0.04	0.05	0.06	0.08	0.1
TEMED(5)	0.001	0.002	0.003	0.004	0.005	0.006	0.008	0.01

(1) Acrylamide solution: *30 per cent acrylamide/bisacrylamide (29:1) solution R.*
(2) 1.0 M Tris (pH 6.8): *1 M tris-hydrochloride buffer solution pH 6.8 R.*
(3) 100 g/L SDS: a 100 g/L solution of *sodium dodecyl sulfate R.*
(4) 100 g/L APS: a 100 g/L solution of *ammonium persulfate R.* Ammonium persulfate provides the free radicals that drive polymerisation of acrylamide and bisacrylamide. Since ammonium persulfate solution decomposes rapidly, fresh solutions must be prepared daily.
(5) TEMED: *tetramethylethylenediamine R.*

of the comb completely. Leave the gel in a vertical position and allow to polymerise at room temperature.

Preparation of the samples

Unless otherwise stated in the specific monograph, the samples can be prepared as follows:

Preparation of the samples (non-reducing conditions) Mix equal volumes of a mixture comprising *water R* plus the preparation to be examined or the reference preparation, and *concentrated SDS-PAGE sample buffer R.*

Preparation of the samples (reducing conditions) Mix equal volumes of a mixture comprising *water R* plus the preparation to be examined or the reference preparation, and *concentrated SDS-PAGE sample buffer for reducing conditions R* containing 2-ME (or DTT) as the reducing agent.

The concentration prescribed in the monograph can vary depending on the protein and staining method.

Sample treatment: keep for 5 min in a water-bath or in a block heater set at 100 °C, then cool. The temperature and time in the monograph may vary as protein cleavage may occur during the heat treatment.

Mounting the gel in the electrophoresis apparatus and electrophoretic separation

After polymerisation is complete (about 30 min), remove the polytetrafluoroethylene comb carefully. Rinse the wells immediately with water or with the *SDS-PAGE running buffer R* to remove any unpolymerised acrylamide.

If necessary, straighten the teeth of the stacking gel with a blunt hypodermic needle attached to a syringe. Remove the clamps on one short side, carefully pull out the tubing and replace the clamps. Proceed similarly on the other short side. Remove the tubing from the bottom part of the gel. Mount the gel in the electrophoresis apparatus. Add the electrophoresis buffers to the top and bottom reservoirs. Remove any bubbles that become trapped at the bottom of the gel between the glass plates. This is best done with a bent hypodermic needle attached to a syringe. Never pre-run the gel before loading the samples, since this will destroy the discontinuity of the buffer systems. Before loading the sample carefully rinse each well with *SDS-PAGE running buffer R.* Prepare the test and reference solutions in the recommended sample buffer and treat as specified in the individual monograph. Apply the appropriate volume of each solution to the stacking gel wells.

Start the electrophoresis using the conditions recommended by the manufacturer of the equipment. Manufacturers of SDS-PAGE equipment may provide gels of different surface area and thickness and electrophoresis running time and current/voltage may vary in order to achieve optimal separation. Check that the dye front is moving into the resolving gel. When the dye is near the bottom of the gel, stop the electrophoresis. Remove the gel assembly from the apparatus and carefully separate the glass plates. Remove the spacers, cut off and discard the stacking gel and immediately proceed with staining.

5-2 SDS-PAGE - GRADIENT CONCENTRATION GELS

Gradient gels (resolving gels) are prepared with an increasing concentration of acrylamide from the top to the bottom. Preparation of gradient gels requires a gradient-forming apparatus. Ready-to-use gradient gels are commercially available with specific recommended protocols.

Gradient gels offer some advantages, as some proteins which co-migrate on fixed-concentration gels can be resolved within gradient gels. During electrophoresis, the proteins migrate until the pore size prevents further progress and a stacking effect therefore occurs, resulting in sharper bands. As shown in Table 2.2.31.-3, gradient gels also allow separation of proteins with a wider range of molecular masses compared to a single fixed concentration gel.

The table gives suggested compositions of the linear gradient, relating the range of acrylamide concentrations to the appropriate protein molecular mass ranges. Note that other gradient shapes (e.g. concave) can be prepared for specific applications.

Table 2.2.31.-3

Acrylamide (per cent)	Protein range (kDa)
5 - 15	20 - 250
5 - 20	10 - 200
10 - 20	10 - 150
8 - 20	8 - 150

Gradient gels are also used for the determination of molecular mass and protein purity.

5-3 DETECTION OF PROTEINS IN GELS

Coomassie and silver staining are the most common protein staining methods and are described in more detail below. Several other commercial stains, detection methods and commercial kits are available. For example, fluorescent stains

are visualised using a fluorescent imager and often provide a linear response over a wide range of protein concentrations, often several orders of magnitude depending on the protein.

Coomassie staining has a protein detection level of approximately 1 µg to 10 µg of protein per band. Silver staining is the most sensitive method for staining proteins in gels and a band containing 10 ng to 100 ng can be detected. These figures are considered robust in the context of these gels. Improved sensitivity of 1 or 2 orders of magnitude has sometimes been reported in the literature.

Coomassie staining responds in a more linear manner than silver staining; however, the response and range depend on the protein and development time. Both Coomassie and silver staining can be less reproducible if staining is stopped in a subjective manner, i.e. the point at which the staining is deemed satisfactory. The use of dynamic ranges of reference proteins is very important as they help assess the intra-experimental sensitivity and linearity. All gel staining steps are carried out while wearing gloves, at room temperature, with gentle shaking (e.g. on an orbital shaker platform) and using any convenient container.

Coomassie staining

Immerse the gel in a large excess of *Coomassie staining solution R* and allow to stand for at least 1 h. Remove the staining solution.

Destain the gel with a large excess of *destaining solution R*. Change the destaining solution several times, until the stained protein bands are clearly distinguishable on a clear background. The more thoroughly the gel is destained, the smaller is the amount of protein that can be detected by the method. Destaining can be speeded up by including a few grams of anion-exchange resin or a small sponge in the *destaining solution R*.

NOTE: the acid-alcohol solutions used in this procedure do not completely fix proteins in the gel. This can lead to losses of some low-molecular-mass proteins during the staining and destaining of thin gels. Permanent fixation is obtainable by allowing the gel to stand in a mixture of 1 volume of trichloroacetic acid R, 4 volumes of methanol R and 5 volumes of water R for 1 h before it is immersed in the Coomassie staining solution R.

Silver staining

Immerse the gel in a large excess of *fixing solution R* and allow to stand for 1 h. Remove the fixing solution, add fresh fixing solution and incubate either for at least 1 h or overnight, if convenient. Discard the fixing solution and wash the gel in a large excess of *water R* for 1 h. Soak the gel for 15 min in a 1 per cent *V/V* solution of *glutaraldehyde R*. Wash the gel twice for 15 min in a large excess of *water R*. Soak the gel in fresh *silver nitrate reagent R* for 15 min, in darkness. Wash the gel three times for 5 min in a large excess of *water R*. Immerse the gel for about 1 min in *developer solution R* until satisfactory staining has been obtained. Stop the development by incubation in the *blocking solution R* for 15 min. Rinse the gel with *water R*.

5-4 RECORDING OF THE RESULTS

Gels are photographed or scanned while they are still wet or after an appropriate drying procedure. Currently, gel scanning systems with data analysis software are commercially available to immediately photograph and analyse the wet gel.

Depending on the staining method used, gels are treated in a slightly different way. For Coomassie staining, after the destaining step, allow the gel to stand in a 100 g/L solution of *glycerol R* for at least 2 h (overnight incubation is possible).

For silver staining, add to the final rinsing a step of 5 min in a 20 g/L solution of *glycerol R*.

Drying of stained SDS polyacrylamide gels is one of the methods used to obtain permanent documentation. This method frequently results in the cracking of gel during drying between cellulose films.

Immerse 2 sheets of porous cellulose film in *water R* and incubate for 5 min to 10 min. Place one of the sheets on a drying frame. Carefully lift the gel and place it on the cellulose film. Remove any trapped air bubbles and pour a few millilitres of *water R* around the edges of the gel. Place the second sheet on top and remove any trapped air bubbles. Complete the assembly of the drying frame. Place in an oven or leave at room temperature until dry.

5-5 MOLECULAR-MASS DETERMINATION

Molecular masses of proteins are determined by comparison of their mobilities with those of several marker proteins of known molecular weight. Mixtures of pre-stained and unstained proteins with precisely known molecular masses blended for uniform staining are available for calibrating gels. They are available in various molecular mass ranges. Concentrated stock solutions of proteins of known molecular mass are diluted in the appropriate sample buffer and loaded on the same gel as the protein sample to be examined.

Immediately after the gel has been run, the position of the bromophenol blue tracking dye is marked to identify the leading edge of the electrophoretic ion front. This can be done by cutting notches in the edges of the gel or by inserting a needle soaked in India ink into the gel at the dye front. After staining, measure the migration distances of each protein band (markers and unknowns) from the top of the resolving gel. Divide the migration distance of each protein by the distance travelled by the tracking dye. The normalised migration distances are referred to as the relative mobilities of the proteins (relative to the dye front), or R_F. Construct a plot of the logarithm of the relative molecular masses (M_r) of the protein standards as a function of the R_F values. Unknown molecular masses can be estimated by linear regression analysis (or more accurately by non-linear regression analysis) or interpolation from the curves of log M_r against R_F if the values obtained for the unknown samples are positioned along the approximately linear part of the graph.

5-6 VALIDATION OF THE TEST

The test is not valid unless the target resolution range of the gel has been demonstrated by the distribution of appropriate molecular mass markers, e.g. across 80 per cent of the length of the gel. The separation obtained for the expected proteins must show a linear relationship between the logarithm of the molecular mass and the R_F. If the plot has a sigmoidal shape, then only data from the linear region of the curve can be used in the calculations. Additional validation requirements with respect to the test sample may be specified in individual monographs.

Sensitivity must also be validated. A reference protein control corresponding to the desired concentration limit that is run in parallel with the test samples can serve to establish system suitability.

5-7 QUANTIFICATION OF IMPURITIES

SDS-PAGE is often used as a limit test for impurities. When impurities are quantified by normalisation to the main band using an integrating densitometer or image analysis, the responses must be validated for linearity. Note that depending on the detection method and protein as described in the introduction of section 5-3, the linear range can vary

but can be assessed within each run by using one or more control samples containing an appropriate range of protein concentration.

Where the impurity limit is specified in the individual monograph, a reference solution corresponding to that level of impurity should be prepared by diluting the test solution. For example, where the limit is 5 per cent, a reference solution would be a 1:20 dilution of the test solution.

No impurity (any band other than the main band) in the electropherogram obtained with the test solution may be more intense than the main band obtained with the reference solution.

Under validated conditions, impurities may be quantified by normalisation to the main band using an integrating densitometer or by image analysis.

G. Capillary Electrophoresis[1]

(Ph. Eur. method 2.2.47)

GENERAL PRINCIPLES

Capillary electrophoresis is a physical method of analysis based on the migration, inside a capillary, of charged analytes dissolved in an electrolyte solution, under the influence of a direct-current electric field.

The migration velocity of an analyte under an electric field of intensity E, is determined by the electrophoretic mobility of the analyte and the electro-osmotic mobility of the buffer inside the capillary. The electrophoretic mobility of a solute (μ_{ep}) depends on the characteristics of the solute (electric charge, molecular size and shape) and those of the buffer in which the migration takes place (type and ionic strength of the electrolyte, pH, viscosity and additives).

The electrophoretic velocity (v_{ep}) of a solute, assuming a spherical shape, is given by the equation:

$$v_{ep} = \mu_{ep} \times E = \left(\frac{q}{6\pi\eta r}\right) \times \left(\frac{V}{L}\right)$$

q = effective charge of the solute,
η = viscosity of the electrolyte solution,
r = Stoke's radius of the solute,
V = applied voltage,
L = total length of the capillary.

When an electric field is applied through the capillary filled with buffer, a flow of solvent is generated inside the capillary, called electro-osmotic flow. The velocity of the electro-osmotic flow depends on the electro-osmotic mobility (μ_{eo}) which in turn depends on the charge density on the capillary internal wall and the buffer characteristics. The electro-osmotic velocity (v_{eo}) is given by the equation:

$$v_{eo} = \mu_{eo} \times E = \left(\frac{\varepsilon\zeta}{\eta}\right) \times \left(\frac{V}{L}\right)$$

ε = dielectric constant of the buffer,
ζ = zeta potential of the capillary surface.

The velocity of the solute (v) is given by:

$$v = v_{ep} + v_{eo}$$

The electrophoretic mobility of the analyte and the electro-osmotic mobility may act in the same direction or in opposite directions, depending on the charge of the solute. In normal capillary electrophoresis, anions will migrate in the opposite direction to the electro-osmotic flow and their velocities will be smaller than the electro-osmotic velocity. Cations will migrate in the same direction as the electro-osmotic flow and their velocities will be greater than the electro-osmotic velocity. Under conditions in which there is a fast electro-osmotic velocity with respect to the electrophoretic velocity of the solutes, both cations and anions can be separated in the same run.

The time (t) taken by the solute to migrate the distance (l) from the injection end of the capillary to the detection point (capillary effective length) is given by the expression:

$$t = \frac{l}{v_{ep} + v_{eo}} = \frac{l \times L}{(\mu_{ep} + \mu_{eo}) \times V}$$

In general, uncoated fused-silica capillaries above pH 3 have negative charge due to ionised silanol groups in the inner wall. Consequently, the electro-osmotic flow is from anode to cathode. The electro-osmotic flow must remain constant from run to run if good reproducibility is to be obtained in the migration velocity of the solutes. For some applications, it may be necessary to reduce or suppress the electro-osmotic flow by modifying the inner wall of the capillary or by changing the concentration, composition and/or pH of the buffer solution.

After the introduction of the sample into the capillary, each analyte ion of the sample migrates within the background electrolyte as an independent zone, according to its electrophoretic mobility. Zone dispersion, that is the spreading of each solute band, results from different phenomena. Under ideal conditions the sole contribution to the solute-zone broadening is molecular diffusion of the solute along the capillary (longitudinal diffusion). In this ideal case the efficiency of the zone, expressed as the number of theoretical plates (N), is given by:

$$N = \frac{(\mu_{ep} + \mu_{eo}) \times V \times l}{2 \times D \times L}$$

D = molecular diffusion coefficient of the solute in the buffer.

In practice, other phenomena such as heat dissipation, sample adsorption onto the capillary wall, mismatched conductivity between sample and buffer, length of the injection plug, detector cell size and unlevelled buffer reservoirs can also significantly contribute to band dispersion.

Separation between 2 bands (expressed as the resolution, R_s) can be obtained by modifying the electrophoretic mobility of the analytes, the electro-osmotic mobility induced in the capillary and by increasing the efficiency for the band of each analyte, according to the equation:

$$R_s = \frac{\sqrt{N}(\mu_{epb} - \mu_{epa})}{4(\overline{\mu}_{ep} + \mu_{eo})}$$

μ_{epa} and μ_{epb} = electrophoretic mobilities of the 2 analytes separated,

μ_{ep} = mean electrophoretic mobility of the 2 analytes $\overline{\mu}_{ep} = {}^1/_2 (\mu_{epb} + \mu_{epa})$.

APPARATUS

An apparatus for capillary electrophoresis is composed of:
— a high-voltage, controllable direct-current power supply;

[1] *This chapter has undergone pharmacopoeial harmonisation. See chapter 5.8 Pharmacopoeial harmonisation.*

- 2 buffer reservoirs, held at the same level, containing the prescribed anodic and cathodic solutions;
- 2 electrode assemblies (the cathode and the anode), immersed in the buffer reservoirs and connected to the power supply;
- a separation capillary (usually made of fused-silica) which, when used with some specific types of detectors, has an optical viewing window aligned with the detector. The ends of the capillary are placed in the buffer reservoirs. The capillary is filled with the solution prescribed in the monograph;
- a suitable injection system;
- a detector able to monitor the amount of substances of interest passing through a segment of the separation capillary at a given time; it is usually based on absorption spectrophotometry (UV and visible) or fluorimetry, but conductimetric, amperometric or mass spectrometric detection can be useful for specific applications; indirect detection is an alternative method used to detect non-UV-absorbing and non-fluorescent compounds;
- a thermostatic system able to maintain a constant temperature inside the capillary is recommended to obtain a good separation reproducibility;
- a recorder and a suitable integrator or a computer.

The definition of the injection process and its automation are critical for precise quantitative analysis. Modes of injection include gravity, pressure or vacuum injection and electrokinetic injection. The amount of each sample component introduced electrokinetically depends on its electrophoretic mobility, leading to possible discrimination using this injection mode.

Use the capillary, the buffer solutions, the preconditioning method, the sample solution and the migration conditions prescribed in the monograph of the considered substance. The employed electrolytic solution is filtered to remove particles and degassed to avoid bubble formation that could interfere with the detection system or interrupt the electrical contact in the capillary during the separation run. A rigorous rinsing procedure should be developed for each analytical method to achieve reproducible migration times of the solutes.

CAPILLARY ZONE ELECTROPHORESIS

PRINCIPLE

In capillary zone electrophoresis, analytes are separated in a capillary containing only buffer without any anticonvective medium. With this technique, separation takes place because the different components of the sample migrate as discrete bands with different velocities. The velocity of each band depends on the electrophoretic mobility of the solute and the electro-osmotic flow in the capillary (see General Principles). Coated capillaries can be used to increase the separation capacity of those substances adsorbing on fused-silica surfaces.

Using this mode of capillary electrophoresis, the analysis of both small (M_r < 2000) and large molecules (2000 < M_r < 100 000) can be accomplished. Due to the high efficiency achieved in capillary zone electrophoresis, separation of molecules having only minute differences in their charge-to-mass ratio can be effected. This separation mode also allows the separation of chiral compounds by addition of chiral selectors to the separation buffer.

OPTIMISATION

Optimisation of the separation is a complex process where several separation parameters can play a major role. The main factors to be considered in the development of separations are instrumental and electrolytic solution parameters.

Instrumental parameters

Voltage A Joule heating plot is useful in optimising the applied voltage and capillary temperature. Separation time is inversely proportional to applied voltage. However, an increase in the voltage used can cause excessive heat production, giving rise to temperature and, as a result thereof, viscosity gradients in the buffer inside the capillary. This effect causes band broadening and decreases resolution.

Polarity Electrode polarity can be normal (anode at the inlet and cathode at the outlet) and the electro-osmotic flow will move toward the cathode. If the electrode polarity is reversed, the electro-osmotic flow is away from the outlet and only charged analytes with electrophoretic mobilities greater than the electro-osmotic flow will pass to the outlet.

Temperature The main effect of temperature is observed on buffer viscosity and electrical conductivity, and therefore on migration velocity. In some cases, an increase in capillary temperature can cause a conformational change in proteins, modifying their migration time and the efficiency of the separation.

Capillary The dimensions of the capillary (length and internal diameter) contribute to analysis time, efficiency of separations and load capacity. Increasing both effective length and total length can decrease the electric fields (working at constant voltage) which increases migration time. For a given buffer and electric field, heat dissipation, and hence sample band-broadening, depend on the internal diameter of the capillary. The latter also affects the detection limit, depending on the sample volume injected and the detection system employed.

Since the adsorption of the sample components on the capillary wall limits efficiency, methods to avoid these interactions should be considered in the development of a separation method. In the specific case of proteins, several strategies have been devised to avoid adsorption on the capillary wall. Some of these strategies (use of extreme pH and adsorption of positively charged buffer additives) only require modification of the buffer composition to prevent protein adsorption. In other strategies, the internal wall of the capillary is coated with a polymer, covalently bonded to the silica, that prevents interaction between the proteins and the negatively charged silica surface. For this purpose, ready-to-use capillaries with coatings consisting of neutral-hydrophilic, cationic and anionic polymers are available.

Electrolytic solution parameters

Buffer type and concentration Suitable buffers for capillary electrophoresis have an appropriate buffer capacity in the pH range of choice and low mobility to minimise current generation.

Matching buffer-ion mobility to solute mobility, whenever possible, is important for minimising band distortion. The type of sample solvent used is also important to achieve on-column sample focusing, which increases separation efficiency and improves detection.

An increase in buffer concentration (for a given pH) decreases electro-osmotic flow and solute velocity.

Buffer pH The pH of the buffer can affect separation by modifying the charge of the analyte or additives, and by changing the electro-osmotic flow. In protein and peptide separation, changing the pH of the buffer from above to below the isoelectric point (pI) changes the net charge of the

solute from negative to positive. An increase in the buffer pH generally increases the electro-osmotic flow.

Organic solvents Organic modifiers (methanol, acetonitrile, etc.) may be added to the aqueous buffer to increase the solubility of the solute or other additives and/or to affect the degree of ionisation of the sample components. The addition of these organic modifiers to the buffer generally causes a decrease in the electro-osmotic flow.

Additives for chiral separations For the separation of enantiomers, a chiral selector is added to the separation buffer. The most commonly used chiral selectors are cyclodextrins, but crown ethers, polysaccharides and proteins may also be used. Since chiral recognition is governed by the different interactions between the chiral selector and each of the enantiomers, the resolution achieved for the chiral compounds depends largely on the type of chiral selector used. In this regard, for the development of a given separation it may be useful to test cyclodextrins having a different cavity size (α-, β-, or γ-cyclodextrin) or modified cyclodextrins with neutral (methyl, ethyl, hydroxyalkyl, etc.) or ionisable (aminomethyl, carboxymethyl, sulfobutyl ether, etc.) groups. When using modified cyclodextrins, batch-to-batch variations in the degree of substitution of the cyclodextrins must be taken into account since it will influence the selectivity. Other factors controlling the resolution in chiral separations are concentration of chiral selector, composition and pH of the buffer and temperature. The use of organic additives, such as methanol or urea can also modify the resolution achieved.

CAPILLARY GEL ELECTROPHORESIS

PRINCIPLE

In capillary gel electrophoresis, separation takes place inside a capillary filled with a gel that acts as a molecular sieve. Molecules with similar charge-to-mass ratios are separated according to molecular size since smaller molecules move more freely through the network of the gel and therefore migrate faster than larger molecules. Different biological macromolecules (for example, proteins and DNA fragments), which often have similar charge-to-mass ratios, can thus be separated according to their molecular mass by capillary gel electrophoresis.

CHARACTERISTICS OF GELS

2 types of gels are used in capillary electrophoresis: permanently coated gels and dynamically coated gels.

Permanently coated gels, such as cross-linked polyacrylamide, are prepared inside the capillary by polymerisation of the monomers. They are usually bonded to the fused-silica wall and cannot be removed without destroying the capillary.

If the gels are used for protein analysis under reducing conditions, the separation buffer usually contains sodium dodecyl sulfate and the samples are denatured by heating in a mixture of sodium dodecyl sulfate and 2-mercaptoethanol or dithiothreitol before injection. When non-reducing conditions are used (for example, analysis of an intact antibody), 2-mercaptoethanol and dithiothreitol are not used.

Separation in cross-linked gels can be optimised by modifying the separation buffer (as indicated in the capillary zone electrophoresis section) and controlling the gel porosity during the gel preparation. For cross-linked polyacrylamide gels, the porosity can be modified by changing the concentration of acrylamide and/or the proportion of cross-linker. As a rule, a decrease in the porosity of the gel leads to a decrease in the mobility of the solutes. Due to the rigidity of these gels, only electrokinetic injection can be used.

Dynamically coated gels are hydrophilic polymers, such as linear polyacrylamide, cellulose derivatives, dextran, etc., which can be dissolved in aqueous separation buffers giving rise to a separation medium that also acts as a molecular sieve. These separation media are easier to prepare than cross-linked polymers. They can be prepared in a vial and filled by pressure in a wall-coated capillary (with no electro-osmotic flow). Replacing the gel before every injection generally improves the separation reproducibility.

The porosity of the gels can be increased by using polymers of higher molecular mass (at a given polymer concentration) or by decreasing the polymer concentration (for a given polymer molecular mass). A reduction in the gel porosity leads to a decrease in the mobility of the solute for the same buffer. Since the dissolution of these polymers in the buffer gives low viscosity solutions, both hydrodynamic and electrokinetic injection techniques can be used.

CAPILLARY ISOELECTRIC FOCUSING

PRINCIPLE

In isoelectric focusing, the molecules migrate under the influence of the electric field, so long as they are charged, in a pH gradient generated by ampholytes having pI values in a wide range (poly-aminocarboxylic acids), dissolved in the separation buffer.

The three basic steps of isoelectric focusing are loading, focusing and mobilisation.

Loading step

Two methods may be employed:

— loading in one step: the sample is mixed with ampholytes and introduced into the capillary either by pressure or vacuum;

— sequential loading: a leading buffer, then the ampholytes, then the sample mixed with ampholytes, again ampholytes alone and finally the terminating buffer are introduced into the capillary. The volume of the sample must be small enough not to modify the pH gradient.

Focusing step

When the voltage is applied, ampholytes migrate toward the cathode or the anode, according to their net charge, thus creating a pH gradient from anode (lower pH) to cathode (higher pH). During this step the components to be separated migrate until they reach a pH corresponding to their isoelectric point (pI) and the current drops to very low values.

Mobilisation step

If mobilisation is required for detection, use one of the following methods.

— in the first method, mobilisation is accomplished during the focusing step under the effect of the electro-osmotic flow; the electro-osmotic flow must be small enough to allow the focusing of the components;

— in the second method, mobilisation is accomplished by applying positive pressure after the focusing step;

— in the third method, mobilisation is achieved after the focusing step by adding salts to the cathode reservoir or the anode reservoir (depending on the direction chosen for mobilisation) in order to alter the pH in the capillary when the voltage is applied. As the pH is changed, the proteins and ampholytes are mobilised in the direction of the reservoir which contains the added salts and pass the detector.

The separation achieved, expressed as ΔpI, depends on the pH gradient (dpH/dx), the number of ampholytes having different pI values, the molecular diffusion coefficient (D), the intensity of the electric field (E) and the variation of the

electrophoretic mobility of the analyte with the pH ($-d\mu/dpH$):

$$\Delta pI = 3 \times \sqrt{\frac{D(dpH/dx)}{E(-d\mu/dpH)}}$$

OPTIMISATION

The main parameters to be considered in the development of separations are:

Voltage

Capillary isoelectric focusing utilises very high electric fields, 300 V/cm to 1000 V/cm in the focusing step.

Capillary

The electro-osmotic flow must be reduced or suppressed depending on the mobilisation strategy (see above). Coated capillaries tend to reduce the electro-osmotic flow.

Solutions

The anode buffer reservoir is filled with a solution with a pH lower than the pI of the most acidic ampholyte and the cathode reservoir is filled with a solution with a pH higher than the pI of the most basic ampholyte. Phosphoric acid for the anode and sodium hydroxide for the cathode are frequently used.

Addition of a polymer, such as methylcellulose, in the ampholyte solution tends to suppress convective forces (if any) and electro-osmotic flow by increasing the viscosity. Commercial ampholytes are available covering many pH ranges and may be mixed if necessary to obtain an expanded pH range. Broad pH ranges are used to estimate the isoelectric point whereas narrower ranges are employed to improve accuracy. Calibration can be done by correlating migration time with isoelectric point for a series of protein markers.

During the focusing step precipitation of proteins at their isoelectric point can be prevented, if necessary, using buffer additives such as glycerol, surfactants, urea or zwitterionic buffers. However, depending on the concentration, urea denatures proteins.

MICELLAR ELECTROKINETIC CHROMATOGRAPHY (MEKC)

PRINCIPLE

In micellar electrokinetic chromatography, separation takes place in an electrolyte solution which contains a surfactant at a concentration above the critical micellar concentration (*cmc*). The solute molecules are distributed between the aqueous buffer and the pseudo-stationary phase composed of micelles, according to the partition coefficient of the solute. The technique can therefore be considered as a hybrid of electrophoresis and chromatography. It is a technique that can be used for the separation of both neutral and charged solutes, maintaining the efficiency, speed and instrumental suitability of capillary electrophoresis. One of the most widely used surfactants in MEKC is the anionic surfactant sodium dodecyl sulfate, although other surfactants, for example cationic surfactants such as cetyltrimethylammonium salts, are also used.

The separation mechanism is as follows. At neutral and alkaline pH, a strong electro-osmotic flow is generated and moves the separation buffer ions in the direction of the cathode. If sodium dodecyl sulfate is employed as the surfactant, the electrophoretic migration of the anionic micelle is in the opposite direction, towards the anode. As a result, the overall micelle migration velocity is slowed down compared to the bulk flow of the electrolytic solution. In the case of neutral solutes, since the analyte can partition between the micelle and the aqueous buffer, and has no electrophoretic mobility, the analyte migration velocity will depend only on the partition coefficient between the micelle and the aqueous buffer. In the electropherogram, the peaks corresponding to each uncharged solute are always between that of the electro-osmotic flow marker and that of the micelle (the time elapsed between these two peaks is called the separation window). For electrically charged solutes, the migration velocity depends on both the partition coefficient of the solute between the micelle and the aqueous buffer, and on the electrophoretic mobility of the solute in the absence of micelle.

Since the mechanism in MEKC of neutral and weakly ionised solutes is essentially chromatographic, migration of the solute and resolution can be rationalised in terms of the retention factor of the solute (k'), also referred to as mass distribution ratio (D_m), which is the ratio of the number of moles of solute in the micelle to those in the mobile phase. For a neutral compound, k' is given by:

$$k' = \frac{t_R - t_0}{t_0 \times \left(1 - \frac{t_R}{t_{mc}}\right)} = K \times \frac{V_S}{V_M}$$

t_R = migration time of the solute,
t_0 = analysis time of an unretained solute (determined by injecting an electro-osmotic flow marker which does not enter the micelle, for instance methanol),
t_{mc} = micelle migration time (measured by injecting a micelle marker, such as Sudan III, which migrates while continuously associated in the micelle),
K = partition coefficient of the solute,
V_S = volume of the micellar phase,
V_M = volume of the mobile phase.

Likewise, the resolution between 2 closely-migrating solutes (R_s) is given by:

$$R_s = \frac{\sqrt{N}}{4} \times \frac{\alpha - 1}{\alpha} \times \frac{k'_b}{k'_b + 1} \times \frac{1 - \left(\frac{t_0}{t_{mc}}\right)}{1 + k'_a \times \left(\frac{t_0}{t_{mc}}\right)}$$

N = number of theoretical plates for one of the solutes,
α = selectivity,
k'_a and k'_b = retention factors for both solutes, respectively ($k'_b > k'_a$).

Similar, but not identical, equations give k' and R_s values for electrically charged solutes.

OPTIMISATION

The main parameters to be considered in the development of separations by MEKC are instrumental and electrolytic solution parameters.

Instrumental parameters

Voltage Separation time is inversely proportional to applied voltage. However, an increase in voltage can cause excessive heat production that gives rise to temperature gradients and viscosity gradients of the buffer in the cross-section of the capillary. This effect can be significant with high conductivity buffers such as those containing micelles. Poor heat dissipation causes band broadening and decreases resolution.

Temperature Variations in capillary temperature affect the partition coefficient of the solute between the buffer and the micelles, the critical micellar concentration and the viscosity of the buffer. These parameters contribute to the migration time of the solutes. The use of a good cooling system

improves the reproducibility of the migration time for the solutes.

Capillary As in capillary zone electrophoresis, the dimensions of the capillary (length and internal diameter) contribute to analysis time and efficiency of separations. Increasing both effective length and total length can decrease the electric fields (working at constant voltage), increase migration time and improve the separation efficiency. The internal diameter controls heat dissipation (for a given buffer and electric field) and consequently the sample band broadening.

Electrolytic solution parameters

Surfactant type and concentration The type of surfactant, in the same way as the stationary phase in chromatography, affects the resolution since it modifies separation selectivity. Also, the log k' of a neutral compound increases linearly with the concentration of surfactant in the mobile phase. Since resolution in MEKC reaches a maximum when k' approaches the value of $\sqrt{t_{mc}/t_0}$, modifying the concentration of surfactant in the mobile phase changes the resolution obtained.

Buffer pH Although pH does not modify the partition coefficient of non-ionised solutes, it can modify the electro-osmotic flow in uncoated capillaries. A decrease in the buffer pH decreases the electro-osmotic flow and therefore increases the resolution of the neutral solutes in MEKC, resulting in a longer analysis time.

Organic solvents To improve MEKC separation of hydrophobic compounds, organic modifiers (methanol, propanol, acetonitrile, etc.) can be added to the electrolytic solution. The addition of these modifiers usually decreases migration time and the selectivity of the separation. Since the addition of organic modifiers affects the critical micellar concentration, a given surfactant concentration can be used only within a certain percentage of organic modifier before the micellisation is inhibited or adversely affected, resulting in the absence of micelles and, therefore, in the absence of partition. The dissociation of micelles in the presence of a high content of organic solvent does not always mean that the separation will no longer be possible; in some cases the hydrophobic interaction between the ionic surfactant monomer and the neutral solutes forms solvophobic complexes that can be separated electrophoretically.

Additives for chiral separations For the separation of enantiomers using MEKC, a chiral selector is included in the micellar system, either covalently bound to the surfactant or added to the micellar separation electrolyte. Micelles that have a moiety with chiral discrimination properties include salts of *N*-dodecanoyl-L-amino acids, bile salts, etc. Chiral resolution can also be achieved using chiral discriminators, such as cyclodextrins, added to the electrolytic solutions which contain micellised achiral surfactants.

Other additives Several strategies can be carried out to modify selectivity, by adding chemicals to the buffer. The addition of several types of cyclodextrins to the buffer can also be used to reduce the interaction of hydrophobic solutes with the micelle, thus increasing the selectivity for this type of compound.

The addition of substances able to modify solute-micelle interactions by adsorption on the latter, is used to improve the selectivity of the separations in MEKC. These additives may be a second surfactant (ionic or non-ionic) which gives rise to mixed micelles or metallic cations which dissolve in the micelle and form co-ordination complexes with the solutes.

QUANTIFICATION

Peak areas must be divided by the corresponding migration time to give the corrected area in order to:

— compensate for the shift in migration time from run to run, thus reducing the variation of the response,

— compensate for the different responses of sample constituents with different migration times.

Where an internal standard is used, verify that no peak of the substance to be examined is masked by that of the internal standard.

CALCULATIONS

From the values obtained, calculate the content of the component or components being examined. When prescribed, the percentage content of one or more components of the sample to be examined is calculated by determining the corrected area(s) of the peak(s) as a percentage of the total of the corrected areas of all peaks, excluding those due to solvents or any added reagents (normalisation procedure). The use of an automatic integration system (integrator or data acquisition and processing system) is recommended.

SYSTEM SUITABILITY

In order to check the behaviour of the capillary electrophoresis system, system suitability parameters are used. The choice of these parameters depends on the mode of capillary electrophoresis used. They are: retention factor (k') (only for micellar electrokinetic chromatography), apparent number of theoretical plates (N), symmetry factor (A_s) and resolution (R_s). In previous sections, the theoretical expressions for N and R_s have been described, but more practical equations that allow these parameters to be calculated from the electropherograms are given below.

APPARENT NUMBER OF THEORETICAL PLATES

The apparent number of theoretical plates (N) may be calculated using the expression:

$$N = 5.54 \times \left(\frac{t_R}{w_h}\right)^2$$

t_R = migration time or distance along the baseline from the point of injection to the perpendicular dropped from the maximum of the peak corresponding to the component,

w_h = width of the peak at half-height.

RESOLUTION

The resolution (R_s) between peaks of similar height of 2 components may be calculated using the expression:

$$R_s = \frac{1.18 \times (t_{R2} - t_{R1})}{w_{h1} + w_{h2}}$$

$$t_{R2} > t_{R1}$$

t_{R1} and t_{R2} = migration times or distances along the baseline from the point of injection to the perpendiculars dropped from the maxima of two adjacent peaks,

w_{h1} and w_{h2} = peak widths at half-height.

When appropriate, the resolution may be calculated by measuring the height of the valley (H_v) between 2 partly resolved peaks in a standard preparation and the height of the smaller peak (H_p) and calculating the peak-to-valley ratio:

$$\frac{p}{v} = \frac{H_p}{H_v}$$

SYMMETRY FACTOR

The symmetry factor (A_s) of a peak may be calculated using the expression:

$$A_s = \frac{w_{0.05}}{2d}$$

$w_{0.05}$ = width of the peak at one-twentieth of the peak height,
d = distance between the perpendicular dropped from the peak maximum and the leading edge of the peak at one-twentieth of the peak height.

Tests for area repeatability (standard deviation of areas or of the area/migration-time ratio) and for migration time repeatability (standard deviation of migration time) are introduced as suitability parameters. Migration time repeatability provides a test for the suitability of the capillary washing procedures. An alternative practice to avoid the lack of repeatability of the migration time is to use migration time relative to an internal standard.

A test for the verification of the signal-to-noise ratio for a standard preparation (or the determination of the limit of quantification) may also be useful for the determination of related substances.

SIGNAL-TO-NOISE RATIO

The detection limit and quantification limit correspond to signal-to-noise ratios of 3 and 10 respectively. The signal-to-noise ratio (S/N) is calculated using the expression:

$$\frac{S}{N} = \frac{2H}{h}$$

H = height of the peak corresponding to the component concerned, in the electropherogram obtained with the prescribed reference solution, measured from the maximum of the peak to the extrapolated baseline of the signal observed over a distance equal to twenty times the width at half-height,
h = range of the background in an electropherogram obtained after injection of a blank, observed over a distance equal to twenty times the width at the half-height of the peak in the electropherogram obtained with the prescribed reference solution and, if possible, situated equally around the place where this peak would be found.

H. Supercritical Fluid Chromatography

(Ph. Eur. method 2.2.45)

Supercritical fluid chromatography (SFC) is a method of chromatographic separation in which the mobile phase is a fluid in a supercritical or a subcritical state. The stationary phase, contained in a column, consists of either finely divided solid particles, such as a silica or porous graphite, a chemically modified stationary phase, as used in liquid chromatography, or, for capillary columns, a cross-linked liquid film evenly coated on the walls of the column.

SFC is based on mechanisms of adsorption or mass distribution.

APPARATUS

The apparatus usually consists of a cooled pumping system, an injector, a chromatographic column, contained in an oven, a detector, a pressure regulator and a data acquisition device (or an integrator or a chart recorder).

Pumping system

Pumping systems are required to deliver the mobile phase at a constant flow rate. Pressure fluctuations are to be minimised, e.g. by passing the pressurised solvent through a pulse-damping device. Tubing and connections are capable of withstanding the pressures developed by the pumping system.

Microprocessor controlled systems are capable of accurately delivering a mobile phase in either constant or varying conditions, according to a defined programme. In the case of gradient elution, pumping systems which deliver solvent(s) from several reservoirs are available and solvent mixing can be achieved on either the low or high-pressure side of the pump(s).

Injectors

Injection may be carried out directly at the head of the column using a valve.

Stationary phases

Stationary phases are contained in columns which have been described in the chapters on *Liquid chromatography* (*2.2.29*) (packed columns) and *Gas chromatography* (*2.2.28*) (capillary columns). A capillary column has a maximum internal diameter (Ø) of 100 µm.

Mobile phases

Usually the mobile phase is carbon-dioxide which may contain a polar modifier such as methanol, 2-propanol or acetonitrile. The composition, pressure (density), temperature and flow rate of the prescribed mobile phase may either be constant throughout the whole chromatographic procedure (isocratic, isodense, isothermic elution) or may vary according to a defined programme (gradient elution of the modifier, pressure (density), temperature or flow rate).

Detectors

Ultraviolet/visible (UV/Vis) spectrophotometers and flame ionisation detectors are the most commonly employed detectors. Light scattering detectors, infrared absorption spectrophotometers, thermal conductivity detectors or other special detectors may be used.

METHOD

Prepare the test solution(s) and the reference solution(s) as prescribed. The solutions must be free from solid particles.

Criteria for assessing the suitability of the system are described in the chapter on *Chromatographic separation techniques* (*2.2.46*). The extent to which adjustments of parameters of the chromatographic system can be made to satisfy the criteria of system suitability are also given in this chapter.

J. Isoelectric Focusing[1]

(Ph. Eur. method 2.2.54)

GENERAL PRINCIPLES

Isoelectric focusing (IEF) is a method of electrophoresis that separates proteins according to their isoelectric point. Separation is carried out in a slab of polyacrylamide or agarose gel that contains a mixture of amphoteric electrolytes (ampholytes). When subjected to an electric field, the ampholytes migrate in the gel to create a pH gradient. In some cases gels containing an immobilised pH gradient, prepared by incorporating weak acids and bases to specific regions of the gel network during the preparation of the gel, are used. When the applied proteins reach the gel fraction that has a pH that is the same as their isoelectric point (pI),

[1] *This chapter has undergone pharmacopoeial harmonisation. See chapter 5.8 Pharmacopoeial harmonisation.*

their charge is neutralised and migration ceases. Gradients can be made over various ranges of pH, according to the mixture of ampholytes chosen.

THEORETICAL ASPECTS

When a protein is at the position of its isoelectric point, it has no net charge and cannot be moved in a gel matrix by the electric field. It may, however, move from that position by diffusion. The pH gradient forces a protein to remain in its isoelectric point position, thus concentrating it; this concentrating effect is called "focusing". Increasing the applied voltage or reducing the sample load result in improved separation of bands. The applied voltage is limited by the heat generated, which must be dissipated. The use of thin gels and an efficient cooling plate controlled by a thermostatic circulator prevents the burning of the gel whilst allowing sharp focusing. The separation is estimated by determining the minimum pI difference (ΔpI), which is necessary to separate 2 neighbouring bands:

$$\Delta pI = 3 \times \sqrt{\frac{D(dpH/dx)}{E(-d\mu/dpH)}}$$

D = diffusion coefficient of the protein,

$\frac{dpH}{dx}$ = pH gradient,

E = intensity of the electric field, in volts per centimetre,

$-\frac{d\mu}{dpH}$ = variation of the solute mobility with the pH in the region close to the pI.

Since D and $-\frac{d\mu}{dpH}$ for a given protein cannot be altered, the separation can be improved by using a narrower pH range and by increasing the intensity of the electric field.

Resolution between protein bands on an IEF gel prepared with carrier ampholytes can be quite good. Improvements in resolution may be achieved by using immobilised pH gradients where the buffering species, which are analogous to carrier ampholytes, are copolymerised within the gel matrix. Proteins exhibiting pIs differing by as little as 0.02 pH units may be resolved using a gel prepared with carrier ampholytes while immobilised pH gradients can resolve proteins differing by approximately 0.001 pH units.

PRACTICAL ASPECTS

Special attention must be paid to sample characteristics and/or preparation. Having salt in the sample can be problematic and it is best to prepare the sample, if possible, in deionised water or 2 per cent ampholytes, using dialysis or gel filtration if necessary.

The time required for completion of focusing in thin-layer polyacrylamide gels is determined by placing a coloured protein (e.g. haemoglobin) at different positions on the gel surface and by applying the electric field: the steady state is reached when all applications give an identical band pattern. In some protocols the completion of the focusing is indicated by the time elapsed after the sample application.

The IEF gel can be used as an identity test when the migration pattern on the gel is compared to a suitable standard preparation and IEF calibration proteins, the IEF gel can be used as a limit test when the density of a band on IEF is compared subjectively with the density of bands appearing in a standard preparation, or it can be used as a quantitative test when the density is measured using a densitometer or similar instrumentation to determine the relative concentration of protein in the bands subject to validation.

APPARATUS

An apparatus for IEF consists of:

- a controllable generator for constant potential, current and power; potentials of 2500 V have been used and are considered optimal under a given set of operating conditions; a supply of up to 30 W of constant power is recommended;
- a rigid plastic IEF chamber that contains a cooled plate, of suitable material, to support the gel;
- a plastic cover with platinum electrodes that are connected to the gel by means of paper wicks of suitable width, length and thickness, impregnated with solutions of anodic and cathodic electrolytes.

ISOELECTRIC FOCUSING IN POLYACRYLAMIDE GELS: DETAILED PROCEDURE

The following method is a detailed description of an IEF procedure in thick polyacrylamide slab gels, which is used unless otherwise stated in the monograph.

PREPARATION OF THE GELS

Mould

The mould (see Figure 2.2.54.-1) is composed of a glass plate (A) on which a polyester film (B) is placed to facilitate handling of the gel, one or more spacers (C), a second glass plate (D) and clamps to hold the structure together.

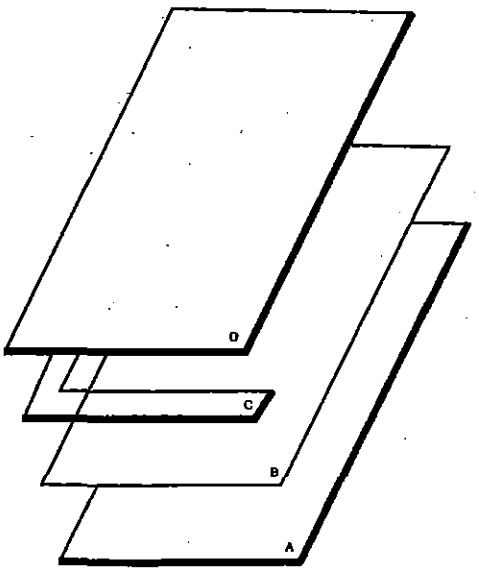


Figure 2.2.54.-1 – *Mould*

7.5 per cent polyacrylamide gel

Dissolve 29.1 g of *acrylamide R* and 0.9 g of *methylenebisacrylamide R* in 100 mL of *water R*.
To 2.5 volumes of this solution, add the mixture of ampholytes specified in the monograph and dilute to 10 volumes with *water R*. Mix carefully and degas the solution.

Preparation of the mould

Place the polyester film on the lower glass plate, apply the spacer, place the second glass plate and fit the clamps. Before use, place the solution on a magnetic stirrer and add 0.25 volumes of a 100 g/L solution of *ammonium persulfate R* and 0.25 volumes of *tetramethylethylenediamine R*. Immediately fill the space between the glass plates of the mould with the solution.

METHOD

Dismantle the mould and, making use of the polyester film, transfer the gel onto the cooled support, wetted with a few millilitres of a suitable liquid, taking care to avoid forming air bubbles. Prepare the test solutions and reference solutions as specified in the monograph. Place strips of paper for sample application, about 10 mm × 5 mm in size, on the gel and impregnate each with the prescribed amount of the test and reference solutions. Also apply the prescribed quantity of a solution of proteins with known isoelectric points as pH markers to calibrate the gel. In some protocols the gel has pre-cast slots where a solution of the sample is applied instead of using impregnated paper strips. Cut 2 strips of paper to the length of the gel and impregnate them with the electrolyte solutions: acid for the anode and alkaline for the cathode. The compositions of the anode and cathode solutions are given in the monograph. Apply these paper wicks to each side of the gel several millimetres from the edge. Fit the cover so that the electrodes are in contact with the wicks (respecting the anodic and cathodic poles). Proceed with the isoelectric focusing by applying the electrical parameters described in the monograph. Switch off the current when the migration of the mixture of standard proteins has stabilised. Using forceps, remove the sample application strips and the 2 electrode wicks. Immerse the gel in *fixing solution for isoelectric focusing in polyacrylamide gel R*. Incubate with gentle shaking at room temperature for 30 min. Drain off the solution and add 200 mL of *destaining solution R*. Incubate with shaking for 1 h. Drain the gel, add *coomassie staining solution R*. Incubate for 30 min. Destain the gel by passive diffusion with *destaining solution R* until the bands are well visualised against a clear background. Locate the position and intensity of the bands in the electropherogram as prescribed in the monograph.

VARIATIONS TO THE DETAILED PROCEDURE (SUBJECT TO VALIDATION)

Where reference to the general method on isoelectric focusing is made, variations in methodology or procedure may be made subject to validation. These include:

- the use of commercially available pre-cast gels and of commercial staining and destaining kits,
- the use of immobilised pH gradients,
- the use of rod gels,
- the use of gel cassettes of different dimensions, including ultra-thin (0.2 mm) gels,
- variations in the sample application procedure, including different sample volumes or the use of sample application masks or wicks other than paper,
- the use of alternate running conditions, including variations in the electric field depending on gel dimensions and equipment, and the use of fixed migration times rather than subjective interpretation of band stability,
- the inclusion of a pre-focusing step,
- the use of automated instrumentation,
- the use of agarose gels.

VALIDATION OF ISO-ELECTRIC FOCUSING PROCEDURES

Where alternative methods to the detailed procedure are employed they must be validated. The following criteria may be used to validate the separation:

- formation of a stable pH gradient of desired characteristics, assessed for example using coloured pH markers of known isoelectric points,
- comparison with the electropherogram provided with the chemical reference substance for the preparation to be examined,
- any other validation criteria as prescribed in the monograph.

SPECIFIED VARIATIONS TO THE GENERAL METHOD

Variations to the general method required for the analysis of specific substances may be specified in detail in monographs. These include:

- the addition of urea in the gel (3 M concentration is often satisfactory to keep protein in solution but up to 8 M can be used): some proteins precipitate at their isoelectric point; in this case, urea is included in the gel formulation to keep the protein in solution; if urea is used, only fresh solutions should be used to prevent carbamylation of the protein;
- the use of alternative staining methods;
- the use of gel additives such as non-ionic detergents (e.g. octylglucoside) or zwitterionic detergents (e.g., CHAPS or CHAPSO), and the addition of ampholyte to the sample, to prevent proteins from aggregating or precipitating.

POINTS TO CONSIDER

Samples can be applied to any area on the gel, but to protect the proteins from extreme pH environments samples should not be applied close to either electrode. During method development the analyst can try applying the protein in 3 positions on the gel (i.e. middle and both ends); the pattern of a protein applied at opposite ends of the gel may not be identical.

A phenomenon known as cathodic drift, where the pH gradient decays over time, may occur if a gel is focused too long. Although not well understood, electroendoosmosis and absorption of carbon dioxide may be factors that lead to cathodic drift. Cathodic drift is observed as focused protein migrating off the cathode end of the gel. Immobilised pH gradients may be used to address this problem.

Efficient cooling (approximately 4 °C) of the bed that the gel lies on during focusing is important. High field strengths used during isoelectric focusing can lead to overheating and affect the quality of the focused gel.

K. Peptide Mapping[1]

(Ph. Eur. method 2.2.55)

Peptide mapping is an identity test for proteins, especially those obtained by rDNA technology. It involves the chemical or enzymatic treatment of a protein resulting in the formation of peptide fragments followed by separation and identification of these fragments in a reproducible manner. It is a powerful test that is capable of identifying almost any single amino acid changes resulting from events such as errors in the reading of complementary DNA (cDNA) sequences or point mutations. Peptide mapping is a comparative procedure because the information obtained, compared to a reference substance similarly treated, confirms the primary structure of the protein, is capable of detecting whether alterations in structure have occurred, and demonstrates process consistency and genetic stability. Each

[1] *This chapter has undergone pharmacopoeial harmonisation. See chapter 5.8 Pharmacopoeial harmonisation.*

Table 2.2.55.-1. – *Examples of cleavage agents*

Type	Agent	Specificity
Enzymatic	Trypsin (EC 3.4.21.4)	*C*-terminal side of Arg and Lys
	Chymotrypsin (EC 3.4.21.1)	*C*-terminal side of hydrophobic residues (e.g. Leu, Met, Ala, aromatics)
	Pepsin (EC 3.4.23.1 and 2)	Non-specific digest
	Lysyl endopeptidase (Lys-C endopeptidase) (EC 3.4.21.50)	*C*-terminal side of Lys
	Glutamyl endopeptidase (from *S. aureus* strain V8) (EC 3.4.21.19)	*C*-terminal side of Glu and Asp
	Peptidyl-Asp metallo-endopeptidase (endoproteinase Asp-N)	*N*-terminal side of Asp
	Clostripain (EC 3.4.22.8)	*C*-terminal side of Arg
Chemical	Cyanogen bromide	*C*-terminal side of Met
	2-Nitro-5-thio-cyanobenzoic acid	*N*-terminal side of Cys
	O-Iodosobenzoic acid	*C*-terminal side of Trp and Tyr
	Dilute acid	Asp and Pro
	BNPS-skatole	Trp

protein presents unique characteristics which must be well understood so that the scientific and analytical approaches permit validated development of a peptide map that provides sufficient specificity.

This chapter provides detailed assistance in the application of peptide mapping and its validation to characterise the desired protein, to evaluate the stability of the expression construct of cells used for recombinant DNA products and to evaluate the consistency of the overall process, to assess product stability as well as to ensure the identity of the protein, or to detect the presence of protein variant.

Peptide mapping is not a general method, but involves developing specific maps for each unique protein. Although the technology is evolving rapidly, there are certain methods that are generally accepted. Variations of these methods will be indicated, when appropriate, in specific monographs.

A peptide map may be viewed as a fingerprint of a protein and is the end product of several chemical processes that provide a comprehensive understanding of the protein being analysed. 4 principal steps are necessary for the development of the procedure: isolation and purification of the protein, if the protein is part of a formulation; selective cleavage of the peptide bonds; chromatographic separation of the peptides; and analysis and identification of the peptides. A test sample is digested and assayed in parallel with a reference substance. Complete cleavage of peptide bonds is more likely to occur when enzymes such as endoproteases (e.g., trypsin) are used, instead of chemical cleavage reagents. A map must contain enough peptides to be meaningful. On the other hand, if there are too many fragments, the map might lose its specificity because many proteins will then have the same profiles.

ISOLATION AND PURIFICATION

Isolation and purification are necessary for analysis of bulk drugs or dosage forms containing interfering excipients and carrier proteins and, when required, will be specified in the monograph. Quantitative recovery of protein from the dosage form must be validated.

SELECTIVE CLEAVAGE OF PEPTIDE BONDS

The selection of the approach used for the cleavage of peptide bonds will depend on the protein under test. This selection process involves determination of the type of cleavage to be employed, enzymatic or chemical, and the type of cleavage agent within the chosen category. Several cleavage agents and their specificity are shown in Table 2.2.55.-1. This list is not all-inclusive and will be expanded as other cleavage agents are identified.

Pretreatment of sample

Depending on the size or the configuration of the protein, different approaches in the pretreatment of samples can be used. If trypsin is used as a cleavage agent for proteins with a molecular mass greater than 100 000 Da, lysine residues must be protected by citraconylation or maleylation; otherwise, too many peptides will be generated.

Pretreatment of the cleavage agent

Pretreatment of cleavage agents, especially enzymatic agents, might be necessary for purification purposes to ensure reproducibility of the map. For example, trypsin used as a cleavage agent will have to be treated with tosyl-L-phenylalanine chloromethyl ketone to inactivate chymotrypsin. Other methods, such as purification of trypsin by high performance liquid chromatography (HPLC) or immobilisation of enzyme on a gel support, have been successfully used when only a small amount of protein is available.

Pretreatment of the protein

Under certain conditions, it might be necessary to concentrate the sample or to separate the protein from excipients and stabilisers used in formulation of the product, if these interfere with the mapping procedure. Physical procedures used for pretreatment can include ultrafiltration, column chromatography and lyophilization. Other pretreatments, such as the addition of chaotropic agents (e.g. urea) can be used to unfold the protein prior to mapping. To allow the enzyme to have full access to cleavage sites and permit some unfolding of the protein, it is often necessary to reduce and alkylate the disulfide bonds prior to digestion.

Digestion with trypsin can introduce ambiguities in the peptide map due to side reactions occurring during the digestion reaction, such as non-specific cleavage, deamidation, disulfide isomerisation, oxidation of methionine

residues, or formation of pyroglutamic groups created from the deamidation of glutamine at the *N*-terminal side of a peptide. Furthermore, peaks may be produced by autohydrolysis of trypsin. Their intensities depend on the ratio of trypsin to protein. To avoid autohydrolysis, solutions of proteases may be prepared at a pH that is not optimal (e.g. at pH 5 for trypsin), which would mean that the enzyme would not become active until diluted with the digest buffer.

Establishment of optimal digestion conditions
Factors that affect the completeness and effectiveness of digestion of proteins are those that could affect any chemical or enzymatic reactions.

pH of the reaction milieu The pH of the digestion mixture is empirically determined to ensure the optimisation of the performance of the given cleavage agent. For example, when using cyanogen bromide as a cleavage agent, a highly acidic environment (e.g. pH 2, formic acid) is necessary; however, when using trypsin as a cleavage agent, a slightly alkaline environment (pH 8) is optimal. As a general rule, the pH of the reaction milieu must not alter the chemical integrity of the protein during the digestion and must not change during the course of the fragmentation reaction.

Temperature A temperature between 25 °C and 37 °C is adequate for most digestions. The temperature used is intended to minimise chemical side reactions. The type of protein under test will dictate the temperature of the reaction milieu, because some proteins are more susceptible to denaturation as the temperature of the reaction increases. For example, digestion of recombinant bovine somatropin is conducted at 4 °C, because at higher temperatures it will precipitate during digestion.

Time If sufficient sample is available, a time course study is considered in order to determine the optimum time to obtain a reproducible map and avoid incomplete digestion. Time of digestion varies from 2 h to 30 h. The reaction is stopped by the addition of an acid which does not interfere in the map or by freezing.

Amount of cleavage agent used Although excessive amounts of cleavage agent are used to accomplish a reasonably rapid digestion time (i.e. 6-20 hours), the amount of cleavage agent is minimised to avoid its contribution to the chromatographic map pattern. A protein to protease ratio between 20:1 and 200:1 is generally used. It is recommended that the cleavage agent is added in 2 or more stages to optimise cleavage. Nonetheless, the final reaction volume remains small enough to facilitate the next step in peptide mapping, the separation step. To sort out digestion artifacts that might interfere with the subsequent analysis, a blank determination is performed, using a digestion control with all the reagents, except the test protein.

CHROMATOGRAPHIC SEPARATION

Many techniques are used to separate peptides for mapping. The selection of a technique depends on the protein being mapped. Techniques that have been successfully used for separation of peptides are shown in Table 2.2.55-2. In this section, a most widely used reversed-phase HPLC method is described as one of the procedures of chromatographic separation.

The purity of solvents and mobile phases is a critical factor in HPLC separation. HPLC-grade solvents and water that are commercially available, are recommended for reversed-phase HPLC. Dissolved gases present a problem in gradient systems where the solubility of the gas in a solvent may be less in a mixture than in a single solvent. Vacuum degassing and agitation by sonication are often used as useful degassing procedures. When solid particles in the solvents are drawn into the HPLC system, they can damage the sealing of pump valves or clog the top of the chromatographic column. Both pre- and post-pump filtration is also recommended.

Table 2.2.55-2. – *Techniques used for the separation of peptides*

Reversed-phase high performance liquid chromatography (HPLC)
Ion-exchange chromatography (IEC)
Hydrophobic interaction chromatography (HIC)
Polyacrylamide gel electrophoresis (PAGE), non-denaturating
Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)
Capillary electrophoresis (CE)
Paper chromatography-high voltage (PCHV)
High voltage-paper electrophoresis (HVPE)

Chromatographic column
The selection of a chromatographic column is empirically determined for each protein. Columns with 10 nm or 30 nm pore size with silica support can give optimal separation. For smaller peptides, *octylsilyl silica gel for chromatography R* (3-10 µm) and *octadecylsilyl silica gel for chromatography R* (3-10 µm) column packings are more efficient than *butylsilyl silica gel for chromatography R* (5-10 µm).

Solvent
The most commonly used solvent is water with acetonitrile as the organic modifier to which not more than 0.1 per cent trifluoroacetic acid is added. If necessary, add propyl alcohol or isopropyl alcohol to solubilise the digest components, provided that the addition does not unduly increase the viscosity of the components.

Mobile phase
Buffered mobile phases containing phosphate are used to provide some flexibility in the selection of pH conditions, since shifts of pH in the 3.0-5.0 range enhance the separation of peptides containing acidic residues (e.g. glutamic and aspartic acids). Sodium or potassium phosphates, ammonium acetate, phosphoric acid at a pH between 2 and 7 (or higher for polymer-based supports) have also been used with acetonitrile gradients. Acetonitrile containing trifluoroacetic acid is used quite often.

Gradient
Gradients can be linear, nonlinear, or include step functions. A shallow gradient is recommended in order to separate complex mixtures. Gradients are optimised to provide clear resolution of 1 or 2 peaks that will become "marker" peaks for the test.

Isocratic elution
Isocratic HPLC systems using a single mobile phase are used on the basis of their convenience of use and improved detector responses. Optimal composition of a mobile phase to obtain clear resolution of each peak is sometimes difficult to establish. Mobile phases for which slight changes in component ratios or in pH significantly affect retention times of peaks in peptide maps must not be used in isocratic HPLC systems.

Other parameters
Temperature control of the column is usually necessary to achieve good reproducibility. The flow rates for the mobile phases range from 0.1-2.0 mL/min, and the detection of peptides is performed with a UV detector at 200-230 nm. Other methods of detection have been used (e.g. post-column derivatisation), but they are not as robust or versatile as UV detection.

Validation

This section provides an experimental means for measuring the overall performance of the test method. The acceptance criteria for system suitability depend on the identification of critical test parameters that affect data interpretation and acceptance. These critical parameters are also criteria that monitor peptide digestion and peptide analysis. An indicator that the desired digestion endpoint has been achieved is shown by comparison with a reference standard, which is treated in the same manner as the test protein. The use of a reference substance in parallel with the test protein is critical in the development and establishment of system suitability limits. In addition, a chromatogram is included with the reference substance for additional comparison purposes. Other indicators may include visual inspection of protein or peptide solubility, the absence of intact protein, or measurement of responses of a digestion-dependent peptide. The critical system suitability parameters for peptide analysis will depend on the particular mode of peptide separation and detection and on the data analysis requirements.

When peptide mapping is used as an identification test, the system suitability requirements for the identified peptides cover selectivity and precision. In this case, as well as when identification of variant protein is done, the identification of the primary structure of the peptide fragments in the peptide map provides both a verification of the known primary structure and the identification of protein variants by comparison with the peptide map of the reference substance for the specified protein. The use of a digested reference substance for a given protein in the determination of peptide resolution is the method of choice. For an analysis of a variant protein, a characterised mixture of a variant and a reference substance can be used, especially if the variant peptide is located in a less-resolved region of the map. The index of pattern consistency can be simply the number of major peptides detected. Peptide pattern consistency can be best defined by the resolution of peptide peaks. Chromatographic parameters, such as peak-to-peak resolution, maximum peak width, peak area, peak tailing factors, and column efficiency, may be used to define peptide resolution. Depending on the protein under test and the method of separation used, single peptide or multiple peptide resolution requirements may be necessary.

The replicate analysis of the digest of the reference substance for the protein under test yields measures of precision and quantitative recovery. Recovery of the identified peptides is generally ascertained by the use of internal or external peptide standards. The precision is expressed as the relative standard deviation (RSD). Differences in the recovery and precision of the identified peptides are to be expected; therefore, the system suitability limits will have to be established for both the recovery and the precision of the identified peptides. These limits are unique for a given protein and will be specified in the individual monograph.

Visual comparison of the relative retentions, the peak responses (the peak area or the peak height), the number of peaks, and the overall elution pattern is completed initially. It is then complemented and supported by mathematical analysis of the peak response ratios and by the chromatographic profile of a 1:1 (*V/V*) mixture of sample and reference substance digest. If all peaks in the sample digest and in the reference substance digest have the same relative retentions and peak response ratios, then the identity of the sample under test is confirmed.

If peaks that initially eluted with significantly different relative retentions are then observed as single peaks in the 1:1 mixture, the initial difference would be an indication of system variability. However, if separate peaks are observed in the 1:1 mixture, this would be evidence of the nonequivalence of the peptides in each peak. If a peak in the 1:1 mixture is significantly broader than the corresponding peak in the sample and reference substance digest, it may indicate the presence of different peptides. The use of computer-aided pattern recognition software for the analysis of peptide mapping data has been proposed and applied, but issues related to the validation of the computer software preclude its use in a compendial test in the near future. Other automated approaches have been used that employ mathematical formulas, models, and pattern recognition. Such approaches are, for example, the automated identification of compounds by IR spectroscopy and the application of diode-array UV spectral analysis for identification of peptides. These methods have limitations due to inadequate resolutions, co-elution of fragments, or absolute peak response differences between reference substance and sample digest fragments.

The numerical comparison of the peak retention times and peak areas or peak heights can be done for a selected group of relevant peaks that have been correctly identified in the peptide maps. Peak areas can be calculated using 1 peak showing relatively small variation as an internal reference, keeping in mind that peak area integration is sensitive to baseline variation and likely to introduce error in the analysis. Alternatively, the percentage of each peptide peak height relative to the sum of all peak heights can be calculated for the sample under test. The percentage is then compared to that of the corresponding peak of the reference substance. The possibility of auto-hydrolysis of trypsin is monitored by producing a blank peptide map, that is, the peptide map obtained when a blank solution is treated with trypsin.

The minimum requirement for the qualification of peptide mapping is an approved test procedure that includes system suitability as a test control. In general, early in the regulatory process, qualification of peptide mapping for a protein is sufficient. As the regulatory approval process for the protein progresses, additional qualifications of the test can include a partial validation of the analytical procedure to provide assurance that the method will perform as intended in the development of a peptide map for the specified protein.

ANALYSIS AND IDENTIFICATION OF PEPTIDES

This section gives guidance on the use of peptide mapping during development in support of regulatory applications.

The use of a peptide map as a qualitative tool does not require the complete characterisation of the individual peptide peaks. However, validation of peptide mapping in support of regulatory applications requires rigorous characterisation of each of the individual peaks in the peptide map. Methods to characterise peaks range from *N*-terminal sequencing of each peak followed by amino acid analysis to the use of mass spectroscopy (MS).

For characterisation purposes, when *N*-terminal sequencing and amino acids analysis are used, the analytical separation is scaled up. Since scale-up might affect the resolution of peptide peaks, it is necessary, using empirical data, to assure that there is no loss of resolution due to scale-up. Eluates corresponding to specific peptide peaks are collected, vacuum-concentrated, and chromatographed again, if necessary. Amino acid analysis of fragments may be limited by the peptide size. If the *N*-terminus is blocked, it may need to be cleared before sequencing. *C*-terminal sequencing of proteins in combination with carboxypeptidase and matrix-

assisted laser desorption ionisation coupled to time-of-flight analyser (MALDI-TOF) can also be used for characterisation purposes.

The use of MS for characterisation of peptide fragments is by direct infusion of isolated peptides or by the use of on-line LC-MS for structure analysis. In general, it includes electrospray and MALDI-TOF-MS, as well as fast-atom bombardment (FAB). Tandem MS has also been used to sequence a modified protein and to determine the type of amino acid modification that has occurred. The comparison of mass spectra of the digests before and after reduction provides a method to assign the disulfide bonds to the various sulfydryl-containing peptides.

If regions of the primary structure are not clearly demonstrated by the peptide map, it might be necessary to develop a secondary peptide map. The goal of a validated method of characterisation of a protein through peptide mapping is to reconcile and account for at least 95 per cent of the theoretical composition of the protein structure.

L. Amino Acid Analysis[1]

(Ph. Eur. method 2.2.56)

Amino acid analysis refers to the methodology used to determine the amino acid composition or content of proteins, peptides, and other pharmaceutical preparations. Proteins and peptides are macromolecules consisting of covalently bonded amino acid residues organised as a linear polymer. The sequence of the amino acids in a protein or peptide determines the properties of the molecule. Proteins are considered large molecules that commonly exist as folded structures with a specific conformation, while peptides are smaller and may consist of only a few amino acids. Amino acid analysis can be used to quantify proteins and peptides, to determine the identity of proteins or peptides based on their amino acid composition, to support protein and peptide structure analysis, to evaluate fragmentation strategies for peptide mapping, and to detect atypical amino acids that might be present in a protein or peptide. It is necessary to hydrolyse a protein/peptide to its individual amino acid constituents before amino acid analysis. Following protein/peptide hydrolysis, the amino acid analysis procedure can be the same as that practiced for free amino acids in other pharmaceutical preparations. The amino acid constituents of the test sample are typically derivatised for analysis.

APPARATUS

Methods used for amino acid analysis are usually based on a chromatographic separation of the amino acids present in the test sample. Current techniques take advantage of the automated chromatographic instrumentation designed for analytical methodologies. An amino acid analysis instrument will typically be a low-pressure or high-pressure liquid chromatograph capable of generating mobile phase gradients that separate the amino acid analytes on a chromatographic column. The instrument must have post-column derivatisation capability, unless the sample is analysed using precolumn derivatisation. The detector is usually an ultraviolet/visible or fluorescence detector depending on the derivatisation method used. A recording device (e.g., integrator) is used for transforming the analogue signal from the detector and for quantitation. It is preferred that instrumentation be dedicated particularly for amino acid analysis.

GENERAL PRECAUTIONS

Background contamination is always a concern for the analyst in performing amino acid analysis. High purity reagents are necessary (e.g., low purity hydrochloric acid can contribute to glycine contamination). Analytical reagents are changed routinely every few weeks using only high-pressure liquid chromatography (HPLC) grade solvents. Potential microbial contamination and foreign material that might be present in the solvents are reduced by filtering solvents before use, keeping solvent reservoirs covered, and not placing amino acid analysis instrumentation in direct sunlight.

Laboratory practices can determine the quality of the amino acid analysis. Place the instrumentation in a low traffic area of the laboratory. Keep the laboratory clean. Clean and calibrate pipets according to a maintenance schedule. Keep pipet tips in a covered box; the analysts may not handle pipet tips with their hands. The analysts may wear powder-free latex or equivalent gloves. Limit the number of times a test sample vial is opened and closed because dust can contribute to elevated levels of glycine, serine, and alanine.

A well-maintained instrument is necessary for acceptable amino acid analysis results. If the instrument is used on a routine basis, it is to be checked daily for leaks, detector and lamp stability, and the ability of the column to maintain resolution of the individual amino acids. Clean or replace all instrument filters and other maintenance items on a routine schedule.

REFERENCE MATERIAL

Acceptable amino acid standards are commercially available for amino acid analysis and typically consist of an aqueous mixture of amino acids. When determining amino acid composition, protein or peptide standards are analysed with the test material as a control to demonstrate the integrity of the entire procedure. Highly purified bovine serum albumin has been used as a protein standard for this purpose.

CALIBRATION OF INSTRUMENTATION

Calibration of amino acid analysis instrumentation typically involves analysing the amino acid standard, which consists of a mixture of amino acids at a number of concentrations, to determine the response factor and range of analysis for each amino acid. The concentration of each amino acid in the standard is known. In the calibration procedure, the analyst dilutes the amino acid standard to several different analyte levels within the expected linear range of the amino acid analysis technique. Then, replicates at each of the different analyte levels can be analysed. Peak areas obtained for each amino acid are plotted versus the known concentration for each of the amino acids in the standard dilution. These results will allow the analyst to determine the range of amino acid concentrations where the peak area of a given amino acid is an approximately linear function of the amino acid concentration. It is important that the analyst prepare the samples for amino acid analysis so that they are within the analytical limits (e.g., linear working range) of the technique employed in order to obtain accurate and repeatable results.

4 to 6 amino acid standard levels are analysed to determine a response factor for each amino acid. The response factor is calculated as the average peak area or peak height per nanomole of amino acid present in the standard.

A calibration file consisting of the response factor for each amino acid is prepared and used to calculate the

[1] *This chapter has undergone pharmacopoeial harmonisation. See chapter 5.8 Pharmacopoeial harmonisation.*

concentration of each amino acid present in the test sample. This calculation involves dividing the peak area corresponding to a given amino acid by the response factor for that amino acid to give the nanomoles of the amino acid. For routine analysis, a single-point calibration may be sufficient; however, the calibration file is updated frequently and tested by the analysis of analytical controls to ensure its integrity.

REPEATABILITY

Consistent high quality amino acid analysis results from an analytical laboratory require attention to the repeatability of the assay. During analysis of the chromatographic separation of the amino acids or their derivatives, numerous peaks can be observed on the chromatogram that correspond to the amino acids. The large number of peaks makes it necessary to have an amino acid analysis system that can repeatedly identify the peaks based on retention time and integrate the peak areas for quantitation. A typical repeatability evaluation involves preparing a standard amino acid solution and analysing many replicates (e.g., 6 analyses or more) of the same standard solution. The relative standard deviation (RSD) is determined for the retention time and integrated peak area of each amino acid. An evaluation of the repeatability is expanded to include multiple assays conducted over several days by different analysts. Multiple assays include the preparation of standard dilutions from starting materials to determine the variation due to sample handling. The amino acid composition of a standard protein (e.g., bovine serum albumin) is often analysed as part of the repeatability evaluation. By evaluating the replicate variation (i.e., RSD), the laboratory can establish analytical limits to ensure that the analyses from the laboratory are under control. It is desirable to establish the lowest practical variation limits to ensure the best results. Areas to focus on to lower the variability of the amino acid analysis include sample preparation, high background spectral interference due to quality of reagents and/or laboratory practices, instrument performance and maintenance, data analysis and interpretation, and analyst performance and habits.
All parameters involved are fully investigated in the scope of the validation work.

SAMPLE PREPARATION

Accurate results from amino acid analysis require purified protein and peptide samples. Buffer components (e.g., salts, urea, detergents) can interfere with the amino acid analysis and are removed from the sample before analysis. Methods that utilise post-column derivatisation of the amino acids are generally not affected by buffer components to the extent seen with pre-column derivatisation methods. It is desirable to limit the number of sample manipulations to reduce potential background contamination, to improve analyte recovery, and to reduce labour. Common techniques used to remove buffer components from protein samples include the following methods: (1) injecting the protein sample onto a reversed-phase HPLC system, removing the protein with a volatile solvent containing a sufficient organic component, and drying the sample in a vacuum centrifuge; (2) dialysis against a volatile buffer or water; (3) centrifugal ultrafiltration for buffer replacement with a volatile buffer or water; (4) precipitating the protein from the buffer using an organic solvent (e.g., acetone); (5) gel filtration.

INTERNAL STANDARDS

It is recommended that an internal standard be used to monitor physical and chemical losses and variations during amino acid analysis. An accurately known amount of internal standard can be added to a protein solution prior to hydrolysis. The recovery of the internal standard gives the general recovery of the amino acids of the protein solution. Free amino acids, however, do not behave in the same way as protein-bound amino acids during hydrolysis, whose rates of release or destruction are variable. Therefore, the use of an internal standard to correct for losses during hydrolysis may give unreliable results. It will be necessary to take this point into consideration when interpreting the results. Internal standards can also be added to the mixture of amino acids after hydrolysis to correct for differences in sample application and changes in reagent stability and flow rates. Ideally, an internal standard is an unnaturally occurring primary amino acid that is commercially available and inexpensive. It should also be stable during hydrolysis, its response factor should be linear with concentration, and it needs to elute with a unique retention time without overlapping other amino acids. Commonly used amino acid standards include norleucine, nitrotyrosine, and α-aminobutyric acid.

PROTEIN HYDROLYSIS

Hydrolysis of protein and peptide samples is necessary for amino acid analysis of these molecules. The glassware used for hydrolysis must be very clean to avoid erroneous results. Glove powders and fingerprints on hydrolysis tubes may cause contamination. To clean glass hydrolysis tubes, boil tubes for 1 h in 1 M hydrochloric acid or soak tubes in concentrated nitric acid or in a mixture of equal volumes of concentrated hydrochloric acid and nitric acid. Clean hydrolysis tubes are rinsed with high-purity water followed by a rinse with HPLC grade methanol, dried overnight in an oven, and stored covered until use. Alternatively, pyrolysis of clean glassware at 500 °C for 4 h may also be used to eliminate contamination from hydrolysis tubes. Adequate disposable laboratory material can also be used.

Acid hydrolysis is the most common method for hydrolysing a protein sample before amino acid analysis. The acid hydrolysis technique can contribute to the variation of the analysis due to complete or partial destruction of several amino acids: tryptophan is destroyed; serine and threonine are partially destroyed; methionine might undergo oxidation; and cysteine is typically recovered as cystine (but cystine recovery is usually poor because of partial destruction or reduction to cysteine). Application of adequate vacuum (less than 200 µm of mercury or 26.7 Pa) or introduction of an inert gas (argon) in the headspace of the reaction vessel can reduce the level of oxidative destruction. In peptide bonds involving isoleucine and valine the amido bonds of Ile-Ile, Val-Val, Ile-Val, and Val-Ile are partially cleaved; and asparagine and glutamine are deamidated, resulting in aspartic acid and glutamic acid, respectively. The loss of tryptophan, asparagine, and glutamine during an acid hydrolysis limits quantitation to 17 amino acids. Some of the hydrolysis techniques described are used to address these concerns. Some of the hydrolysis techniques described (i.e., Methods 4-11) may cause modifications to other amino acids. Therefore, the benefits of using a given hydrolysis technique are weighed against the concerns with the technique and are tested adequately before employing a method other than acid hydrolysis.

A time-course study (i.e., amino acid analysis at acid hydrolysis times of 24 h, 48 h and 72 h) is often employed to analyse the starting concentration of amino acids that are partially destroyed or slow to cleave. By plotting the observed concentration of labile amino acids (e.g., serine and threonine) versus hydrolysis time, the line can be

extrapolated to the origin to determine the starting concentration of these amino acids. Time-course hydrolysis studies are also used with amino acids that are slow to cleave (e.g., isoleucine and valine). During the hydrolysis time course, the analyst will observe a plateau in these residues. The level of this plateau is taken as the residue concentration. If the hydrolysis time is too long, the residue concentration of the sample will begin to decrease, indicating destruction by the hydrolysis conditions.

An acceptable alternative to the time-course study is to subject an amino acid calibration standard to the same hydrolysis conditions as the test sample. The amino acid in free form may not completely represent the rate of destruction of labile amino acids within a peptide or protein during the hydrolysis. This is especially true for peptide bonds that are slow to cleave (e.g., Ile-Val bonds). However, this technique will allow the analyst to account for some residue destruction. Microwave acid hydrolysis has been used and is rapid but requires special equipment as well as special precautions. The optimal conditions for microwave hydrolysis must be investigated for each individual protein/peptide sample. The microwave hydrolysis technique typically requires only a few minutes, but even a deviation of one minute may give inadequate results (e.g., incomplete hydrolysis or destruction of labile amino acids). Complete proteolysis, using a mixture of proteases, has been used but can be complicated, requires the proper controls, and is typically more applicable to peptides than proteins.

During initial analyses of an unknown protein, experiments with various hydrolysis time and temperature conditions are conducted to determine the optimal conditions.

METHOD 1

Acid hydrolysis using hydrochloric acid containing phenol is the most common procedure used for protein/peptide hydrolysis preceding amino acid analysis. The addition of phenol to the reaction prevents the halogenation of tyrosine.

Hydrolysis solution

6 M hydrochloric acid containing 0.1 per cent to 1.0 per cent of phenol.

Procedure

Liquid phase hydrolysis Place the protein or peptide sample in a hydrolysis tube, and dry (the sample is dried so that water in the sample will not dilute the acid used for the hydrolysis). Add 200 µL of hydrolysis solution per 500 µg of lyophilised protein. Freeze the sample tube in a dry ice-acetone bath, and flame seal *in vacuo*. Samples are typically hydrolysed at 110 °C for 24 h *in vacuo* or in an inert atmosphere to prevent oxidation. Longer hydrolysis times (e.g., 48 h and 72 h) are investigated if there is a concern that the protein is not completely hydrolysed.

Vapour phase hydrolysis This is one of the most common acid hydrolysis procedures, and it is preferred for microanalysis when only small amounts of the sample are available. Contamination of the sample from the acid reagent is also minimised by using vapour phase hydrolysis. Place vials containing the dried samples in a vessel that contains an appropriate amount of hydrolysis solution. The hydrolysis solution does not come in contact with the test sample. Apply an inert atmosphere or vacuum (less than 200 µm of mercury or 26.7 Pa) to the headspace of the vessel, and heat to about 110 °C for a 24 h hydrolysis time. Acid vapour hydrolyses the dried sample. Any condensation of the acid in the sample vials is to be minimised. After hydrolysis, dry the test sample *in vacuo* to remove any residual acid.

METHOD 2

Tryptophan oxidation during hydrolysis is decreased by using mercaptoethanesulfonic acid as the reducing acid.

Hydrolysis solution

2.5 M mercaptoethanesulfonic acid solution.

Vapour phase hydrolysis

Dry about 1 µg to 100 µg of the protein/peptide under test in a hydrolysis tube. Place the hydrolysis tube in a larger tube with about 200 µL of the hydrolysis solution. Seal the larger tube *in vacuo* (about 50 µm of mercury or 6.7 Pa) to vaporise the hydrolysis solution. Heat the hydrolysis tube to 170-185 °C for about 12.5 min. After hydrolysis, dry the hydrolysis tube *in vacuo* for 15 min to remove the residual acid.

METHOD 3

Tryptophan oxidation during hydrolysis is prevented by using thioglycollic acid (TGA) as the reducing acid.

Hydrolysis solution

7 M hydrochloric acid containing 1 per cent of phenol, 10 per cent of trifluoroacetic acid and 20 per cent of thioglycollic acid.

Vapour phase hydrolysis

Dry about 10 µg to 50 µg of the protein/peptide under test in a sample tube. Place the sample tube in a larger tube with about 200 µL of the hydrolysis solution. Seal the larger tube *in vacuo* (about 50 µm of mercury or 6.7 Pa) to vaporise the TGA. Heat the sample tube to 166 °C for about 15-30 min. After hydrolysis, dry the sample tube *in vacuo* for 5 min to remove the residual acid. Recovery of tryptophan by this method may be dependent on the amount of sample present.

METHOD 4

Cysteine/cystine and methionine oxidation is performed with performic acid before the protein hydrolysis.

Oxidation solution

Use performic acid freshly prepared by mixing 1 volume of hydrogen peroxide solution (30 per cent) and 9 volumes of anhydrous formic acid and incubating at room temperature for 1 h.

Procedure

Dissolve the protein/peptide sample in 20 µL of anhydrous formic acid and heat at 50 °C for 5 min; then add 100 µL of the oxidation solution. Allow the oxidation to proceed for 10-30 min. In this reaction, cysteine is converted to cysteic acid and methionine is converted to methionine-sulfone. Remove the excess reagent from the sample in a vacuum centrifuge. The oxidised protein can then be acid hydrolysed using Method 1 or Method 2. This technique may cause modifications to tyrosine residues in the presence of halides.

METHOD 5

Cysteine/cystine oxidation is accomplished during the liquid phase hydrolysis with sodium azide.

Hydrolysis solution

To 6 M hydrochloric acid containing 0.2 per cent of phenol, add sodium azide to obtain a final concentration of 2 g/L. The added phenol prevents halogenation of tyrosine.

Liquid phase hydrolysis

Conduct the protein/peptide hydrolysis at about 110 °C for 24 h. During the hydrolysis, the cysteine/cystine present in the sample is converted to cysteic acid by the sodium azide present in the hydrolysis solution. This technique allows better tyrosine recovery than Method 4, but it is not quantitative for methionine. Methionine is converted to a

mixture of the parent methionine and its 2 oxidative products, methionine-sulfoxide and methionine-sulfone.

METHOD 6

Cysteine/cystine oxidation is accomplished with dimethyl sulfoxide (DMSO).

Hydrolysis solution

To 6 M hydrochloric acid containing 0.1 per cent to 1.0 per cent of phenol, add dimethyl sulfoxide to obtain a final concentration of 2 per cent *V/V*.

Vapour phase hydrolysis

Conduct the protein/peptide hydrolysis at about 110 °C for 24 h. During the hydrolysis, the cysteine/cystine present in the sample is converted to cysteic acid by the DMSO present in the hydrolysis solution. As an approach to limit variability and compensate for partial destruction, it is recommended to evaluate the cysteic acid recovery from oxidative hydrolysis of standard proteins containing 1-8 mol of cysteine per mole of protein. The response factors from protein/peptide hydrolysates are typically about 30 per cent lower than those for non-hydrolysed cysteic acid standards. Because histidine, methionine, tyrosine, and tryptophan are also modified, a complete compositional analysis is not obtained with this technique.

METHOD 7

Cysteine/cystine reduction and alkylation is accomplished by a vapour phase pyridylethylation reaction.

Reducing solution

Transfer 83.3 µL of pyridine, 16.7 µL of 4-vinylpyridine, 16.7 µL of tributylphosphine, and 83.3 µL of water to a suitable container and mix.

Procedure

Add the protein/peptide (between 1 and 100 µg) to a hydrolysis tube, and place in a larger tube. Transfer the reducing solution to the large tube, seal *in vacuo* (about 50 µm of mercury or 6.7 Pa), and heat at about 100 °C for 5 min. Then remove the inner hydrolysis tube, and dry it in a vacuum desiccator for 15 min to remove residual reagents. The pyridylethylated sample can then be acid hydrolysed using previously described procedures. The pyridylethylation reaction is performed simultaneously with a protein standard sample containing 1-8 mol of cysteine per mole of protein to evaluate the pyridylethyl-cysteine recovery. Longer incubation times for the pyridylethylation reaction can cause modifications to the α-amino terminal group and the ε-amino group of lysine in the protein.

METHOD 8

Cysteine/cystine reduction and alkylation is accomplished by a liquid phase pyridylethylation reaction.

Stock solutions

Prepare and filter 3 solutions: 1 M Tris-hydrochloride pH 8.5 containing 4 mM disodium edetate (stock solution A), 8 M guanidine hydrochloride (stock solution B), and 10 per cent of 2-mercaptoethanol (stock solution C).

Reducing solution

Prepare a mixture of 1 volume of stock solution A and 3 volumes of stock solution B to obtain a buffered solution of 6 M guanidine hydrochloride in 0.25 M tris-hydrochloride.

Procedure

Dissolve about 10 µg of the test sample in 50 µL of the reducing solution, and add about 2.5 µL of stock solution C. Store under nitrogen or argon for 2 h at room temperature in the dark. To achieve the pyridylethylation reaction, add about 2 µL of 4-vinylpyridine to the protein solution, and incubate for an additional 2 h at room temperature in the dark. Desalt the protein/peptide by collecting the protein/peptide fraction from a reversed-phase HPLC separation. The collected sample can be dried in a vacuum centrifuge before acid hydrolysis.

METHOD 9

Cysteine/cystine reduction and alkylation is accomplished by a liquid phase carboxymethylation reaction.

Stock solutions

Prepare as directed for Method 8.

Carboxymethylation solution

Prepare a 100 g/L solution of iodoacetamide in alcohol.

Buffer solution

Use the reducing solution, prepared as described for Method 8.

Procedure

Dissolve the test sample in 50 µL of the buffer solution, and add about 2.5 µL of stock solution C. Store under nitrogen or argon for 2 h at room temperature in the dark. Add the carboxymethylation solution in a ratio 1.5 fold per total theoretical content of thiols, and incubate for an additional 30 min at room temperature in the dark. If the thiol content of the protein is unknown, then add 5 µL of 100 mM iodoacetamide for every 20 nmol of protein present. The reaction is stopped by adding excess of 2-mercaptoethanol. Desalt the protein/peptide by collecting the protein/peptide fraction from a reversed-phase HPLC separation. The collected sample can be dried in a vacuum centrifuge before acid hydrolysis. The *S*-carboxyamidomethyl-cysteine formed will be converted to *S*-carboxymethyl-cysteine during acid hydrolysis.

METHOD 10

Cysteine/cystine is reacted with dithiodiglycolic acid or dithiodipropionic acid to produce a mixed disulfide. The choice of dithiodiglycolic acid or dithiodipropionic acid depends on the required resolution of the amino acid analysis method.

Reducing solution

A 10 g/L solution of dithiodiglycolic acid (or dithiodipropionic acid) in 0.2 M sodium hydroxide.

Procedure

Transfer about 20 µg of the test sample to a hydrolysis tube, and add 5 µL of the reducing solution. Add 10 µL of isopropyl alcohol, and then remove all of the sample liquid by vacuum centrifugation. The sample is then hydrolysed using Method 1. This method has the advantage that other amino acid residues are not derivatised by side reactions, and that the sample does not need to be desalted prior to hydrolysis.

METHOD 11

Asparagine and glutamine are converted to aspartic acid and glutamic acid, respectively, during acid hydrolysis. Asparagine and aspartic acid residues are added and represented by *Asx*, while glutamine and glutamic acid residues are added and represented by *Glx*. Proteins/peptides can be reacted with bis (1,1-trifluoroacetoxy)iodobenzene (BTI) to convert the asparagine and glutamine residues to diaminopropionic acid and diaminobutyric acid residues, respectively, upon acid hydrolysis. These conversions allow the analyst to determine the asparagine and glutamine content of a protein/peptide in the presence of aspartic acid and glutamic acid residues.

Reducing solutions

Prepare and filter 3 solutions: a solution of 10 mM trifluoroacetic acid (Solution A), a solution of 5 M guanidine hydrochloride and 10 mM trifluoroacetic acid (Solution B),

and a freshly prepared solution of dimethylformamide containing 36 mg of BTI per millilitre (Solution C).

Procedure

In a clean hydrolysis tube, transfer about 200 µg of the test sample, and add 2 mL of Solution A or Solution B and 2 mL of Solution C. Seal the hydrolysis tube *in vacuo*. Heat the sample at 60 °C for 4 h in the dark. The sample is then dialysed with water to remove the excess reagents. Extract the dialysed sample 3 times with equal volumes of butyl acetate, and then lyophilise. The protein can then be acid hydrolysed using previously described procedures. The α,β-diaminopropionic and α,γ-diaminobutyric acid residues do not typically resolve from the lysine residues upon ion-exchange chromatography based on amino acid analysis. Therefore, when using ion-exchange as the mode of amino acid separation, the asparagine and glutamine contents are the quantitative difference in the aspartic acid and glutamic acid content assayed with underivatised and BTI-derivatised acid hydrolysis. The threonine, methionine, cysteine, tyrosine, and histidine assayed content can be altered by BTI derivatisation; a hydrolysis without BTI will have to be performed if the analyst is interested in the composition of these other amino acid residues of the protein/peptide.

METHODOLOGIES OF AMINO ACID ANALYSIS: GENERAL PRINCIPLES

Many amino acid analysis techniques exist, and the choice of any one technique often depends on the sensitivity required from the assay. In general, about one-half of the amino acid analysis techniques employed rely on the separation of the free amino acids by ion-exchange chromatography followed by post-column derivatisation (e.g., with ninhydrin or *o*-phthalaldehyde). Post-column derivatisation techniques can be used with samples that contain small amounts of buffer components, (such as salts and urea) and generally require between 5 µg and 10 µg of protein sample per analysis. The remaining amino acid techniques typically involve pre-column derivatisation of the free amino acids (e.g., phenyl isothiocyanate; 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate or *o*-phthalaldehyde; (dimethylamino) azobenzenesulfonyl chloride; 9-fluorenylmethyl chloroformate; and 7-fluoro-4-nitrobenzo-2-oxa-1,3-diazole) followed by reversed-phase HPLC. Pre-column derivatisation techniques are very sensitive and usually require between 0.5 µg and 1.0 µg of protein sample per analysis but may be influenced by buffer salts in the samples. Pre-column derivatisation techniques may also result in multiple derivatives of a given amino acid, which complicates the result interpretation. Post-column derivatisation techniques are generally influenced less by performance variation of the assay than pre-column derivatisation techniques.

The following methods may be used for quantitative amino acid analysis. Instruments and reagents for these procedures are available commercially. Furthermore, many modifications of these methodologies exist with different reagent preparations, reaction procedures, chromatographic systems, etc. Specific parameters may vary according to the exact equipment and procedure used. Many laboratories will use more than one amino acid analysis technique to exploit the advantages offered by each. In each of these methods, the analogue signal is visualised by means of a data acquisition system, and the peak areas are integrated for quantification purposes.

METHOD 1 - POST-COLUMN NINHYDRIN DERIVATISATION

Ion-exchange chromatography with post-column ninhydrin derivatisation is one of the most common methods employed for quantitative amino acid analysis. As a rule, a lithium-based cation-exchange system is employed for the analysis of the more complex physiological samples, and the faster sodium-based cation-exchange system is used for the more simplistic amino acid mixtures obtained with protein hydrolysates (typically containing 17 amino acid components). Separation of the amino acids on an ion-exchange column is accomplished through a combination of changes in pH and cation strength. A temperature gradient is often employed to enhance separation.

When the amino acid reacts with ninhydrin, the reactant has a characteristic purple or yellow colour. Amino acids, except imino acid, give a purple colour, and show an absorption maximum at 570 nm. The imino acids such as proline give a yellow colour, and show an absorption maximum at 440 nm. The post-column reaction between ninhydrin and amino acids eluted from the column is monitored at 440 nm and 570 nm, and the chromatogram obtained is used for the determination of amino acid composition.

The detection limit is considered to be 10 pmol for most of the amino acid derivatives, but 50 pmol for the proline derivative. Response linearity is obtained in the range of 20-500 pmol with correlation coefficients exceeding 0.999. To obtain good composition data, samples larger than 1 µg before hydrolysis are best suited for this amino acid analysis of protein/peptide.

METHOD 2 - POST-COLUMN OPA DERIVATISATION

o-Phthalaldehyde (OPA) reacts with primary amines in the presence of thiol compound, to form highly fluorescent isoindole products. This reaction is used for the post-column derivatisation in analysis of amino acids by ion-exchange chromatography. The rule of the separation is the same as Method 1.

Although OPA does not react with secondary amines (imino acids such as proline) to form fluorescent substances, the oxidation with sodium hypochlorite or chloramine T allows secondary amines to react with OPA. The procedure employs a strongly acidic cation-exchange column for separation of free amino acids followed by post-column oxidation with sodium hypochlorite or chloramine T and post-column derivatisation using OPA and a thiol compound such as *N*-acetyl-L-cysteine or 2-mercaptoethanol. The derivatisation of primary amino acids is not noticeably affected by the continuous supply of sodium hypochlorite or chloramine T.

Separation of the amino acids on an ion-exchange column is accomplished through a combination of changes in pH and cation strength. After post-column derivatisation of eluted amino acids with OPA, the reactant passes through the fluorometric detector. Fluorescence intensity of OPA-derivatised amino acids are monitored with an excitation wavelength of 348 nm and an emission wavelength of 450 nm.

The detection limit is considered to be a few tens of picomole level for most of the OPA-derivatised amino acids. Response linearity is obtained in the range of a few picomole level to a few tens of nanomole level. To obtain good compositional data, samples larger than 500 ng of protein/peptide before hydrolysis are recommended.

METHOD 3 - PRE-COLUMN PITC DERIVATISATION

Phenylisothiocyanate (PITC) reacts with amino acids to form phenylthiocarbamyl (PTC) derivatives which can be detected with high sensitivity at 254 nm. Therefore, pre-column derivatisation of amino acids with PITC followed by a reversed-phase HPLC separation with UV detection is used to analyse the amino acid composition.

After the reagent is removed under vacuum, the derivatised amino acids can be stored dry and frozen for several weeks with no significant degradation. If the solution for injection is kept cold, no noticeable loss in chromatographic response occurs after 3 days.

Separation of the PTC-amino acids on a reversed-phase HPLC with an octadecylsilyl (ODS) column is accomplished through a combination of changes in concentrations of acetonitrile and buffer ionic strength. PTC-amino acids eluted from the column are monitored at 254 nm.

The detection limit is considered to be 1 pmol for most of the PTC-amino acids. Response linearity is obtained in the range of 20-500 pmol with correlation coefficients exceeding 0.999. To obtain good compositional data, samples larger than 500 ng of protein/peptide before hydrolysis are recommended.

METHOD 4 - PRE-COLUMN AQC DERIVITISATION

Pre-column derivatisation of amino acids with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) followed by reversed-phase HPLC separation with fluorometric detection is used.

AQC reacts with amino acids to form stable, fluorescent unsymmetric urea derivatives (AQC-amino acids) which are readily amenable to analysis by reversed-phase HPLC. Therefore, pre-column derivatisation of amino acids with AQC followed by reversed-phase HPLC separation with fluorimetric detection is used to analyse the amino acid composition.

Separation of the AQC-amino acids on a reversed-phase HPLC with an ODS column is accomplished through a combination of changes in concentrations of acetonitrile and buffer ionic strengh. Selective fluorescence detection of the derivatives with an excitation wavelength at 250 nm and an emission wavelength at 395 nm allows for the direct injection of the reaction mixture with no significant interference from the only major fluorescent reagent by-product, 6-aminoquinoline. Excess reagent is rapidly hydrolysed ($t_{1/2}$<15 s) to yield 6-aminoquinoline, *N*-hydroxysuccinimide and carbon dioxide, and after 1 min no further derivatisation can take place.

Peak areas for AQC-amino acids are essentially unchanged for at least 1 week at room temperature. Therefore AQC-amino acids have more than sufficient stability to allow for overnight automated chromatographic analysis.

The detection limit is considered to range from about 40 fmol to 320 fmol for each amino acid, except for cystein. The detection limit for cystein is approximately 800 fmol. Response linearity is obtained in the range of 2.5-200 µM with correlation coefficients exceeding 0.999. Good compositional data can be obtained from the analysis of derivatised protein hydrolysates derived from as little as 30 ng of protein/peptide.

METHOD 5 - PRE-COLUMN OPA DERIVATISATION

Pre-column derivatisation of amino acids with *o*-phthalaldehyde (OPA) followed by reversed-phase HPLC separation with fluorometric detection is used. This technique does not detect amino acids that exist as secondary amines (e.g., proline).

OPA in conjunction with a thiol reagent reacts with primary amine groups to form highly fluorescent isoindole products. 2-Mercaptoethanol or 3-mercaptopropionic acid can be used as the thiol. OPA itself does not fluoresce and consequently produces no interfering peaks. In addition, its solubility and stability in aqueous solution, along with the rapid kinetics for the reaction, make it amenable to automated derivatisation and analysis using an autosampler to mix the sample with the reagent. However, lack of reactivity with secondary amino acids has been a predominant drawback. This method does not detect amino acids that exist as secondary amines (e.g., proline). To compensate for this drawback, this technique may be combined with another technique described in Method 7 or Method 8.

Pre-column derivatisation of amino acids with OPA is followed by a reversed-phase HPLC separation. Because of the instability of the OPA-amino acid derivative, HPLC separation and analysis are performed immediately following derivatisation. The liquid chromatograph is equipped with a fluorometric detector for the detection of derivatised amino acids. Fluorescence intensity of OPA-derivatised amino acids is monitored with an excitation wavelength of 348 nm and an emission wavelength of 450 nm.

Detection limits as low as 50 fmol via fluorescence have been reported, although the practical limit of analysis remains at 1 pmol.

METHOD 6 - PRE-COLUMN DABS-CL DERIVATISATION

Pre-column derivatisation of amino acids with (dimethylamino)azobenzenesulfonyl chloride (DABS-Cl) followed by reversed-phase HPLC separation with visible light detection is used.

DABS-Cl is a chromophoric reagent employed for the labelling of amino acids. Amino acids labelled with DABS-Cl (DABS-amino acids) are highly stable and show an absorption maximum at 436 nm.

DABS-amino acids, all naturally occurring amino acid derivatives, can be separated on an ODS column of a reversed-phase HPLC by employing gradient systems consisting of acetonitrile and aqueous buffer mixture. Separated DABS-amino acids eluted from the column are detected at 436 nm in the visible region.

This method can analyse the imino acids such as proline together with the amino acids at the same degree of sensitivity, DABS-Cl derivatisation method permits the simultaneous quantification of tryptophan residues by previous hydrolysis of the protein/peptide with sulfonic acids such as mercaptoethanesulfonic acid, *p*-toluenesulfonic acid or methanesulfonic acid described in Method 2 under Protein hydrolysis. The other acid-labile residues, asparagine and glutamine, can also be analysed by previous conversion into diaminopropionic acid and diaminobutyric acid, respectively, by treatment of protein/peptide with BTI described in Method 11 under Protein hydrolysis.

The non-proteinogenic amino acid norleucine cannot be used as an internal standard in this method as this compound is eluted in a chromatographic region crowded with peaks of primary amino acids. Nitrotyrosine can be used as an internal standard because it is eluted in a clean region.

The detection limit of DABS-amino acid is about 1 pmol. As little as 2-5 pmol of an individual DABS-amino acid can be quantitatively analysed with reliability, and only 10-30 ng

of the dabsylated protein hydrolysate is required for each analysis.

METHOD 7 - PRE-COLUMN FMOC-CL DERIVATISATION

Pre-column derivatisation of amino acids with 9-fluorenylmethyl chloroformate (FMOC-Cl) followed by reversed-phase HPLC separation with fluorometric detection is used.

FMOC-Cl reacts with both primary and secondary amino acids to form highly fluorescent products. The reaction proceeds under mild conditions in aqueous solution and is completed in 30 s. The derivatives are stable, only the histidine derivative showing any breakdown. Although FMOC-Cl is fluorescent itself, the reagent excess and fluorescent side-products can be eliminated without loss of FMOC-amino acids.

FMOC-amino acids are separated by a reversed-phase HPLC using an ODS column. The separation is carried out by gradient elution varied linearly from a mixture of 10 volumes of acetonitrile, 40 volumes of methanol and 50 volumes of acetic acid buffer to a mixture of 50 volumes of acetonitrile and 50 volumes of acetic acid buffer and 20 amino acid derivatives are separated in 20 min. Each derivative eluted from the column is monitored by a fluorometric detector set at an excitation wavelength of 260 nm and an emission wavelength of 313 nm.

The detection limit is in the low femtomole range. A linearity range of 0.1-50 µM is obtained for most of the amino acids.

METHOD 8 - PRE-COLUMN NBD-F DERIVATISATION

Pre-column derivatisation of amino acids with 7-fluoro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-F) followed by reversed-phase HPLC separation with fluorometric detection is used.

NBD-F reacts with both primary and secondary amino acids to form highly fluorescent products. Amino acids are derivatised with NBD-F by heating to 60 °C for 5 min.

NBD-amino acid derivatives are separated on an ODS column of a reversed-phase HPLC by employing a gradient elution system consisting of acetonitrile and aqueous buffer mixture, and 17 amino acid derivatives are separated in 35 min. ε-Aminocaproic acid can be used as an internal standard, because it is eluted in a clean chromatographic region. Each derivative eluted from the column is monitored by a fluorometric detector set at an excitation wavelength of 480 nm and an emission wavelength of 530 nm.

The sensitivity of this method is almost the same as for the pre-column OPA derivatisation method (Method 5), excluding proline to which OPA is not reactive, and might be advantageous for NBD-F against OPA. The detection limit for each amino acid is about 10 fmol. Profile analysis can be achieved with about 1.5 mg of protein hydrolysates in the pre-column reaction mixture.

DATA CALCULATION AND ANALYSIS

When determining the amino acid content of a protein/peptide hydrolysate, it should be noted that the acid hydrolysis step destroys tryptophan and cysteine. Serine and threonine are partially destroyed by acid hydrolysis, while isoleucine and valine residues may be only partially cleaved. Methionine can undergo oxidation during acid hydrolysis, and some amino acids (e.g., glycine and serine) are common contaminants. Application of adequate vacuum (less than 200 µm of mercury or 26.7 Pa) or introduction of inert gas (argon) in the headspace of the reaction vessel during vapour phase hydrolysis can reduce the level of oxidative destruction. Therefore, the quantitative results obtained for cysteine, tryptophan, threonine, isoleucine, valine, methionine, glycine, and serine from a protein/peptide hydrolysate may be variable and may warrant further investigation and consideration.

Amino Acid Mole Percent

This is the number of specific amino acid residues per 100 residues in a protein. This result may be useful for evaluating amino acid analysis data when the molecular mass of the protein under investigation is unknown. This information can be used to corroborate the identity of a protein/peptide and has other applications. Carefully identify and integrate the peaks obtained as directed for each procedure. Calculate the mole percent for each amino acid present in the test sample using the formula:

$$\frac{100r_U}{r}$$

in which r_U is the peak response, in nanomoles, of the amino acid under test; and r is the sum of peak responses, in nanomoles, for all amino acids present in the test sample. Comparison of the mole percent of the amino acids under test to data from known proteins can help establish or corroborate the identity of the sample protein.

Unknown Protein Samples

This data analysis technique can be used to estimate the protein concentration of an unknown protein sample using the amino acid analysis data. Calculate the mass, in micrograms, of each recovered amino acid using the formula:

$$\frac{mM_r}{1000}$$

in which m is the recovered quantity, in nanomoles, of the amino acid under test; and M_r is the average molecular mass for that amino acid, corrected for the mass of the water molecule that was eliminated during peptide bond formation. The sum of the masses of the recovered amino acids will give an estimate of the total mass of the protein analysed after appropriate correction for partially and completely destroyed amino acids. If the molecular mass of the unknown protein is available (i.e., by SDS-PAGE analysis or mass spectroscopy), the amino acid composition of the unknown protein can be predicted. Calculate the number of residues of each amino acid using the formula:

$$\frac{m}{\left(\frac{1000M}{M_n}\right)}$$

in which m is the recovered quantity, in nanomoles, of the amino acid under test; M is the total mass, in micrograms, of the protein; and M_n is the molecular mass of the unknown protein.

Known protein samples

This data analysis technique can be used to investigate the amino acid composition and protein concentration of a protein sample of known molecular mass and amino acid composition using the amino acid analysis data. When the composition of the protein being analysed is known, one can exploit the fact that some amino acids are recovered well, while other amino acid recoveries may be compromised because of complete or partial destruction (e.g., tryptophan, cysteine, threonine, serine, methionine), incomplete bond cleavage (i.e., for isoleucine and valine) and free amino acid contamination (i.e., by glycine and serine).

Because those amino acids that are recovered best represent the protein, these amino acids are chosen to quantify the

amount of protein. Well-recovered amino acids are, typically, aspartate-asparagine, glutamate-glutamine, alanine, leucine, phenylalanine, lysine, and arginine. This list can be modified based on experience with one's own analysis system. Divide the quantity, in nanomoles, of each of the well-recovered amino acids by the expected number of residues for that amino acid to obtain the protein content based on each well-recovered amino acid. Average the protein content results calculated. The protein content determined for each of the well-recovered amino acids should be evenly distributed about the mean. Discard protein content values for those amino acids that have an unacceptable deviation from the mean. Typically greater than 5 per cent variation from the mean is considered unacceptable. Recalculate the mean protein content from the remaining values to obtain the protein content of the sample. Divide the content of each amino acid by the calculated mean protein content to determine the amino acid composition of the sample by analysis.

Calculate the relative compositional error, in percentage, using the formula:

$$\frac{100m}{m_S}$$

in which m is the experimentally determined quantity, in nanomoles per amino acid residue, of the amino acid under test; and m_S is the known residue value for that amino acid. The average relative compositional error is the average of the absolute values of the relative compositional errors of the individual amino acids, typically excluding tryptophan and cysteine from this calculation. The average relative compositional error can provide important information on the stability of analysis run over time. The agreement in the amino acid composition between the protein sample and the known composition can be used to corroborate the identity and purity of the protein in the sample.

M. Glycan Analysis of Glycoproteins

(Ph. Eur. method 2.2.59)

1 INTRODUCTION

Glycan analysis is a test to analyse glycan moieties of glycoproteins. It may involve:

— whole glycoprotein analysis;
— separation and detection of protein glycoforms;
— analysis of glycopeptides obtained after enzymatic treatment of the glycoprotein;
— analysis of released glycans obtained after chemical or enzymatic treatment of the glycoprotein.

Monosaccharide analysis may complement information obtained by glycan analysis.

Glycosylation can play a predominant role in determining the function, pharmacokinetics, pharmacodynamics, stability, and immunogenicity of biotherapeutics. Glycosylation, unlike transcription, is a non-template-driven enzymatic modification process that results in glycan heterogeneity. The manufacturing procedure also has an influence on glycan heterogeneity. Glycoprotein glycan analysis may therefore be an important test to identify variations in the glycosylation pattern of the glycoprotein and/or monitor the consistency of the glycosylation pattern during production.

Glycan analysis can be a comparative procedure, because the information obtained, compared to a similarly treated reference substance, confirms product consistency.

This chapter provides approaches used for glycoprotein glycan analysis and requirements for the application of methods and validation of methods.

Glycan analysis is not a single general method, but involves the application of specific procedures and the development of specific glycan maps for each unique glycoprotein. Specific procedures are therefore indicated in relevant specific monographs.

1-1 PROTEIN GLYCOSYLATION

There are 3 main types of enzymatic glycosylation found in proteins:

— *N*-glycosylation, which involves the addition of oligosaccharides to the nitrogen on the terminal amide group of asparagine;
— *O*-glycosylation, which involves the addition of oligosaccharides to the hydroxyl groups of serine, threonine, and/or hydroxyproline;
— *C*-glycosylation, which involves the addition of an α-mannopyranose to the C2-carbon of the indole ring of tryptophan.

Non-enzymatic additions, also known as glycation, can occur when proteins are incubated with reducing sugars.

This chapter describes analytical methods for the *N*- and *O*-linked glycosylations, which are the most commonly found in glycoprotein medicinal products.

1-2 HETEROGENEITY OF THE PROTEIN GLYCOSYLATION

Different levels of glycan heterogeneity can appear during the production of glycoproteins. This heterogeneity may result from variations:

— in the degree of occupancy (full, partial, unoccupied);
— in the type of glycosylation (*N*- or *O*-linked);
— in the oligosaccharide structures (extensions, branching and linkage).

This heterogeneity in glycosylation results in a set of glycoforms for one specific glycoprotein. These variations arise because, unlike transcription and translation, glycosylation is a non-template post-translational modification process. The glycosylation pattern at a given site depends on many factors including the cell-specific and/or growth-dependent availability of glycosyltransferases and exo-glycosidases found in the Golgi apparatus and endoplasmic reticulum. Protein glycosylation is also influenced by the protein structure, the production process, the host-vector expression system and the cell culture conditions.

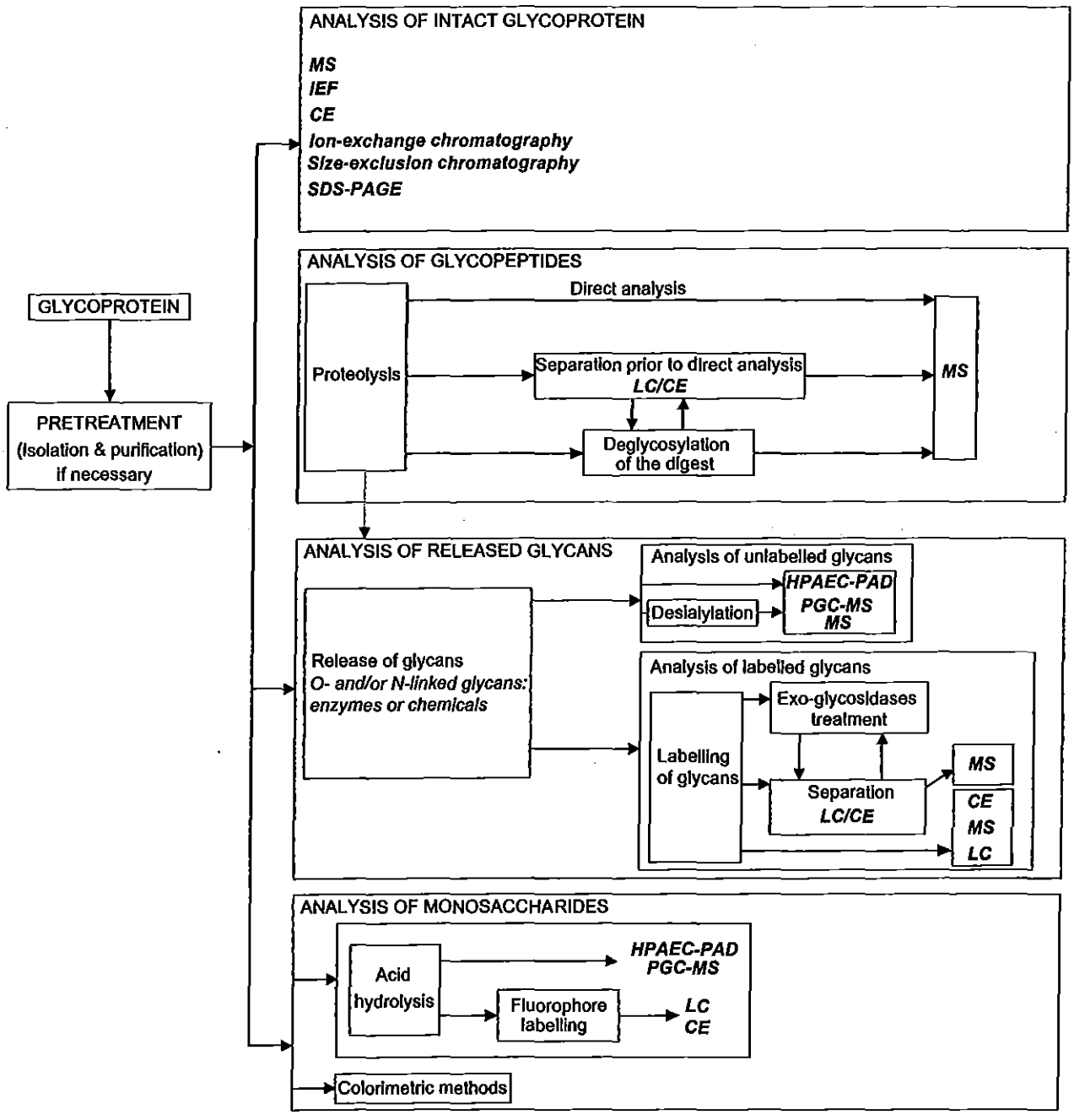
2 GLYCAN ANALYSIS PROCEDURES

Heterogeneity in glycosylation can be assessed by 4 distinct and complementary approaches:

— analysis of the intact glycoprotein;
— analysis of glycopeptides;
— analysis of released glycans;
— monosaccharide analysis.

The present section provides methods and general requirements used for glycan analysis of glycoproteins containing *N*- and *O*-linked glycans.

Glycan analysis is usually a multistep process. There are numerous methodologies for glycan analysis. This variety is a consequence of the diversity and complexity of glycan structures, of the available technologies and detection systems, and of the wide range of approaches depending on the level of information required.



CE: Capillary electrophoresis
HPAEC-PAD: High-pH anion-exchange chromatography with pulsed amperometric detection
IEF: Isoelectric focusing
LC: Liquid chromatography
MS: Mass spectrometry
PGC: Porous graphite chromatography
SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis

Figure 2.2.59.-1. – *Overview of glycan analysis procedures*

Figure 2.2.59.-1 provides an overview of glycan analysis analytical procedures that can be employed to apply the chosen approach(es). Many variations of the same techniques and conditions are available depending on the glycan structures and origin.

Isolation and purification

Isolation and purification may be necessary for analysis of bulk drug substances or dosage forms containing interfering excipients, and, when required, will be described in the specific monograph.

2-1 ANALYSIS OF INTACT GLYCOPROTEIN

Analysis of the intact glycoprotein provides information on the overall pattern of glycosylation of the glycoprotein.

This approach provides limited information when the molecule is large and contains multiple glycosylation sites.

Methods such as capillary electrophoresis (CE) (*2.2.47*) and mass spectrometry (MS) (*2.2.43*) can be used. Size-based techniques, such as size-exclusion chromatography (*2.2.30*) and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (*2.2.31*), may provide

information on the glycosylation status of a protein. If the degree of sialylation significantly contributes to the biological activity of the glycoprotein, ion-exchange chromatography (*2.2.46*), isoelectric focusing (IEF) (*2.2.54*) or CE (*2.2.47*) may be performed to monitor sialylation. The technique must be chosen according to its suitability to provide a reliable correlation between the degree of sialylation and the bioactivity of the product.

2-2 ANALYSIS OF GLYCOPEPTIDES

Analysis of glycopeptides provides information on site-specific glycosylation properties, on the degree of occupancy, and on the oligosaccharide structures. It involves proteolytic digestion of the glycoprotein. Approaches to site-specific cleavage of the protein backbone are given in general chapter *2.2.55. Peptide mapping.*

After proteolysis of the glycoprotein, the following approaches can be chosen.

Direct analysis by MS (2.2.43) Care should be taken that the glycopeptide signal is not suppressed due to the presence of other peptides, where glycopeptides represent a minor portion of the total peptide mixture and where signal intensities are lower than those of non-glycosylated peptides.

Separation prior to analysis by MS This additional step overcomes the problems raised above. Enrichment or fractionation techniques can be used either in parallel with or sequentially to direct analysis. Separation techniques such as liquid chromatography (LC) (*2.2.29*) and CE (*2.2.47*) are suitable. These techniques may be interfaced with MS to allow online MS measurements.

Deglycosylation of the glycopeptides Identification of the different glycosylation sites of a glycoprotein is made possible by comparing peptide maps obtained by proteolytic digestion of the intact glycoprotein to those obtained when the glycoprotein is deglycosylated previously or following proteolytic digestion. The peptide mass gives information about the glycosylation sites and by calculating the mass difference between the intact glycopeptide and the deglycosylated glycopeptide, it is possible to obtain information about the attached glycans concerning composition and heterogeneity. Approaches to deglycosylation of the protein backbone are given in section 2-3-1. A separation step can be performed after or before deglycosylation.

2-3 ANALYSIS OF RELEASED GLYCANS

Analysis of released glycans provides a convenient way to obtain information on the various populations of glycans present on the protein (bi-, tri-, and tetra-antennary profile). The degree of sialylation can also be addressed at this stage. Depending on the chosen method, prior derivatisation/labelling may be needed to allow the detection of the glycans.

Analysis of released glycans generally involves the release and purification of glycans from the reaction mixture, followed by the labelling/derivatisation of the glycans, where needed; the glycans are then profiled (fractionation or separation).

2-3-1 Release of glycans

The selection of the approach used for the release of glycans will depend on the glycoprotein under test. The cleavage agent to be employed is chosen according to the type of cleavage needed and level of information required. Enzymatic or chemical cleavage may be used. Table 2.2.59.-1 gives a non-exhaustive list of enzymatic cleavage agents and their specificity.

Digestion efficiency is generally dependent on the accessibility of the glycans on the protein and hence the protein can be denatured to maximise glycosylation site exposure, unless it is desirable to distinguish between surface and buried glycans.

Chemical cleavage agents might also be used, using for example hydrazine or alkaline borohydride for β-elimination.

Table 2.2.59.-1. – *Examples of enzymatic cleavage agents*

Agents	Specificity
***N*-linked glycans release**	
Peptide-N^4-(*N*-acetyl-β-glucosaminyl)asparagine amidase (EC 3.5.1.52)	Hydrolysis of an N^4-(acetyl-β-D-glucosaminyl)asparagine residue in which the glucosamine residue may be further glycosylated, to yield a (substituted) *N*-acetyl-β-D-glucosaminylamine and a peptide containing an aspartate residue
- Peptide *N*-glycosidase F (PNGase F)	Release of *N*-glycan chain but no release of *N*-glycan chain containing (α1-3)-linked core fucose
- Peptide *N*-glycosidase A (PNGase A)	Release of *N*-glycan chain containing (α1-3)-linked core fucose
Mannosyl-glycoprotein endo-β-*N*-acetylglucosaminidase (EC 3.2.1.96)	Endohydrolysis of the *N,N'*-diacetylchitobiosyl unit in high-mannose glycopeptides/ glycoproteins containing the -[Man(GlcNAc)$_2$] Asn structure
- Endo-β-*N*-acetylglucosaminidase F (endo F)	Release of high-mannose, hybrid and complex oligosaccharides
- Endo-β-*N*-acetylglucosaminidase H (endo H)	Release of high-mannose, hybrid oligosaccharides
***O*-linked glycans release**	
Glycopeptide α-*N*-acetylgalactosaminidase (EC 3.2.1.97)*	Hydrolysis of terminal D-galactosyl-*N*-acetyl-α-D-galactosaminidic residues
* This enzyme has limited usage because of its high substrate specificity.	

2-3-2 Analysis of glycans

Released glycans can be analysed or profiled by chromatographic, electrophoretic and mass spectrometric techniques, and in general by a combination of these techniques. The choice of the method can be grouped according to the nature of the glycans and level of information required.

Analysis of glycans provides information on the various populations of glycans present on the protein (high-mannose, hybrid, complex). Information on the relative amounts of branched structures might be obtained by analysis of desialylated glycans.

A separation step may be required. It implies the use of LC (*2.2.29*) and CE (*2.2.47*) as intermediate techniques. LC (*2.2.29*) can be used preparatively with individual fractions being collected (usually labelling is required) or can be directly coupled to MS (*2.2.43*).

2-3-2-1 Analysis of unlabelled glycans

Native glycans can be analysed by high-pH anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD), porous graphite chromatography (PGC) and MS (*2.2.43*).

HPAEC-PAD has high sensitivity and can also separate some linkage isomers. Response factors of the different signals are not equal for the different oligosaccharide structures. Absolute quantification of the glycan is not possible unless an oligosaccharide reference library is available. Quantification can be obtained by comparison with a well-characterised reference standard of the substance being tested, or by relating the peak area of each glycan to the total peak area of all glycans in the map.

PGC can also be used to separate native glycans because of its higher selectivity compared to the conventional non-polar columns. A PGC-electrospray-ionisation-MS approach can be applied for direct glycan analysis.

2-3-2-2 Analysis of labelled glycans

Labelling of glycans

The type of derivatisation carried out will depend on the method used to detect glycans: UV or fluorescent.

Derivatisation with fluorescent labels is the most commonly used technique for labelling glycans at their reducing end by reductive amination. One label can be attached to every single mono- and oligo-saccharide, allowing the determination of molar quantities. Table 2.2.59.-2 gives a non-exhaustive list of commonly used fluorescent labels and suitable analytical techniques.

Table 2.2.59.-2. – *Examples of fluorescent labels and suitable techniques*

Name	Acronym	Analytical techniques
2-Aminobenzoic acid	2-AA	LC (*2.2.29*), MS (*2.2.43*)
2-Aminobenzamide	2-AB	LC (*2.2.29*), MS (*2.2.43*)
2-Aminopyridine	2-AP	LC (*2.2.29*), MS (*2.2.43*)
2-Amino-9(10*H*)-acridinone	AMAC	Gel electrophoresis (*2.2.23*)
Trisodium 8-aminopyrene-1,3,6-trisulfonic acid	APTS	CE (*2.2.47*)

Permethylation of glycans may also be used when MS (*2.2.43*) is used alone for detection. It is based on the methylation of the oligosaccharides.

Analysis of labelled glycans

Labelled glycans can be analysed by analytical techniques such as LC (*2.2.29*), CE (*2.2.47*) and MS (*2.2.43*).

According to the separation properties of the glycans, glycans can be profiled and quantified by several LC (*2.2.29*) systems using an appropriate label: reversed-phase (separation by hydrophobicity), normal-phase (separation by size), and anion-exchange (separation by charge) LC.

2-4 MONOSACCHARIDE ANALYSIS

Monosaccharide analysis provides information on the monosaccharide composition of a glycoprotein. Analysis of monosaccharides can be performed using either colorimetric or separation methods.

2-4-1 Colorimetric methods

The colorimetric methods, which are based on chemical staining, provide information on the quantity of specific classes of sugars such as sialic acids, neutral sugars and hexosamines.

2-4-2 Separation methods

The separation methods generate quantitative information on the overall monosaccharide composition. The methods require acid hydrolysis pre-treatment of the oligosaccharide chains of the intact glycoprotein or released glycans, prior to analysis. To release sialic acids, mild acid hydrolysis or enzymatic treatment is employed. The hydrolysis step is a significant source of variability and may require product-specific validation.

Methods for separation and quantification of monosaccharides include:

— the use of HPAEC-PAD and PGC-MS, which allow the determination of molar quantities of native monosaccharides (sialic acids, neutral sugars and alcohol sugars);

— fluorophore labelling of monosaccharides followed by separation methods such as reversed-phase or ion-exchange chromatography, or CE.

3 EVALUATION AND ANALYSIS OF DATA

Data obtained from analytical methods for the analysis of glycans can be analysed and evaluated for 3 different purposes:

— confirmation of identity of individual structures or families of structures;

— confirmation of compliance of the substance being tested with qualitative requirements;

— confirmation of compliance of the substance being tested with quantitative requirements.

Specific considerations with respect to reference standards and method development of each level of analysis are set out in sections 4 and 5 respectively.

3-1 CONFIRMATION OF IDENTITY OF INDIVIDUAL STRUCTURES OR FAMILIES OF STRUCTURES

The analytical target for a glycan analysis method may be an individual monosaccharide (e.g. sialic acid, fucose), a defined oligosaccharide structure (e.g. tetra-sialylated, tetra-antennary glycan) or a family of structures sharing a common analytical feature (e.g. tetra-sialylated glycans, tri-antennary glycans, glycoprotein isoforms with the same charge). Confirmation of the identity of the analytical target is an essential step in the analysis and evaluation of data, and can be achieved absolutely, by verification of molecular structure, or comparatively, by comparison with an appropriate reference standard.

3-1-1 Absolute confirmation of identity

Absolute confirmation of the identity of glycan structures is typically achieved during product development, and should not necessarily be the target of routine analysis. Identity of the analytical target will be assigned by reference to a known molecular property of the molecule. Such absolute identification of individual structures can require multi-step approaches using enzymatic and chemical reactions, separation techniques and online or offline detection methods, and will most commonly use the charge-to-mass ratio of a molecular ion, determined using a suitable mass spectrometric method as the final basis for structure assignment.

3-1-2 Comparative confirmation of identity

During routine application of the analytical method, the identity of the analytical target may be confirmed by comparison with process or system suitability reference standards. These may be generated from known, well-characterised glycoproteins, which may be of the same general class as the product being tested (e.g. fetuin for complex *N*-linked glycoproteins), or may be derived from a well-characterised batch of the product being tested, which has been established as a reference standard. The following

considerations apply to comparative assignment of structural identity:
- in the case of a validated high reproducibility of the retention times, the absolute retention times can be used for correct assignment;
- alternatively, a glycan marker can be injected at the beginning and at the end of the testing sequence and checked for any drifts in the retention times; based on these reference chromatograms the glycans of the test samples can be assigned;
- in cases where no standard is available to assign all glycan peaks in the test sample, absolute or normalised retention times can be used to monitor and label unidentified glycan peaks.

3-2 CONFIRMATION OF COMPLIANCE OF THE SUBSTANCE BEING TESTED WITH QUALITATIVE REQUIREMENTS

At this level of evaluation, the analytical results obtained with the product being tested are evaluated to demonstrate compliance with specifications. Typically this is achieved by comparison with data obtained in parallel using a reference standard of the substance being tested. In evaluating the data it is necessary:
- to establish that the analytical result obtained using the reference standard is broadly comparable to the expected result, to verify the suitability of the system; for example, in a glycan mapping procedure, this would be achieved by comparison of the map obtained with the reference substance with a provided specimen map obtained during establishment of the reference substance, and by ensuring compliance with all stated system suitability criteria;
- to demonstrate similarity of the maps obtained with the reference substance and the test substance, using any specific compliance criteria given in the specific monograph.

3-3 CONFIRMATION OF COMPLIANCE OF THE SUBSTANCE BEING TESTED WITH QUANTITATIVE REQUIREMENTS

3-3-1 Quantitative measurement of analyte levels and expression of results

In some cases, e.g. measurement of sialic acid or other monosaccharides, data can be expressed in order to obtain a molar ratio of sialic acid to glycoprotein. Data is calculated by reference to a reference standard for sialic acid and to a validated method of protein determination. Either the internal or external standard method may be used (see general chapter *2.2.46. Chromatographic separation techniques*).

3-3-2 Quantitative expressions of separation profile

Profiles or distribution patterns may be expressed numerically in a number of ways, including the normalisation procedure; the percentage content of each analytical target, e.g. glycan entity, is calculated by determining the response of the glycan entity as a percentage of the total response of all the entities, excluding those due to solvents or any added reagents, and those below the disregard limit. In addition, numerical expressions such as the *Z* number, which are method- and product-specific and defined in specific monographs, can be used.

4 REFERENCE STANDARDS

Reference standards for glycan analysis serve 2 functions: the verification of the suitability of the system and the confirmation that the article under test complies with specified requirements.

The reference standards used for system suitability may be:
- a reference substance for the substance being tested;
- glycan moities liberated from a fully characterised reference standard of the substance being tested;
- well-characterised glycan moities liberated from glycoproteins (e.g. fetuin, IgG);
- glycan markers characterised for identity and purity.

The reference standard used for compliance of the glycoprotein under test is a preparation of the substances being tested. It is noted that glycan analysis procedures described in specific monographs prescribe the use of a reference standard for the substance being tested and for which the glycan analysis procedure has been validated.

5 POINTS TO CONSIDER IN METHOD DEVELOPMENT

This section provides means for measuring the overall performance of the method during development. The extent of method development and analytical validation is selected on the basis of their suitability for a specific product. Depending on the chosen approach, several steps are necessary for glycan analysis, for example:
- isolation and purification (or desalting) of the glycoprotein;
- enzymatic (or chemical) treatment of the glycoprotein to selectively release either *N*- or *O*-linked glycans from the protein backbone;
- isolation and purification of the released glycans;
- verification of released sialic acid and monosaccharide residues;
- chromophore labelling of the released glycans;
- separation of the glycans, native or fluorescence labelled;
- glycan identification and quantification (e.g. determination of the *Z* number);
- determination of site occupancy based on relative quantities of glycosylated and non-glycosylated peptides.

Protein isolation and purification

Isolation and purification of the glycoprotein from its matrix may be necessary to remove all interfering substances (e.g. excipients, salts) and, when required, will be specified in the specific monograph. This must be performed in a reproducible manner in order to guarantee a quantitative recovery of the protein.

Release and isolation of oligosaccharides

The approach chosen for the release of glycans will depend on the protein under test and will be based on the types of glycosylation, i.e. *N*- or *O*-linked glycosylation. Non-compendial approaches available for the release of glycans must be optimised in order to ascertain a quantitative profiling of all glycan entities. Factors that impact cleavage efficiency, such as enzyme-to-protein concentration ratio, temperature, reaction time course, and denaturation of protein prior to digestion, must be optimised.

It is noteworthy that the enzymatic/chemical reaction must not alter the glycan composition, e.g. not destroy sialic acid residues. Where there is more than one glycosylation site, the enzymatic treatment should proportionally release all oligosaccharide moieties attached to the protein, independent of their structure and their individual position in the protein. Reproducible recovery of all glycan entities from the reaction mixture must be confirmed.

Derivatisation of released glycans

Derivatisation is usually carried out according to non-compendial protocols. Therefore, the reproducible derivatisation of all glycan entities must be verified. This may be achieved through optimisation of the reaction conditions such as amount of the derivatisation reagent, reaction temperature and time. The derivatisation reaction must not

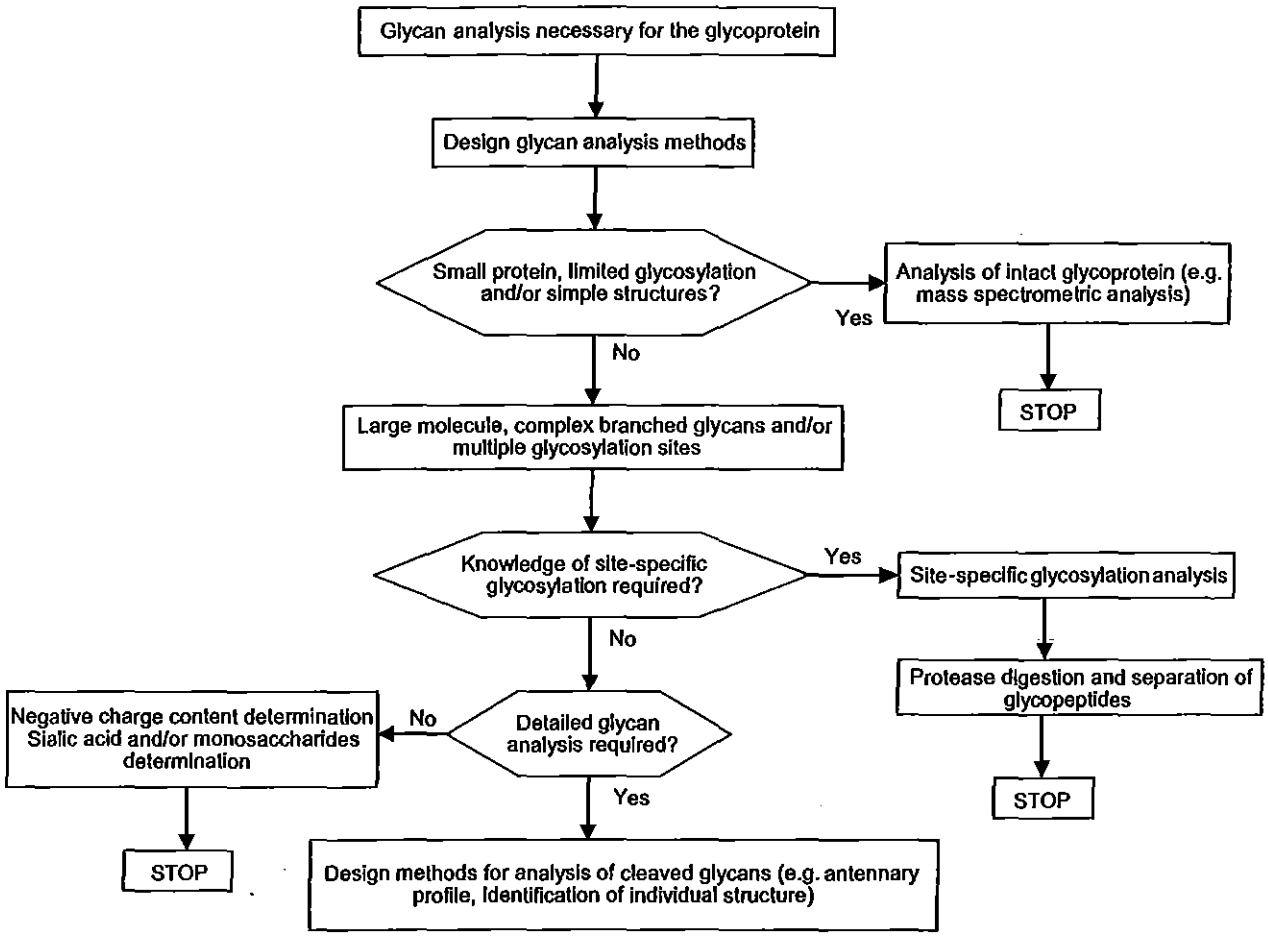


Figure 2.2.59.-2. – *Guidance on methods to be used when glycan analysis is required*

change the glycan composition, e.g. not destroy sialic acid residues.

Separation, identification and system suitability

The methods employed for glycan analysis must be capable of detecting and separating different glycan moieties to ascertain a reliable identification and quantification.

The acceptance criteria for system suitability, which also cover glycan cleavage, recovery and analysis, depend on the critical test parameters that affect the outcome of the result.

A comparison between the glycan map of the substance under test and that of a reference substance, being treated in the same conditions, is an indicator to evaluate the performance of the analytical procedure. In order to further confirm the obtained results, the analyses may be repeated with an orthogonal method. The use of a reference standard (e.g. reference substance of the product being examined, system suitability glycan marker) is essential in the establishment of system suitability parameters and validation of the analytical procedure.

Reproducibility of quantitative expression (e.g. *Z* number estimation) of glycan profiles must be verified.

Determination of site occupancy based on relative quantities of glycosylated and non-glycosylated peptides

Where site occupancy is estimated by comparison of glycosylated and non-glycosylated peptides from an enzymatically digested glycoprotein, reproducible cleavage of both forms of the peptide must be demonstrated.

6 GLYCAN ANALYSIS DECISION-MAKING FRAMEWORK

This decision-making framework is given for information and does not constitute a mandatory part of the European Pharmacopoeia.

The choice of procedures used to analyse glycans is established according to the level of information required to ensure the quality of the glycoprotein and is set up during the development phase of the product.

Figure 2.2.59.-2 provides guidance in the choice of methods to be used when glycan analysis is required.

Appendix IV

A. Clarity of Solution

(Ph. Eur. method 2.2.1)

Opalescence is the effect of light being absorbed or scattered by submicroscopic particles or optical density inhomogeneities. The absence of any particles or inhomogeneities in a solution results in a clear solution.

A liquid is considered *clear* if its clarity is the same as that of *water R* or of the solvent used, or if its opalescence is not more pronounced than that of reference suspension I (see Table 2.2.1.-1), when examined under the conditions described below.

Requirements in monographs are expressed in terms of the visual method by comparing with the defined reference suspensions (see Table 2.2.1.-1). However, instrumental methods may also be used for determining compliance with monograph requirements once the suitability of the instrument has been established as described below and calibration with reference suspensions I-IV and with *water R* or the solvent used has been performed.

VISUAL METHOD

Using identical test-tubes of colourless, transparent, neutral glass with a flat base and an internal diameter of 15-25 mm, compare the liquid to be examined with a reference suspension freshly prepared as described below. Ensure that the depths of the layers in the 2 test-tubes are the same (about 40 mm).

Compare the liquids in diffused daylight 5 min after preparation of the reference suspension, viewing vertically against a black background.

System suitability The diffusion of light must be such that reference suspension I can readily be distinguished from *water R*, and that reference suspension II can readily be distinguished from reference suspension I (see Table 2.2.1.-1).

INSTRUMENTAL METHOD

The instrumental assessment of clarity and opalescence provides a more discriminatory test that does not depend on the visual acuity of the analyst. Numerical results are more useful for process control and quality monitoring, especially in stability studies. For example, previous numerical data on stability can be extrapolated to determine whether a given batch of a preparation will exceed shelf-life limits prior to the expiry date.

TURBIDIMETRY AND NEPHELOMETRY

When a suspension is viewed at right angles to the direction of the incident light, the system appears opalescent due to the scattering of light by the particles of the suspension (Tyndall effect). A certain portion of the light beam entering a turbid liquid is transmitted, another portion is absorbed and the remaining portion is scattered by the suspended particles. The light-scattering effect of suspended particles can be measured either indirectly by observation of the transmitted light (turbidimetry) or directly by measuring the scattered light (nephelometry). Turbidimetry and nephelometry are more reliable in low turbidity ranges, where there is a linear relationship between turbidity values and detector signals. As the degree of turbidity increases, not all the particles are exposed to the incident light and the scattered or the transmitted radiation of other particles is hindered on its way to the detector.

For quantitative measurements, the construction of calibration curves is essential. Linearity must be based on at least 4 levels of concentrations. Reference suspensions must show a sufficiently stable degree of turbidity and must be produced under well-defined conditions.

MEASUREMENTS IN RATIO MODE

The determination of opalescence of coloured liquids is done using instruments with ratio mode, since colour provides a negative interference, attenuating both incident and scattered light and lowering the turbidity value. The effect is so great, even for moderately coloured samples, that conventional nephelometers cannot be used.

In turbidimetry or nephelometry with ratio mode, the ratio of the transmission measurement to the 90° scattered light measurement is determined. This procedure compensates for the light that is diminished by the colour of the sample. Instruments with ratio mode use as light source a tungsten lamp with spectral sensitivity at about 550 nm operating at a filament colour temperature of 2700 K. Other suitable light sources may also be used. Silicon photodiodes and photomultipliers are commonly used as detectors and record changes in light scattered or transmitted by the sample. The light scattered at 90 ± 2.5° is measured by the primary detector. Other detectors measure back and forward scatter (reflected light) as well as transmitted light. The results are obtained by calculating the ratio of the 90° scattered light measured to the sum of the components of forward scattered and transmitted light values.

The instruments used are calibrated against standards of known turbidity and are capable of automatic measurement of turbidity. The test results are obtained directly from the instrument and compared to the specifications in the individual monograph.

Alternatively, the influence of the colour of the sample may also be eliminated by using an infrared light-emitting diode (IR LED) having an emission maximum at 860 nm with a 60 nm spectral bandwidth as the light source of the instrument.

INSTRUMENT REQUIREMENTS

Instruments complying with the following characteristics and verified using reference suspensions as described below may be used instead of visual examination for determination of compliance with monograph requirements.

- *Measuring unit*: NTU (nephelometric turbidity units). NTU is based on the turbidity of a primary standard of formazin. FTU (formazin turbidity units) or FNU (formazin nephelometric units) are also used, and are equivalent to NTU in regions of low turbidity (up to 40 NTU). These units are used in all 3 instrumental methods (nephelometry, turbidimetry and in ratio mode).
- *Measuring range*: 0.01-1100 NTU.
- *Resolution*: 0.01 NTU within the range 0-9.99 NTU; 0.1 NTU within the range 10.0-99.9 NTU; and 1 NTU for the range > 100 NTU.
- *Accuracy*: ± (10 per cent of reading + 0.01 NTU) within the range 0-20 NTU; ± 7.5 per cent within the range 20-1100 NTU.
- *Repeatability*: ± 0.05 NTU within the range 0-20 NTU; ± 2 per cent of the reading within the range 20-1100 NTU.

Instruments with measuring range or resolution, accuracy and repeatability capabilities other than those mentioned above may be used provided they are sufficiently validated and are capable for the intended use.

CONTROL OF INSTRUMENT PERFORMANCE

— *Calibration*: performed with at least 4 reference suspensions of formazin covering the measuring range of interest. Reference suspensions described in this chapter or suitable reference standards calibrated against the primary reference suspensions may be used.

— *Stray light*: < 0.15 NTU within the range 0-10 NTU; < 0.5 NTU within the range 10-1100 NTU. Stray light is defined as that light that reaches the nephelometric detector without being a result of scatter from the sample. Stray light is always a positive interference and is a significant source of error in low-range turbidity measurements. Sources of stray light include: imperfections in and scratches on sample cells, internal reflections of the optical system, contamination of the optics or sample cell chamber with dust, and electronic noise. Instrument design can also affect stray light. The influence of stray light becomes negligible in ratio mode measurements.

The test methodology for the specific substance/product to be analysed must also be verified to demonstrate its analytical capability. The instrument and methodology shall be consistent with the attributes of the substance to be examined.

Measurements of standards and samples should be carried out under the same temperature conditions, preferably between 20 °C and 25 °C.

REFERENCE SUSPENSIONS

Formazin has several desirable characteristics that make it an excellent turbidity standard. It can be reproducibly prepared from assayed raw materials. The physical characteristics make it a desirable light-scatter calibration standard. The formazin polymer consists of chains of different lengths, which fold into random configurations. This results in a wide variety of particle shapes and sizes, which allows the analysis of different particle sizes and shapes that are found in real samples. Stabilised formazin suspensions that can be used to prepare stable, diluted turbidity standards are commercially available and may be used after comparison with the standards prepared as described.

All steps of the preparation of reference suspensions as described below are carried out at 25 ± 3 °C.

Hydrazine sulfate solution

Dissolve 1.0 g of *hydrazine sulfate R* in *water R* and dilute to 100.0 mL with the same solvent. Allow to stand for 4-6 h.

Primary opalescent suspension (formazin suspension)

In a 100 mL ground-glass-stoppered flask, dissolve 2.5 g of *hexamethylenetetramine R* in 25.0 mL of *water R*. Add 25.0 mL of the hydrazine sulfate solution. Mix and allow to stand for 24 h. This suspension is stable for 2 months, provided it is stored in a glass container free from surface defects. The suspension must not adhere to the glass and must be mixed thoroughly before use.

Standard of opalescence

Dilute 15.0 mL of the primary opalescent suspension to 1000.0 mL with *water R*. This suspension is freshly prepared and may be stored for up to 24 h.

Reference suspensions

Prepare the reference suspensions according to Table 2.2.1.-1. Mix and shake before use.

Table 2.2.1.-1

	I	II	III	IV
Standard of opalescence	5.0 mL	10.0 mL	30.0 mL	50.0 mL
Water R	95.0 mL	90.0 mL	70.0 mL	50.0 mL

Measurements of reference suspensions I-IV in ratio mode show a linear relationship between the concentrations and measured NTU values (see Table 2.2.1.-2).

Table 2.2.1.-2

Formazin suspensions	Opalescent values (NTU)
Reference suspension I	3
Reference suspension II	6
Reference suspension III	18
Reference suspension IV	30
Standard of opalescence	60
Primary opalescent suspension	4000

B. Colour of Solution[1]

(Ph. Eur. method 2.2.2)

◊A solution is *colourless* if it has the appearance of *water R* or the solvent used for the preparation of the solution to be examined, or is not more intensely coloured than reference solution B_9.

Report the results together with the method used (method I, method II or method III).

VISUAL METHODS

The examination of the degree of coloration of liquids in the range brown-yellow-red is carried out using one of the 2 methods below, as prescribed in the monograph.

METHOD I

Using identical tubes of colourless, transparent, neutral glass with an external diameter of 12 mm, compare 2.0 mL of the liquid to be examined with 2.0 mL of *water R*, of the solvent used for the preparation of the solution to be examined, or of the reference solution (see Tables of reference solutions) prescribed in the monograph. Compare the colours in diffuse daylight, viewing horizontally against a white background.

METHOD II

Using identical tubes of colourless, transparent, neutral glass with a flat base and an internal diameter of 15-25 mm, compare the liquid to be examined with *water R*, with the solvent used for the preparation of the solution to be examined, or with the reference solution (see Tables of reference solutions) prescribed in the monograph, the depth of the layer being 40 mm. Compare the colours in diffuse daylight, viewing vertically against a white background.

PREPARATION OF REFERENCE SOLUTIONS

Primary solutions

Yellow solution Dissolve 46 g of *ferric chloride R* in about 900 mL of a mixture of 25 mL of *hydrochloric acid R* and 975 mL of *water R* and dilute to 1000.0 mL with the same mixture. Titrate and adjust the solution to contain 45.0 mg of $FeCl_3,6H_2O$ per millilitre by adding the same acidic mixture. Protect the solution from light.

Titration. In a 250 mL conical flask fitted with a ground-glass stopper, introduce 10.0 mL of the solution, 15 mL of

[1] *This chapter has undergone pharmacopoeial harmonisation. See chapter 5.8. Pharmacopoeial harmonisation.*

water R, 5 mL of *hydrochloric acid R* and 4 g of *potassium iodide R*, close the flask, allow to stand in the dark for 15 min and add 100 mL of *water R*. Titrate the iodine released with *0.1 M sodium thiosulfate*, using 0.5 mL of *starch solution R*, added towards the end of the titration, as indicator.

1 mL of *0.1 M sodium thiosulfate* is equivalent to 27.03 mg of $FeCl_3,6H_2O$.

Red solution Dissolve 60 g of *cobalt chloride R* in about 900 mL of a mixture of 25 mL of *hydrochloric acid R* and 975 mL of *water R* and dilute to 1000.0 mL with the same mixture. Titrate and adjust the solution to contain 59.5 mg of $CoCl_2,6H_2O$ per millilitre by adding the same acidic mixture.

Titration. In a 250 mL conical flask fitted with a ground-glass stopper, introduce 5.0 mL of the solution, 5 mL of *dilute hydrogen peroxide solution R* and 10 mL of a 300 g/L solution of *sodium hydroxide R*. Boil gently for 10 min, allow to cool and add 60 mL of *dilute sulfuric acid R* and 2 g of *potassium iodide R*. Close the flask and dissolve the precipitate by shaking gently. Titrate the iodine released with *0.1 M sodium thiosulfate*, using 0.5 mL of *starch solution R*, added towards the end of the titration, as indicator. The end-point is reached when the solution turns pink.

1 mL of *0.1 M sodium thiosulfate* is equivalent to 23.79 mg of $CoCl_2,6H_2O$.

Blue primary solution Dissolve 63 g of *copper sulfate pentahydrate R* in about 900 mL of a mixture of 25 mL of *hydrochloric acid R* and 975 mL of *water R* and dilute to 1000.0 mL with the same mixture. Titrate and adjust the solution to contain 62.4 mg of $CuSO_4,5H_2O$ per millilitre by adding the same acidic mixture.

Titration. Into a 250 mL conical flask fitted with a ground-glass stopper, introduce 10.0 mL of the solution, 50 mL of *water R*, 12 mL of *dilute acetic acid R* and 3 g of *potassium iodide R*. Titrate the iodine released with *0.1 M sodium thiosulfate*, using 0.5 mL of *starch solution R*, added towards the end of the titration, as indicator. The end-point is reached when the solution shows a slight pale brown colour.

1 mL of *0.1 M sodium thiosulfate* is equivalent to 24.97 mg of $CuSO_4,5H_2O$.

Standard solutions

Using the 3 primary solutions, prepare the 5 standard solutions as follows.

Table 2.2.2.-1.

Standard solution	Volumes in millilitres			
	Yellow solution	Red solution	Blue solution	Hydrochloric acid (10 g/L HCl)
B (brown)	3.0	3.0	2.4	1.6
BY (brownish-yellow)	2.4	1.0	0.4	6.2
Y (yellow)	2.4	0.6	0.0	7.0
GY (greenish-yellow)	9.6	0.2	0.2	0.0
R (red)	1.0	2.0	0.0	7.0

Reference solutions for Methods I and II

Using the 5 standard solutions, prepare the following reference solutions.

Table 2.2.2.-2. – *Reference solutions B*

Reference solution	Volumes in millilitres	
	Standard solution B	Hydrochloric acid (10 g/L HCl)
B_1	75.0	25.0
B_2	50.0	50.0
B_3	37.5	62.5
B_4	25.0	75.0
B_5	12.5	87.5
B_6	5.0	95.0
B_7	2.5	97.5
B_8	1.5	98.5
B_9	1.0	99.0

Table 2.2.2.-3. – *Reference solutions BY*

Reference solution	Volumes in millilitres	
	Standard solution BY	Hydrochloric acid (10 g/L HCl)
BY_1	100.0	0.0
BY_2	75.0	25.0
BY_3	50.0	50.0
BY_4	25.0	75.0
BY_5	12.5	87.5
BY_6	5.0	95.0
BY_7	2.5	97.5

Table 2.2.2.-4. – *Reference solutions Y*

Reference solution	Volumes in millilitres	
	Standard solution Y	Hydrochloric acid (10 g/L HCl)
Y_1	100.0	0.0
Y_2	75.0	25.0
Y_3	50.0	50.0
Y_4	25.0	75.0
Y_5	12.5	87.5
Y_6	5.0	95.0
Y_7	2.5	97.5

Table 2.2.2.-5. – *Reference solutions GY*

Reference solution	Volumes in millilitres	
	Standard solution GY	Hydrochloric acid (10 g/L HCl)
GY_1	25.0	75.0
GY_2	15.0	85.0
GY_3	8.5	91.5
GY_4	5.0	95.0
GY_5	3.0	97.0
GY_6	1.5	98.5
GY_7	0.75	99.25

Table 2.2.2.-6. – *Reference solutions R*

Reference solution	Volumes in millilitres	
	Standard solution R	Hydrochloric acid (10 g/L HCl)
R_1	100.0	0.0
R_2	75.0	25.0
R_3	50.0	50.0
R_4	37.5	62.5
R_5	25.0	75.0
R_6	12.5	87.5
R_7	5.0	95.0

Storage

For Method I, the reference solutions may be stored in sealed tubes of colourless, transparent, neutral glass of 12 mm external diameter, protected from light.

For Method II, prepare the reference solutions immediately before use from the standard solutions.◊

INSTRUMENTAL METHOD – METHOD III

PRINCIPLE

The observed colour of an object depends primarily on its light-absorbing characteristics. However, a variety of conditions such as light-source differences, spectral energy of the illuminant, visual sensitivity of the observer, size differences, background differences and directional differences affect the perception of colour. Hue, lightness (or brightness) and saturation are 3 attributes of the colour. Instrumental measurement under defined conditions allows numerical expression of a colour. The base of any instrumental measurement of colour is that the human eye has been shown to detect colour via 3 types of receptors.

Instrumental methods for measurement of colour provide more objective data than the subjective viewing of colours by a small number of individuals. With adequate maintenance and calibration, instrumental methods can provide accurate, precise and consistent measurements of colour that do not drift with time. Through extensive colour-matching experiments with human subjects having normal colour vision, distribution coefficients (weighting factors) have been established for each wavelength in the visible spectrum, giving the relative amount of stimulation of each receptor type caused by the light of that wavelength.

The International Commission on Illumination (CIE) has developed models taking into account the light source and the angle at which the observer is looking at the target (field of view). In a visual test for coloration of solutions, there are requirements that lead to the use of a 2° angle and diffuse daylight (illuminant C). The mean sensitivity of the human eye is represented by the distribution coefficients $\bar{x}_\lambda$, $\bar{y}_\lambda$ and $\bar{z}_\lambda$ (Figure 2.2.2.-1).

For any colour, the amount of stimulation of each receptor type is defined by the set of tristimulus values (*XYZ*).

The relationship between the distribution coefficients and the tristimulus values (*X*, *Y* and *Z*) is given by the following equations, expressed in terms of integrals:

$$X = k\int_0^\infty f_\lambda \bar{x}_\lambda S_\lambda d\lambda$$

$$Y = k\int_0^\infty f_\lambda \bar{y}_\lambda S_\lambda d\lambda$$

$$Z = k\int_0^\infty f_\lambda \bar{z}_\lambda S_\lambda d\lambda$$

$$k = 100 / \int_0^\infty \bar{y}_\lambda S_\lambda d\lambda$$

k = normalising constant characterising the stimulation of one receptor type and the used illumination;

S_λ = relative spectral power distribution of the illuminant;

$\bar{x}_\lambda$, $\bar{y}_\lambda$ and $\bar{z}_\lambda$ = colour matching distribution coefficients for CIE 2° Standard Observer;

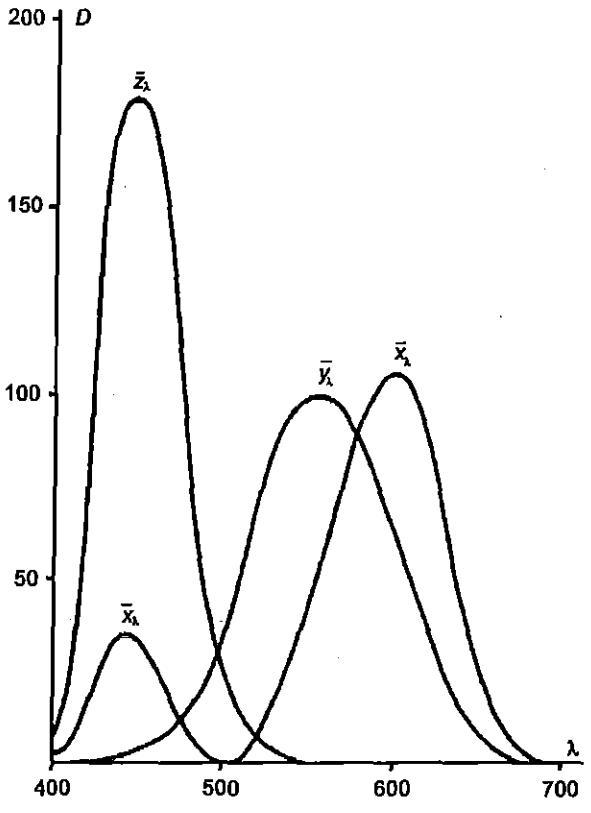


Figure 2.2.2.-1. – *Mean sensitivity of the human eye represented by distribution coefficients, CIE 2° Standard Observer (D = distribution coefficient; λ = wavelength in nanometres)*

f_λ = spectral transmittance T_λ of the material;

λ = wavelength, in nanometres.

In practical calculations of tristimulus values, the integration is approximated by a summation, as follows:

$$X = k\sum_\lambda T_\lambda \bar{x}_\lambda S_\lambda \Delta\lambda$$

$$Y = k\sum_\lambda T_\lambda \bar{y}_\lambda S_\lambda \Delta\lambda$$

$$Z = k\sum_\lambda T_\lambda \bar{z}_\lambda S_\lambda \Delta\lambda$$

$$k = \frac{100}{\sum_\lambda S_\lambda \bar{y}_\lambda \Delta\lambda}$$

The tristimulus values can be used to calculate the CIE *Lab* colour space co-ordinates: *L*(lightness or brightness)*, *a*(red-green)* and *b*(yellow-blue)*; these are defined by:

$$L^* = 116f(Y/Y_n) - 16$$

$$a^* = 500[f(X/X_n) - f(Y/Y_n)]$$

$$b^* = 200[f(Y/Y_n) - f(Z/Z_n)]$$

where X_n, Y_n and Z_n are the tristimulus values of *water R* and

$f(X/X_n) = (X/X_n)^{1/3}$ if $X/X_n > (6/29)^3$

otherwise $f(X/X_n) = 841/108(X/X_n) + 4/29$;

$f(Y/Y_n) = (Y/Y_n)^{1/3}$ if $Y/Y_n > (6/29)^3$

otherwise $f(Y/Y_n) = 841/108(Y/Y_n) + 4/29$;

$f(Z/Z_n) = (Z/Z_n)^{1/3}$ if $Z/Z_n > (6/29)^3$

otherwise $f(Z/Z_n) = 841/108(Z/Z_n) + 4/29$.

In the spectrophotometric method, transmittance values are obtained at discrete wavelengths throughout the visible spectrum. These values are then used to calculate the tristimulus values through the use of weighting factors $\bar{x}_\lambda$, $\bar{y}_\lambda$, and $\bar{z}_\lambda$ for a 2° Standard Observer and CIE standard illuminant C (see the current International Commission on Illumination publication, CIE).

SPECTROPHOTOMETRIC METHOD

Using a suitable spectrophotometer according to the manufacturer's instructions, determine the transmittance (T) at least over the range 400-700 nm, at intervals of not greater than 10 nm. Express the result as a percentage. Calculate the tristimulus values X, Y, and Z and the colour co-ordinates L^*, a^* and b^*.

DETERMINATION OF COLORATION

Calibrate the instrument according to the manufacturer's recommendations. Carry out system performance tests prior to each measurement or at regular intervals, depending on the use of the apparatus. For this purpose, use certified reference materials within the measurement range.

Operate the apparatus according to the manufacturer's instructions and test the sample solution and reference solution(s) under the same conditions (e.g. path length of the cuvette, temperature).

For transmittance measurements, use *water R* as the standard, assigning it a transmittance of 100.0 per cent at all wavelengths in the visible spectrum. Then the weighting factors $\bar{x}_\lambda$, $\bar{y}_\lambda$ and $\bar{z}_\lambda$ for CIE standard illuminant C are used to calculate the tristimulus values corresponding to colour co-ordinates $L^* = 100$, $a^* = 0$ and $b^* = 0$.

Reference measurements can be made using the colour co-ordinates of *water R* or freshly prepared pharmacopoeial reference solutions, or using the respective colour co-ordinates stored in the instrument manufacturer's database, provided the latter have been obtained under the same testing conditions.

If the test solution is turbid or hazy, it is filtered or centrifuged. If the test solution is not filtered or centrifuged, any haziness or turbidity is reported with the results. Air bubbles are to be avoided or, where applicable, removed.

The instrumental method is used to compare 2 solutions in terms of their colour or colour difference, or a deviation from a defined colour. Calculate the colour difference (ΔE^*_{tr}) between the test solution (t) and a reference solution (r) using the following equation:

$$\Delta E^*_{tr} = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2}$$

where ΔL^*, Δa^* and Δb^* are the differences in colour co-ordinates.

The CIE *LCh* colour co-ordinates may be used instead of the CIE *Lab* colour co-ordinates.

Assessment of location within the $L^*a^*b^*$ colour space

Instruments may provide information on the actual location of the test solution within the $L^*a^*b^*$ colour space. Using appropriate algorithms, correspondence to pharmacopoeial reference solutions (such as 'test solution equals reference solution XY', 'test solution close to reference solution XY' or 'test solution between reference solutions XY and XZ') can be obtained.

Appendix V

A. Determination of Melting Point

Method I

(Ph. Eur. method 2.2.14)

The melting point determined by the capillary method is the temperature at which the last solid particle of a compact column of a substance in a tube passes into the liquid phase (i.e. clear point). The melting point determined by this method is specific to the methodology (e.g. heating rate) described in this chapter. Similarly, whenever the use of certified reference materials is required, their certified values refer to the described analytical procedure.

When prescribed in the monograph, the same apparatus and method are used for the determination of other factors, such as meniscus formation or melting range, that characterise the melting behaviour of a substance.

Equipment The equipment consists of a metal heating block with 1 or more compartments for capillary tubes, or of a suitable glass vessel containing a liquid bath (e.g. water, liquid paraffin or silicone oil) and fitted with a suitable means of heating and stirring. The equipment is equipped with a temperature sensor or a suitable certified thermometer allowing readings at least to the nearest 0.1 °C.

Samples are introduced into the equipment in glass capillary tubes. The dimensions are chosen according to the manufacturer's requirements, typically with an external diameter of 1.3-1.5 mm and a wall thickness of 0.1-0.3 mm. In some equipment glass slides are used instead of capillary tubes.

The equipment is capable of heating samples at a rate of 1 °C/min or less. The accuracy of the equipment is at most ± 0.5 °C.

Detection can be performed either visually or instrumentally. In the case of instrumental detection, this is generally performed by image recording and subsequent analysis or by a photodetector that measures the transmitted or reflected light from the sample.

Method The substance is previously treated as described in the monograph. Coarse crystals are to be avoided as they might lead to false results. If necessary, samples are crushed into a fine powder. Unless otherwise prescribed, dry the finely powdered substance *in vacuo* over *anhydrous silica gel R* for 24 h. Introduce a sufficient quantity into a capillary tube to give a compact column as described by the instrument manufacturer (e.g. 4-6 mm in height). Raise the temperature of the apparatus to about 5 °C below the presumed melting point. Allow the temperature to stabilise and then introduce the capillary tube into the instrument. Finally, adjust the rate of heating to about 1 °C/min unless otherwise prescribed.

In the case of instrumental detection, follow the instrument manufacturer's requirements for the determination of the melting point. For visual detection, record the temperature at which the last particle of the substance to be examined passes into the liquid phase.

Samples can be measured in parallel if the instrument allows multiple sample processing.

System suitability Carry out a system suitability test before the measurements for example by choosing a suitable reference material with a melting point close to that expected for the substance to be examined.

Qualification / Calibration of the equipment The qualification / calibration is carried out periodically according to the instrument manufacturer's requirements, using at least 2 certified reference materials. These are selected to cover the temperature range that is used on the equipment.
Use capillary tubes with the same dimensions as those used for sample measurement.

Guidance on how to compare results obtained from certified reference materials with values from the certificates can be found on the European Reference Materials (ERM) website (Application note 1).

Method II

(No Ph. Eur. method)

Apparatus

(*a*) A glass heating vessel of suitable construction and capacity containing one of the following, or another suitable liquid, to a height of not less than 14 cm.

(i) A liquid paraffin of sufficiently high boiling point.

(ii) A silicone fluid of sufficiently high boiling point.

(iii) Water.

(*b*) A suitable stirring device capable of rapidly mixing the liquid.

(*c*) An accurately standardised thermometer suitable for the substance being examined complying with the requirements of British Standard 1365:1990 (Specification for short-range short-stem thermometers) for thermometers designated by one of the following Schedule Marks.

Schedule mark at each (max)	Range °C	Graduated	Diameter of stem mm	Overall length mm
SA 55C/80	−10 to 55	0.5°	5.5 to 8	200
SA 105C/80	45 to 105	0.5°	5.5 to 8	200
SA 155C/80	95 to 155	0.5°	5.5 to 8	200
SA 205C/80	145 to 205	0.5°	5.5 to 8	200
SA 225C/80	195 to 255	0.5°	5.5 to 8	200
SA 305C/80	245 to 305	0.5°	5.5 to 8	200
SA 360C/80	295 to 360	0.5°	5.5 to 8	200

(*d*) Thin-walled capillary glass tubes of hard glass, closed at one end, with a wall thickness of 0.10 to 0.15 mm, at least 12 cm in length and of internal diameter 0.9 to 1.1 mm. The tubes should preferably be kept sealed at both ends and cut as required.

Method Dry a small quantity of the finely powdered substance at a temperature considerably below its melting point or at a pressure of 2 kPa over a suitable desiccant, unless otherwise directed. Transfer a portion to a dry capillary tube and pack the powder by tapping on a hard surface so as to form a tightly packed column 4 to 6 mm in height. Heat a suitable liquid in the heating vessel and regulate the rate of rise of temperature, prior to the introduction of the capillary tube, to 3° per minute, unless otherwise directed, stirring constantly. When the temperature reaches 10° below the lowest figure of the range for the substance being tested, adjust the height of the thermometer so that the immersion mark is at the level of the surface of the liquid and insert the capillary tube so that the closed end is near the middle of the bulb of the thermometer. Note the temperature at which the liquefaction of the substance

occurs, which is indicated by the formation of a definite meniscus or, for substances that decompose, the temperature at which frothing begins. Correct the observed temperature for any error in the calibration of the thermometer and for the difference, if any, between the temperature of the emergent stem of the thermometer and the temperature of the emergent stem under the conditions of standardisation of the thermometer. The temperature of the emergent stem is determined by placing the bulb of a second thermometer in contact with the emergent stem at a point approximately midway along the mercury thread in the emergent stem.

The correction to be applied is given by the following equation:

$$t_c = 0.00016n(t_s - t_d)$$

where t_c = correction to be added to the observed temperature of the melting point,

t_s = mean temperature of the emergent column when standardised,

t_d = mean temperature of the emergent column at the observed melting point,

n = number of °C over which the exposed column extends.

The corrected temperature is regarded as the melting point of the substance. When the melting point in the monograph is expressed as a range, the melting point of the substance being tested must fall within that range.

Method III

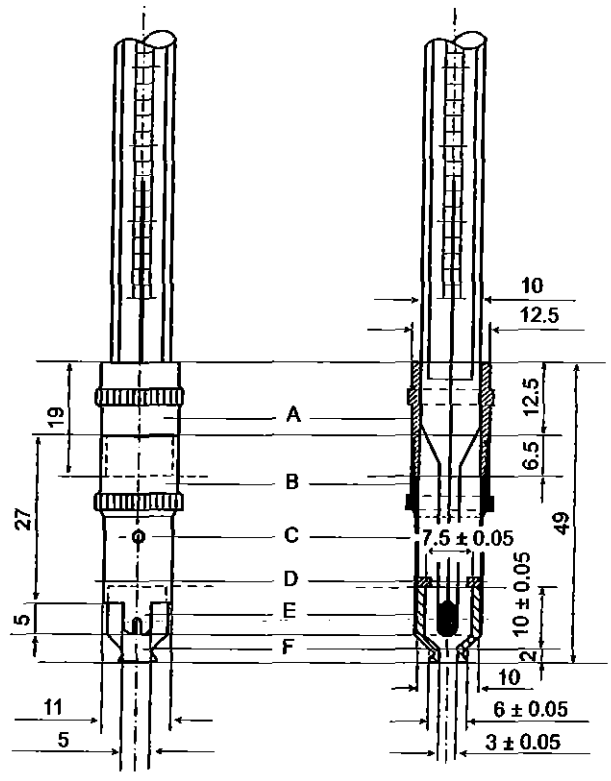
(Ph. Eur. method 2.2.17)

The drop point is the temperature at which the first drop of the melting substance to be examined falls from a cup under defined conditions.

When a monograph does not specify the method to be used, apply method A. Any change from method A to method B is validated.

METHOD A

Equipment The equipment (see Figure 2.2.17.-1) consists of 2 metal sheaths (*A* and *B*) screwed together. Sheath *A* is fixed to a thermometer. A metal cup is loosely fixed to the lower part of sheath *B* by means of 2 tightening bands. Fixed supports 2 mm long determine the exact position of the cup, and in addition are used to centre the thermometer. A hole pierced in the wall of sheath *B* is used to balance the pressure. The draining surface of the cup must be flat and the edges of the outflow orifice must be at right angles to it. The thermometer has the form and size shown in Figure 2.2.17.-1; it covers a range from 0 °C to 110 °C and on its scale a distance of 1 mm represents a difference of 1 °C. The thermometer bulb has a diameter of 3.5 ± 0.2 mm and a height of 6.0 ± 0.3 mm.
The equipment is placed in the axis of a test-tube about 200 mm long and with an external diameter of about 40 mm. It is fixed to the test-tube by means of a laterally grooved stopper through which the thermometer passes. The opening of the cup is placed about 15 mm from the bottom of the test-tube. The whole device is immersed in a beaker with a capacity of about 1 L, filled with water. The bottom of the test-tube is placed about 25 mm from the bottom of the beaker. The water level reaches the upper part of sheath *A*. A stirrer is used to ensure that the temperature of the water remains uniform.



A. upper metal sheath C. pressure-balancing hole E. tightening bands
B. lower metal sheath D. fixed supports F. metal sample cup

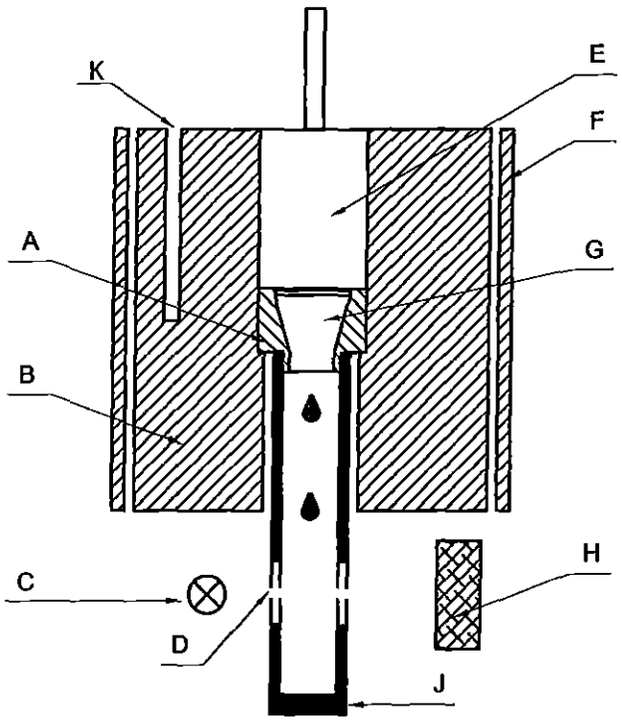
Figure 2.2.17.-1. – *Equipment for the determination of drop point (dimensions in millimetres)*

Method Prepare the substance to be examined as described in the monograph. Fill the cup to the brim with the substance to be examined. Remove the excess substance at both ends of the cup with a spatula. When sheaths *A* and *B* have been assembled, press the cup into its housing in sheath *B* until it touches the supports. Remove with a spatula the substance pushed out by the thermometer. Place the equipment in the water-bath as described above. Heat the water-bath and, when the temperature is at about 10 °C below the presumed drop point, adjust the heating rate to about 1 °C/min. Note the temperature at the fall of the first drop. Carry out at least 3 determinations, each time with a fresh sample of the substance. The difference between the readings must not exceed 3 °C. The mean of 3 readings is the drop point of the substance.

METHOD B - AUTOMATED METHOD

Equipment The equipment (see Figure 2.2.17.-2) consists of a cartridge assembly comprising a cup holder into which the sample cup containing the sample is loosely fixed, and a collector sleeve with a horizontal light slit, which is fixed below the cup. This assembly is placed in a heating block. The block is a metal cylinder with a cylindrical hole along its vertical axis into which the cartridge assembly is placed. There is another, narrower cylindrical vertical hole in which a temperature sensor sits. This is positioned level with the sample cup. The heating block is surrounded by an electrical heating element. Below the heating block a lamp is mounted such that a beam of light shines through the light slit in the collector sleeve, and onto a photo-sensor mounted opposite. The heating element is capable of maintaining the heating

block at a pre-defined temperature, and of heating at a slow and steady, pre-defined rate.



A. cup holder
B. heating block
C. light source
D. light slit
E. cartridge assembly
F. heating element
G. sample cup
H. photo-sensor
J. collector sleeve
K. temperature probe

Figure 2.2.17.-2. – *Example of automated drop point equipment*

Method Prepare the substance to be examined as described in the monograph, then proceed as follows or according to the manufacturer's instructions. Remove the excess substance at both ends of the cup with a spatula. Press the cup into the cup holder, and then press the collector sleeve onto the cup. Place the cartridge assembly in the heating block. Set the instrument to the initial isothermal conditions and the rate for subsequent heating as described in the monograph. Start the temperature programme. When the first drop of molten sample falls through the hole at the bottom of the sample cup, thus interrupting the light beam, a signal from the photo-sensor causes the temperature of the heating block to be recorded automatically.

Calibration Use the equipment according to the manufacturer's instructions and carry out the prescribed calibrations and system performance tests at regular intervals, depending on the use of the equipment and the substances to be examined. Benzoic acid and benzophenone are usually used as certified reference materials. Other materials may be used provided they show no polymorphism. Proceed as follows or according to the manufacturer's instructions. Prepare 3 sample cups for each of 2 certified reference materials. Place the sample cups on a clean surface. Into each sample cup, introduce a small quantity of the sample and press it down with a rod (diameter about 4.5 mm). Check that the opening is completely blocked. Fill the sample cup about half full and compact the sample with a rod (diameter about 9 mm). Fill the sample cup and compact, adding more sample and compacting again if necessary, until the sample cup is completely packed.

Temperature programme for benzoic acid: start temperature = 118.0 °C; heating rate = 0.2 °C/min; end temperature = 126.0 °C. After inserting the cup at 118 °C, a waiting time of 30 s is set before heating starts.

Temperature programme for benzophenone: start temperature = 44.0 °C; heating rate = 0.2 °C/min; end temperature = 56.0 °C. After inserting the cup at 44 °C, a waiting time of 30 s is set before heating starts.

Check the 3 single results: the test is valid if the 3 results are within 0.3 °C of the mean value.

Calculate the corrected mean temperature (T_2) using the following expression:

$$T_1 - F$$

T_1 = mean drop point temperature of 3 samples, in °C;
F = compensation for the difference in temperature between the sample and the point in the heating block where the temperature is measured; this will vary depending upon the design of the automatic drop point instrument and is provided by the manufacturer.

Taking into account the drop point of the certified reference material (T_0), the accuracy of the temperature scale is satisfactory if $|T_2 - T_0|$ is not greater than 0.3 °C.

Method IV

(Ph. Eur. method 2.2.15)

For certain substances, the following method is used to determine the melting point (also referred to as slip point and rising melting point when determined by this method).

Use glass capillary tubes open at both ends, about 80 mm long, having an external diameter of 1.4 mm to 1.5 mm and an internal diameter of 1.0 mm to 1.2 mm.

Introduce into each of 5 capillary tubes a sufficient amount of the substance, previously treated as described, to form in each tube a column about 10 mm high and allow the tubes to stand for the appropriate time and at the prescribed temperature.

Unless otherwise prescribed, substances with a waxy consistency are carefully and completely melted on a water-bath before introduction into the capillary tubes. Allow the tubes to stand at 2-8 °C for 2 h.

Attach one of the tubes to a thermometer graduated in 0.5 °C so that the substance is close to the bulb of the thermometer. Introduce the thermometer with the attached tube into a beaker so that the distance between the bottom of the beaker and the lower part of the bulb of the thermometer is 1 cm. Fill the beaker with water to a depth of 5 cm. Increase the temperature of the water gradually at a rate of 1 °C/min.

The temperature at which the substance begins to rise in the capillary tube is regarded as the melting point.

Repeat the operation with the other 4 capillary tubes and calculate the result as the mean of the 5 readings.

Method V

(Ph. Eur. method 2.2.16)

The instantaneous melting point is calculated using the following expression:

$$\frac{t_1 + t_2}{2}$$

in which t_1 is the first temperature and t_2 the second temperature read under the conditions stated below.

Apparatus The apparatus consists of a metal block resistant to the substance to be examined, of good heat-conducting capacity, such as brass, with a carefully polished plane upper surface. The block is uniformly heated throughout its mass by means of a micro-adjustable gas heater or an electric heating device with fine adjustment. The block has a cylindrical cavity, which is wide enough to accomodate a thermometer that is maintained in the same position during the calibration of the apparatus and the determination of the melting point of the substance to be examined. The cylindrical cavity is parallel to the upper polished surface of the block and about 3 mm from it. The apparatus is calibrated using appropriate substances of known melting point.

Method Heat the block at a suitably rapid rate to a temperature about 10 °C below the presumed melting temperature, then adjust the heating rate to about 1 °C/min. At regular intervals drop a few particles of powdered and, where appropriate, dried substance, prepared as for the capillary tube method, onto the block in the vicinity of the thermometer bulb, cleaning the surface after each test. Record the temperature t_1 at which the substance melts instantaneously for the first time in contact with the metal. Stop the heating. During cooling drop a few particles of the substance at regular intervals on the block, cleaning the surface after each test. Record the temperature t_2 at which the substance ceases to melt instantaneously when it comes in contact with the metal.

Calibration of the apparatus The apparatus may be calibrated using melting point reference substances such as those of the World Health Organization or other appropriate substances.

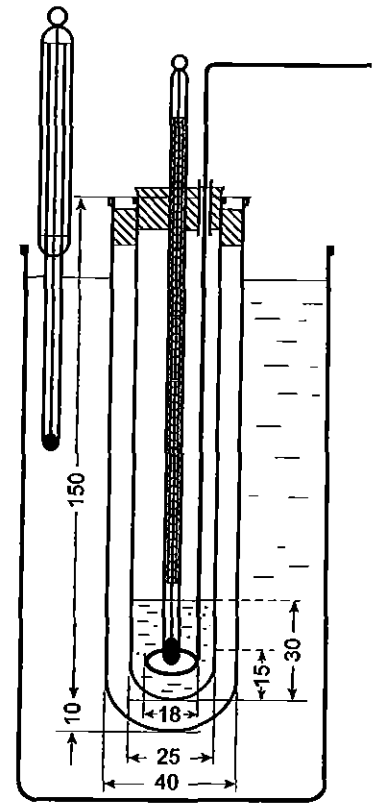


Figure 2.2.18.-1. – *Apparatus for the determination of freezing point*

Dimensions in millimetres

B. Determination of Freezing Point

(Ph. Eur. method 2.2.18)

The freezing point is the maximum temperature occurring during the solidification of a supercooled liquid.

Apparatus The apparatus (see Figure 2.2.18.-1) consists of a test-tube about 25 mm in diameter and 150 mm long placed inside a test-tube about 40 mm in diameter and 160 mm long. The inner tube is closed by a stopper which carries a thermometer about 175 mm long and graduated in 0.2 °C fixed so that the bulb is about 15 mm above the bottom of the tube. The stopper has a hole allowing the passage of the stem of a stirrer made from a glass rod or other suitable material formed at one end into a loop of about 18 mm overall diameter at right angles to the rod. The inner tube with its jacket is supported centrally in a 1 L beaker containing a suitable cooling liquid to within 20 mm of the top. A thermometer is supported in the cooling bath.

Method Place in the inner tube sufficient quantity of the liquid or previously melted substance to be examined, to cover the thermometer bulb and determine the approximate freezing point by cooling rapidly. Place the inner tube in a bath about 5 °C above the approximate freezing point until all but the last traces of crystals are melted. Fill the beaker with water or a saturated solution of sodium chloride, at a temperature about 5 °C lower than the expected freezing point, insert the inner tube into the outer tube, ensuring that some seed crystals are present, and stir thoroughly until solidification takes place. Note the highest temperature observed during solidification.

C. Determination of Distillation Range

(Ph. Eur. method 2.2.11)

The distillation range is the temperature interval, corrected for a pressure of 101.3 kPa, within which a liquid or a specified fraction of a liquid, distils under the following conditions.

Apparatus The apparatus (see Figure 2.2.11.-1) consists of a distillation flask (*A*), a straight tube condenser (*B*) which fits on to the side arm of the flask and a plain-bend adaptor (*C*) attached to the end of the condenser. The lower end of the condenser may, alternatively, be bent to replace the adaptor. A thermometer is inserted in the neck of the flask so that the upper end of the bulb is 5 mm lower than the junction of the lower wall of the lateral tube.
The thermometer can be read to the nearest 0.2 °C and covers a range of at least 50 °C. During the determination, the flask, including its neck, is protected from draughts by a suitable screen.

Method Place in the flask (*A*) 50.0 mL of the liquid to be examined and a few pieces of porous material. Collect the distillate in a 50 mL cylinder graduated in millilitres. Cooling by circulating water is essential for liquids distilling below 150 °C. Heat the flask so that boiling is rapidly achieved and note the temperature at which the first drop of distillate falls into the cylinder. Adjust the heating to give a regular rate of distillation of 2-3 mL/min and note the temperature when the whole or the prescribed fraction of the liquid, measured at 20 °C, has distilled.

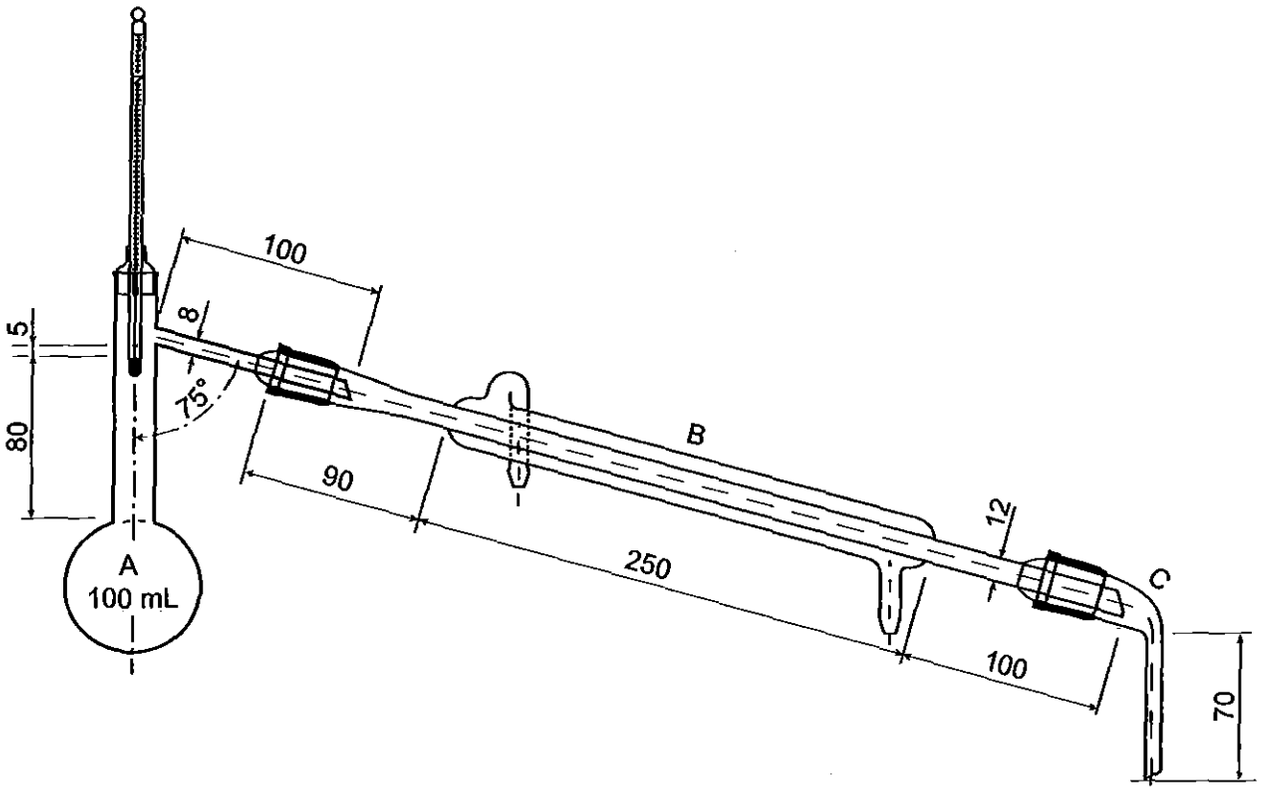


Figure 2.2.11.-1. – *Apparatus for the determination of distillation range*
Dimensions in millimetres

Correct the observed temperatures for barometric pressure by means of the formula:

$$t_1 = t_2 + k(101.3 - b)$$

t_1 = the corrected temperature,
t_2 = the observed temperature, at the barometric pressure b,
k = the correction factor taken from Table 2.2.11.-1 unless the factor is given,
b = the barometric pressure, expressed in kilopascals, during the distillation.

Table 2.2.11.-1. – *Temperature correction in relation to the pressure*

Distillation temperature	Correction factor k
up to 100 °C	0.30
above 100 °C up to 140 °C	0.34
above 140 °C up to 190 °C	0.38
above 190 °C up to 240 °C	0.41
above 240 °C	0.45

D. Determination of Boiling Point

(Ph. Eur. method 2.2.12)

The boiling point is the corrected temperature at which the vapour pressure of a liquid is equal to 101.3 kPa.

Apparatus The apparatus is that used for distillation range (*2.2.11*) with the exception that the thermometer is inserted in the neck of the flask so that the lower end of the bulb is level with the lower end of the neck of the distillation flask and that the flask is placed on a plate of isolating material pierced by a hole 35 mm in diameter.

Method Place in the flask (*A*) 20 mL of the liquid to be examined and a few pieces of porous material. Heat the flask so that boiling is rapidly achieved and record the temperature at which liquid runs from the side-arm into the condenser.

Correct the observed temperature for barometric pressure by means of the formula:

$$t_1 = t_2 + k(101.3 - b)$$

t_1 = the corrected temperature,
t_2 = the observed temperature at barometric pressure b,
k = the correction factor as shown in Table 2.2.11.-1 under Distillation Range,
b = the barometric pressure, in kilopascals, at the time of the determination.

E. Determination of Refractive Index

(Ph. Eur. method 2.2.6)

The refractive index of a medium with reference to air is equal to the ratio of the sine of the angle of incidence of a beam of light in air to the sine of the angle of refraction of the refracted beam in the given medium.

Unless otherwise prescribed, the refractive index is measured at 20 ± 0.5 °C, with reference to the wavelength of the D-line of sodium (λ = 589.3 nm); the symbol is then n_D^{20}.

Refractometers normally determine the critical angle. In such apparatus the essential part is a prism of known refractive index in contact with the liquid to be examined.

Calibrate the apparatus using certified reference materials.

When white light is used, the refractometer is provided with a compensating system. The apparatus gives readings accurate to at least the third decimal place and is provided

with a means of operation at the temperature prescribed. The thermometer is graduated at intervals of 0.5 °C or less.

F. Determination of Optical Rotation and Specific Optical Rotation

(Ph. Eur. method 2.2.7)

PRINCIPLE

Optical rotation (also known as optical activity) is the property displayed by chiral substances of rotating the plane of polarisation of linearly polarised light.

Optical rotation is considered to be positive (+) for dextrorotatory substances (i.e. those that rotate the plane of polarisation in a clockwise direction when viewed in the direction facing the oncoming light beam) and negative (−) for laevorotatory substances (i.e. anticlockwise rotation).

The angle of optical rotation α of a liquid is the angle of rotation of the plane of polarisation, expressed in degrees (°), at the wavelength of the D-line of sodium (λ = 589.3 nm) measured at 20 °C through the liquid when using a path length of 1.00 dm.

The specific optical rotation $[\alpha]_D^{20}$ of a substance in solution is calculated from the angle of optical rotation, as defined above, with reference to a path length of 1.00 dm and a concentration of the substance to be examined of 1 g/mL. The specific optical rotation of a substance in solution is always expressed with reference to a given solvent and concentration.

As some equipment may not use sodium lamps, the wavelength of measurement is given as 589 nm instead of 589.3 nm.

In certain cases specified in the monograph, the angle of optical rotation is measured at other temperatures, other wavelengths and/or in cells with a path length other than 1.00 dm.

In the conventional system adopted by the Pharmacopoeia, the specific optical rotation is expressed by its value without units; the actual units, degree millilitres per decimetre gram [(°)·ml·dm^{-1}·g^{-1}] are understood.

EQUIPMENT

The polarimeter typically consists of:

- a light source, for example a sodium discharge lamp, a light-emitting diode (LED) or another light source capable of providing radiation at the desired wavelength (589 nm unless otherwise prescribed in the monograph); if the light source is polychromatic, a means of isolating the required wavelength is necessary, e.g. an optical filter;
- a polariser and an analyser;
- a sample cell with a path length of 1.00 dm, unless otherwise specified in the monograph;
- a detection system to measure the angle of optical rotation, which must be capable of giving readings to at least the nearest 0.01°, unless otherwise specified in the monograph;
- a temperature control system that indicates the temperature with a readability of 0.1 °C; it may be embedded in the polarimeter (e.g. a Peltier system) or be an external unit (e.g. a cycle-cryostat), and must be able to maintain the temperature of the liquid to within ± 0.5 °C of that prescribed.

EQUIPMENT PERFORMANCE

The accuracy of the scale is checked near the value to be measured or over an appropriate range, usually by means of certified quartz plates. Other certified reference materials may also be suitable (e.g. sucrose solutions).

Optical rotation measurements may be used to quantify the amount of an enantiomer or the ratio of enantiomers present in a sample. For that purpose, the linearity must be checked, for example using sucrose solutions.

PROCEDURE

Determine the zero of the polarimeter and the angle of rotation of the liquid at a wavelength of 589 nm and a temperature of 20 ± 0.5 °C, unless otherwise prescribed. The zero of the polarimeter is determined with the sample cell closed.

For neat liquids, the zero is determined with an empty sample cell.

For solutions, the zero is determined with the sample cell filled with the same solvent as that used for the solution to be examined and measured at the same temperature. The sample preparation procedure is prescribed in the monograph.

Calculate the specific optical rotation at temperature t and wavelength λ using the following formulae.

For neat liquids, the density of the liquid is taken into account:

$$[\alpha]_\lambda^t = \frac{\alpha}{l \cdot \rho_t}$$

For solutions:

$$[\alpha]_\lambda^t = \frac{1000\alpha}{l \cdot c}$$

α	=	angle of rotation measured at temperature t and wavelength λ, in degrees;
l	=	path length of the polarimeter sample cell, in decimetres;
ρ_t	=	density determined at the temperature of measurement t, in grams per cubic centimetre; for the purposes of the Pharmacopoeia, density is replaced by relative density (*2.2.5*);
c	=	concentration of the solution, in grams per litre.

When the limits for optical rotation or specific optical rotation are expressed as the dried substance, the anhydrous substance or the solvent-free substance, the result must be corrected for loss on drying (*2.2.32*), water content (*2.5.12* or *2.5.32*) or content of solvent as appropriate.

G. Determination of Weight per Millilitre, Density, Relative Density and Apparent Density

Weight per millilitre

(No Ph. Eur. method)

The *weight per millilitre* of a liquid is the weight in g of 1 mL of a liquid when weighed in air at 20°, unless otherwise specified in the monograph.

The *weight per millilitre* is determined by dividing the weight in air, expressed in g, of the quantity of liquid that fills a pycnometer at the specified temperature by the capacity,

expressed in mL, of the pycnometer at the same temperature. The capacity of the pycnometer is ascertained from the weight in air, expressed in g, of the quantity of *water* required to fill the pycnometer at that temperature. The weight of a litre of *water* at specified temperatures when weighed against brass weights in air of density 0.0012 g per mL is given in the following table. Ordinary deviations in the density of air from the above value, here taken as the mean, do not affect the result of a determination in the significant figures prescribed for Pharmacopoeial substances.

Temperature	Weight of a litre of water
°C	g
20	997.18
25	996.02
30	994.62

Density

(No Ph. Eur. method)

The *density*, ρ_{20}, of a substance is the ratio of its mass to its volume at 20°. It is expressed in kg m^{-3}.

The *density* is determined by dividing the weight in air of the quantity of the liquid being examined that fills a pycnometer at 20° by the weight in air of *water* required to fill the pycnometer after making allowance for the thrust of the air.

The *density* is calculated from the expression

$$\rho_{20} = \frac{998.2(M_1 + A)}{M_2 + A}$$

where M_1 = weight in air (apparent mass) in grams of the substance being examined,
M_2 = weight in air (apparent mass) in grams of *water*,
A = the correction factor for the thrust of the air, $0.0012M_2$
998.2 = the density of water at 20° in kg m^{-3}.

In most cases, the correction for the thrust of the air may be disregarded.

Relative density

(Ph. Eur. method 2.2.5)

The relative density $d^{t_1}_{t_2}$ of a substance is the ratio of the mass of a certain volume of a substance at temperature t_1 to the mass of an equal volume of water at temperature t_2.

Unless otherwise indicated, the relative density d^{20}_{20} is used. Relative density is also commonly expressed as d^{20}_{4}.

Density ρ_{20}, defined as the mass of a unit volume of the substance at 20 °C may also be used, expressed in kilograms per cubic metre or grams per cubic centimetre (1 kg·m^{-3} = 10^{-3} g·cm^{-3}). These quantities are related by the following equations where density is expressed in grams per cubic centimetre:

$$\rho_{20} = 0.998203 \times d^{20}_{20} \quad \text{or} \quad d^{20}_{20} = 1.00180 \times \rho_{20}$$

$$\rho_{20} = 0.999972 \times d^{20}_{4} \quad \text{or} \quad d^{20}_{4} = 1.00003 \times \rho_{20}$$

$$d^{20}_{4} = 0.998230 \times d^{20}_{20}$$

Relative density or density is measured according to the number of decimals prescribed in the monograph using a density bottle (solids or liquids), a hydrostatic balance (solids), a hydrometer (liquids) or a digital density meter with an oscillating transducer (liquids and gases). When the determination is made by weighing, the buoyancy of air is disregarded, which may introduce an error of 1 unit in the 3rd decimal place. When using a density meter, the buoyancy of air has no influence.

Oscillating transducer density meter The apparatus consists of:

- — a U-shaped tube, usually of borosilicate glass, which contains the liquid to be examined;
- — a magneto-electrical or piezo-electrical excitation system that causes the tube to oscillate as a cantilever oscillator at a characteristic frequency depending on the density of the liquid to be examined;
- — a means of measuring the oscillation period (T), which may be converted by the apparatus to give a direct reading of density, or used to calculate density using the constants A and B described below.

The resonant frequency (f) is a function of the spring constant (c) and the mass (m) of the system:

$$f^2 = \frac{1}{T^2} = \frac{c}{m} \times \frac{1}{4\pi^2}$$

Hence:

$$T^2 = \left(\frac{M}{c} + \frac{\rho \times V}{c}\right) \times 4\pi^2$$

M = mass of the tube;
V = inner volume of the tube.

Introduction of 2 constants $A = c/(4\pi^2 \times V)$ and $B = M/V$, leads to the classical equation for the oscillating transducer:

$$\rho = A \times T^2 - B$$

The constants A and B are determined by operating the instrument with the U-tube filled with 2 different samples of known density, for example, degassed *water R* and air. Control measurements are made daily using degassed *water R*. The results displayed for the control measurement using degassed *water R* shall not deviate from the reference value (ρ_{20} = 0.998203 g·cm^{-3}, d^{20}_{20} = 1.000000) by more than its specified error. For example, an instrument specified to ± 0.0001 g·cm^{-3} shall display 0.9982 ± 0.0001 g·cm^{-3} in order to be suitable for further measurement. Otherwise a re-adjustment is necessary. Calibration with certified reference materials is carried out regularly. Measurements are made using the same procedure as for calibration. The liquid to be examined is equilibrated in a thermostat at 20 °C before introduction into the tube, if necessary, to avoid the formation of bubbles and to reduce the time required for measurement.

Factors affecting accuracy include:

- — temperature uniformity throughout the tube;
- — non-linearity over a range of density;
- — parasitic resonant effects;
- — viscosity, whereby solutions with a higher viscosity than the calibrant have a density that is apparently higher than the true value.

The effects of non-linearity and viscosity may be avoided by using calibrants that have density and viscosity close to those of the liquid to be examined (± 5 per cent for density, ± 50 per cent for viscosity). The density meter may have functions for automatic viscosity correction and for

correction of errors arising from temperature changes and non-linearity.

Precision is a function of the repeatability and stability of the oscillator frequency, which is dependent on the stability of the volume, mass and spring constant of the cell.

Density meters are able to achieve measurements with an error of the order of 1×10^{-3} g·cm^{-3} to 1×10^{-5} g·cm^{-3} and a repeatability of 1×10^{-4} g·cm^{-3} to 1×10^{-6} g·cm^{-3}.

Apparent density

(No Ph. Eur. method)

The term 'Apparent density' is used in the monographs for Dilute Ethanols, Industrial Methylated Spirit and Industrial Methylated Spirit (Ketone-free). It is defined as weight in air per unit volume and expressed in kg m^{-3}. It is named 'density' in the *Laboratory Alcohol Table for Laboratory Use* (HM Customs and Excise 1979).

The *apparent density* is calculated from the following expression:

apparent density = $997.2 \times d_{20}^{20}$

where d_{20}^{20} is the *relative density* of the substance being examined and 997.2 is the weight in air in kg of 1 cubic metre of water.

H. Viscosity

(Ph. Eur. method 2.2.8)

The *dynamic* viscosity or *viscosity coefficient* η is the tangential force per unit surface, known as *shearing stress* τ and expressed in pascals, necessary to move, parallel to the sliding plane, a layer of liquid of 1 square metre at a rate (v) of 1 metre per second relative to a parallel layer at a distance (x) of 1 metre.

The ratio dv/dx is a speed gradient giving the *rate of shear D* expressed in reciprocal seconds (s^{-1}), so that $\eta = \tau/D$.

The unit of dynamic viscosity is the pascal second (Pa·s). The most commonly used submultiple is the millipascal second (mPa·s).

The *kinematic viscosity* v, expressed in square metres per second, is obtained by dividing the dynamic viscosity η by the density ρ expressed in kilograms per cubic metre, of the liquid measured at the same temperature, i.e. $v = \eta/\rho$. The kinematic viscosity is usually expressed in square millimetres per second.

A capillary viscometer may be used for determining the viscosity of Newtonian liquids and a rotating viscometer for determining the viscosity of Newtonian and non-Newtonian liquids. Other viscometers may be used provided that the accuracy and precision are at least as satisfactory as those obtained with the viscometers described in the related chapters.

Method I

(No Ph. Eur. method)

Apparatus

The apparatus consists of a glass U-tube viscometer (Fig.5H–1) made of clear borosilicate glass and constructed in accordance with the dimensions shown in the figure and in Table 5H–1. The monograph states the size of viscometer to be used.

Method

Fill the viscometer with the liquid being examined through tube L to slightly above the mark G, using a long pipette to minimise wetting the tube above the mark. Place the tube vertically in a water bath and when it has attained the specified temperature, adjust the volume of the liquid so that the bottom of the meniscus settles at the mark G. Adjust the liquid to a point about 5 mm above the mark E. After releasing pressure or suction, measure the time taken for the bottom of the meniscus to fall from the top edge of mark E to the top edge of mark F.

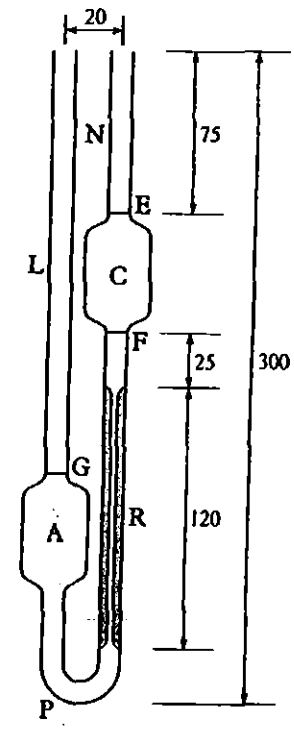


Fig. 5H–1
U-Tube Viscometer

Calculate, as required, either the *kinematic viscosity* (v) in square millimetres per second (mm^2 s^{-1}) from the expression:

$$v = Kt,$$

or the *dynamic viscosity* (η) in millipascal seconds (mPa s) from the expression:

$$\eta = K\rho t,$$

where t = time in seconds for the meniscus to fall from E to F,

ρ = mass/volume (g cm^{-3}) obtained by multiplying the *relative density*, Appendix V G, of the fluid being examined by 0.9982.

The constant (K) of the instrument is determined using the appropriate European Pharmacopoeia reference liquid for viscometers.

Method II (Capillary Viscometer Method)

(Ph. Eur. method 2.2.9)

PRINCIPLE

The determination of viscosity is carried out using a suspended-level (Ubbelohde-type) capillary viscometer of appropriate size at a temperature of 20.0 ± 0.1 °C, unless otherwise prescribed. The time required for the level of the liquid to drop from one mark to the other is measured.

Table 5H–1 U-Tube Viscometer – Dimensions

Size	Nominal viscometer constant	Kinematic viscosity range	Inside diameter of tube R	Outside diameter of tubes[1]		Volume of bulb C	Vertical distance F to G	Outside diameter of bulbs A and C
				L and P	N			
	mm^2s^{-2}	mm^2s^{-1}	mm(±2%)	mm	mm	mm (±5%)	mm	mm
A[2]	0.003	0.9 to 3	0.50	8 to 9	6 to 7	5.0	91±4	21 to 23
B	0.01	2.0 to 10	0.71	8 to 9	6 to 7	5.0	87±4	21 to 23
C	0.03	6 to 30	0.88	8 to 9	6 to 7	5.0	83±4	21 to 23
D	0.1	20 to 100	1.40	9 to 10	7 to 8	10.0	78±4	25 to 27
E	0.3	60 to 300	2.00	9 to 10	7 to 8	10.0	73±4	25 to 27
F	1.0	200 to 1000	2.50	9 to 10	7 to 8	10.0	70±4	25 to 27
G	3.0	600 to 3000	4.00	10 to 11	9 to 10	20.0	60±3	32 to 35
H	10.0	2000 to 10,000	6.10	10 to 11	9 to 10	20.0	50±3	32 to 35

[1]Use 1 to 1.25 mm wall tubing for L, N and P.
[2]300 s minimum flow time; 200 s minimum flow time for all other sizes.

EQUIPMENT

The principal components of an Ubbelohde-type capillary viscometer[1]. are shown in Figure 2.2.9.-1.

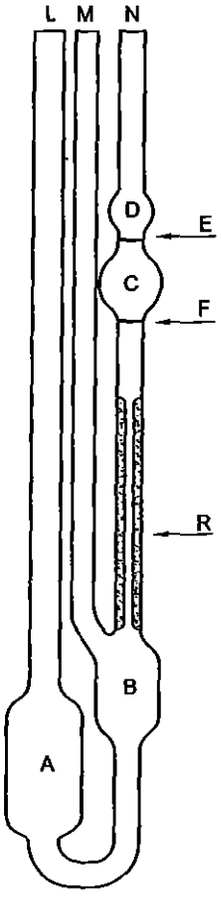


Figure 2.2.9.-1. – *Suspended-level (Ubbelohde-type) capillary viscometer*

PROCEDURE

Select a capillary viscometer of appropriate size to obtain a minimum flow time of 200 s.

[1] *The European Pharmacopoeia describes the system proposed by the International Organization for Standardization (ISO)*

Calibration

Capillary viscometers are calibrated at regular intervals as defined in the quality management system and dictated by the frequency of use of the equipment and the application.

Calibrate the instrument at the temperature used for the measurement by using at least 2 certified reference materials matching the viscosity range of the viscometer.

Calculate the viscometer constant (k) in square millimetres per second squared, using the following expression:

$$k = \frac{\eta}{\rho t}$$

η = dynamic viscosity of the certified reference material, in millipascal seconds;
ρ = density of the certified reference material, in milligrams per cubic millimetre;
t = flow time for the certified reference material to drop from the upper mark to the lower mark, in seconds.

Calculate the mean of the values obtained.

Method

Charge the viscometer (Figure 2.2.9.-1) through tube *L* with a sufficient quantity of the liquid to be examined (previously brought to 20 °C unless otherwise prescribed) to fill bulb *A* while ensuring that the level of liquid in bulb *B* is below the exit to ventilation tube *M*. Immerse the viscometer in the upright position in a water-bath at 20.0 ± 0.1 °C (unless otherwise prescribed) and allow to stand for not less than 30 min to allow the temperature to reach equilibrium. Close tube *M* and draw the level of the liquid in tube *N* up to a level about 8 mm above mark *E*. Keep the liquid at this level by closing tube *N* and opening tube *M*. Open tube *N* and, using a stopwatch, measure the time required, to at least the nearest 1/5 of a second, for the level of the liquid to drop from mark *E* to mark *F*.

The flow time of the liquid to be examined is the mean of 3 consecutive measurements. The result is valid if the relative standard deviation of the 3 measurements is not more than 2.0 per cent.

Calculation

Calculate the kinematic viscosity (ν) (*2.2.8*), in square millimetres per second, using the following expression:

$$\nu = kt$$

k = viscometer constant, in square millimetres per second squared;
t = flow time of the liquid to be examined, in seconds.

Calculate the dynamic viscosity (η) (*2.2.8*), in millipascal seconds, using the following expression:

$$\eta = k\rho t$$

ρ = density of the liquid to be examined at the temperature used for the viscosity measurement, in milligrams per cubic millimetre.

The density may be obtained by multiplying the relative density of the liquid to be examined by 0.99820 (measurement at 20 °C) or 0.99704 (measurement at 25 °C).

Method III (Rotating Viscometer Method)

(Ph. Eur. method 2.2.10)

The principle of the method is to measure the force acting on a rotor (torque) when it rotates at a constant angular velocity (rotational speed) in a liquid. Rotating viscometers are used for measuring the viscosity of Newtonian (shear-independent viscosity) or non-Newtonian liquids (shear dependent viscosity or apparent viscosity). Rotating viscometers can be divided in 2 groups, namely absolute and relative viscometers. In absolute viscometers the flow in the measuring geometry is well defined. The measurements result in absolute viscosity values, which can be compared with any other absolute values. In relative viscometers the flow in the measuring geometry is not defined. The measurements result in relative viscosity values, which cannot be compared with absolute values or other relative values if not determined by the same relative viscometer method.

Different measuring systems are available for given viscosity ranges as well as several rotational speeds.

APPARATUS

The following types of instruments are most common.

CONCENTRIC CYLINDER VISCOMETERS (ABSOLUTE VISCOMETERS)

In the concentric cylinder viscometer (coaxial double cylinder viscometer or simply coaxial cylinder viscometer), the viscosity is determined by placing the liquid in the gap between the inner cylinder and the outer cylinder. Viscosity measurement can be performed by rotating the inner cylinder (Searle type viscometer) or the outer cylinder (Couette type viscometer), as shown in Figures 2.2.10.-1 and 2.2.10.-2, respectively. For laminar flow, the viscosity (or apparent viscosity) η expressed in pascal-seconds is given by the following formula:

$$\eta = \frac{1}{\omega}\left(\frac{M}{4\pi h}\right)\left(\frac{1}{R_i^2} - \frac{1}{R_o^2}\right) = k\frac{M}{\omega}$$

M = torque in newton-metres acting on the cylinder surface,
ω = angular velocity in radians per second,
h = height of immersion in metres of the inner cylinder in the liquid medium,
R_i = radius in metres of the inner cylinder,
R_o = radius in metres of the outer cylinder,
k = constant of the apparatus, expressed in radians per cubic metre.

For non-Newtonian liquids it is indispensable to specify the shear stress (τ) or the shear rate (γ) at which the viscosity is measured. Under narrow gap conditions (conditions satisfied in absolute viscometers), there is a proportional relationship between M and τ and also between ω and γ:

$$\tau = AM \qquad \gamma = B\omega$$

where A and B are constants for the instrument and are calculated from the following expressions:

— *for concentric surface:*

$$A = \frac{1}{4\pi h}\frac{R_i^2 + R_0^2}{R_i^2 R_0^2} \qquad B = \frac{R_i^2 + R_0^2}{R_0^2 - R_i^2}$$

— *for cone-plates:*

$$A = \frac{3}{2\pi R^3} \qquad B = \frac{1}{\alpha}$$

M = torque in Newton-metres acting on the cone or cylinder surface,
ω = angular velocity in radians per second,
R_i = radius in metres of the inner cylinder,
R_0 = radius in metres of the outer cylinder,
R = radius in metres of the cone,
h = height of immersion in metres of the inner cylinder in the liquid medium,
α = angle in radians between the flat disk and the cone,
τ = shear stress in pascals (Pa),
γ = shear rate in reciprocal seconds (s^{-1}).

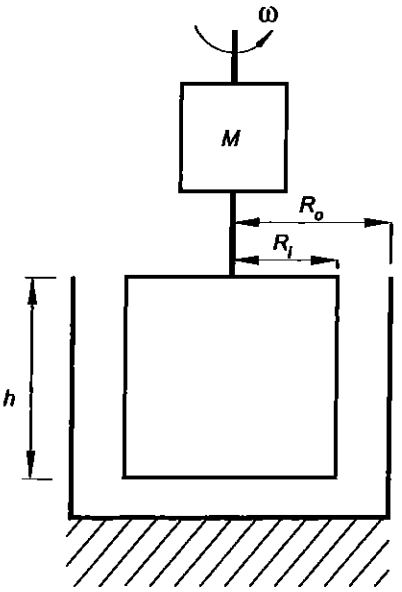


Figure 2.2.10.-1

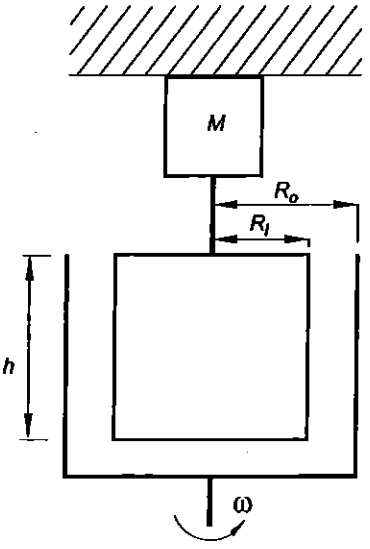


Figure 2.2.10.-2

CONE-PLATE VISCOMETERS (ABSOLUTE VISCOMETERS)

In the cone-plate viscometer, the liquid is introduced into the gap between a flat disc and a cone forming a define angle. Viscosity measurement can be performed by rotating the cone or the flat disc, as shown in Figures 2.2.10.-3 and 2.2.10.-4, respectively. For laminar flow, the viscosity (or apparent viscosity) η expressed in pascal-seconds is given by the following formula:

$$\eta = \left(\frac{M}{\omega}\right)\left(\frac{3\alpha}{2\pi R^3}\right) = k\frac{M}{\omega}$$

M = torque in Newton-metres acting on the flat disc or cone surface,
ω = angular velocity in radians per second,
α = angle in radians between the flat disc and the cone,
R = radius in metres of the cone,
k = constant of the apparatus, expressed in radians per cubic metre.

Constants A, B of the apparatus (see under concentric cylinder viscometers).

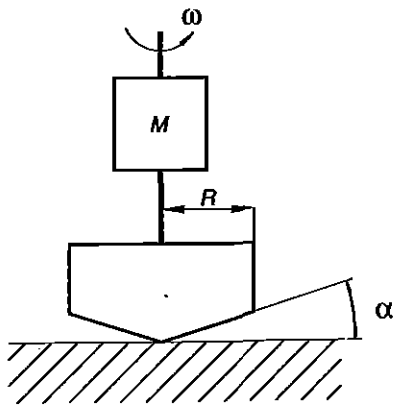


Figure 2.2.10.-3

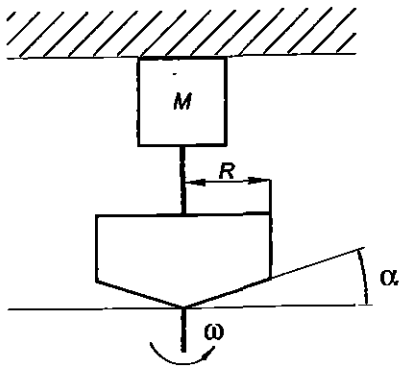


Figure 2.2.10.-4

SPINDLE VISCOMETERS (RELATIVE VISCOMETERS)

In the spindle viscometer, the viscosity is determined by rotating a spindle (for example, cylinder- or disc-shaped, as shown in Figures 2.2.10.-5 and 2.2.10.-6, respectively) immersed in the liquid. Relative values of viscosity (or apparent viscosity) can be directly calculated using conversion factors from the scale reading at a given rotational speed.

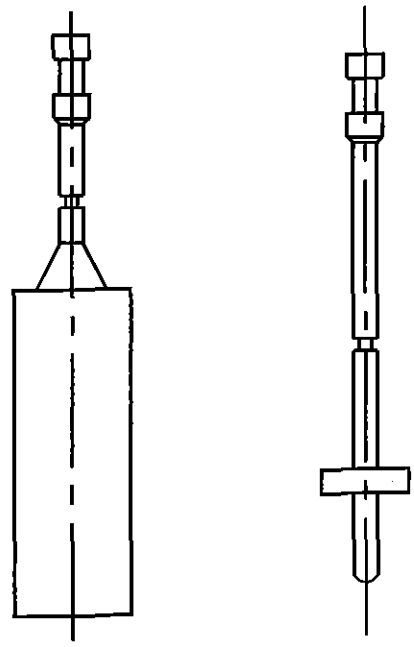


Figure 2.2.10.-5

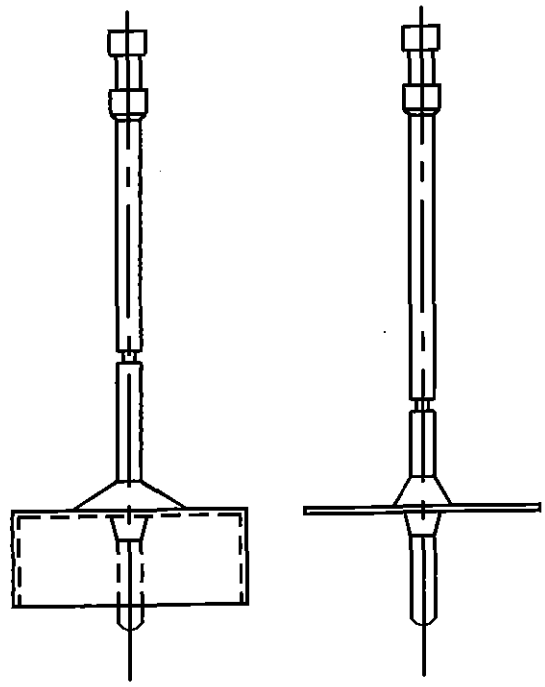


Figure 2.2.10.-6

In a general way, the constant k of the apparatus may be determined at various speeds of rotation using a certified viscometer calibration liquid. The viscosity η then corresponds to the formula:

$$\eta = k\frac{M}{\omega}$$

METHOD

Measure the viscosity (or apparent viscosity) according to the instructions for the operation of the rotating viscometer. The temperature for measuring the viscosity is indicated in the monograph. For non-Newtonian systems, the monograph indicates the type of viscometer to be used and if absolute viscometers are used the angular velocity or the shear rate at

which the measurement is made. If it is impossible to obtain the indicated shear rate exactly, use a shear rate slightly higher and a shear rate slightly lower and interpolate.

With relative viscometers the shear rate is not the same throughout the sample and therefore it cannot be defined. Under these conditions, the viscosity of non-Newtonian liquids determined from the previous formula has a relative character, which depends on the type of spindle and the angular velocity as well as the dimensions of the sample container (Ø = minimum 80 mm) and the depth of immersion of the spindle. The values obtained are comparable only if the method is carried out under experimental conditions that are rigorously the same.

Method IV (Falling Ball and Automatic Rolling Ball Viscometer Methods)

(Ph. Eur. method 2.2.49)

The determination of dynamic viscosity of Newtonian liquids using a falling ball or rolling ball viscometer is performed at 20.0 ± 0.1 °C, unless otherwise prescribed in the monograph. The time required for a test ball to fall or roll in the liquid to be examined from one ring mark or sensor to the other is determined.

METHOD A - FALLING BALL VISCOMETER

Apparatus The falling ball viscometer consists of: a glass tube enclosed in a mantle, which allows precise control of temperature; 6 balls made of glass, nickel-iron or steel with different densities and diameters. The tube is fixed in such a way that the axis is inclined by 10 ± 1° with regard to the vertical. The tube has 2 ring marks that define the distance the ball has to fall. Commercially available apparatuses are supplied with tables giving the constants, the density of the balls and the suitability of the different balls for the expected viscosity range.

Method Fill the clean, dry tube of the viscometer, previously brought to 20.0 ± 0.1 °C, with the liquid to be examined, avoiding bubbles. Choose the ball that is suitable for the viscosity range of the liquid so as to obtain a falling time (run time) not less than 30 s. Place it in the tube, close the tube and maintain the solution at 20.0 ± 0.1 °C for at least 15 min. Let the ball run through the liquid between the 2 ring marks once without measurement. Let it run again and measure with a stop-watch, to at least the nearest 1/5 s, the time required for the ball to fall from the upper to the lower ring mark. Repeat the test run at least 3 times to obtain a minimum of 4 run times. The result is only valid if the relative standard deviation determined on the run times is not greater than 2.0 per cent .

Use the mean of the run times to calculate the dynamic viscosity (η) in millipascal seconds using the formula:

$$\eta = k(\rho_1 - \rho_2) \times t$$

k = constant, in square millimetres per second squared;
ρ_1 = density of the ball used, in grams per cubic centimetre;
ρ_2 = density of the liquid to be examined, in grams per cubic centimetre, obtained by multiplying its relative density (d_{20}^{20}) by 0.9982;
t = mean of the run times of the ball, in seconds.

METHOD B – AUTOMATIC ROLLING BALL VISCOMETER

Apparatus The automatic rolling ball viscometer consists of: several capillaries made of glass or other suitable materials with different diameters enclosed in a thermostatically controlled block, which allows precise control of the temperature; balls made of stainless steel (optionally coated) or other suitable materials; a motor drive that positions the capillary at an inclination angle from 10.0 ± 0.2° to 80.0 ± 0.2° with regard to the vertical. The apparatus has at least 2 sensors to measure the run time of the ball over a predefined distance. Commercially available apparatuses are supplied with tables giving the constants, the density of the balls and the suitability of the different capillaries for the expected viscosity range. Temperature control, control of the inclination angle, run-time determination, repetitions of runs and calculations of the mean and relative standard deviation are performed by the apparatus software.

Method Set the instrument software to perform a measurement with at least 4 run times (2 forward/backward cycles) and a maximum relative standard deviation of 0.5 per cent. Choose the capillary, the ball and the inclination angle that are suitable for the expected viscosity range of the liquid to be examined so as to obtain a rolling time (run time) not less than 20 s over the 100 mm distance or a proportional time over other distances. If no digital density meter is connected to the viscometer, ensure that the density value of the liquid to be examined has been specified in the apparatus software. Fill the clean, dry capillary of the viscometer with the liquid to be examined, avoiding bubbles. Start the measurement immediately after filling the capillary.

The instrument calculates automatically the dynamic viscosity (η) in millipascal seconds and the kinematic viscosity (ν) in square millimetres per second.

Additional points for monographs of the British Pharmacopoeia

The apparatus and methods described in Methods I and II are in agreement in all essentials with International Standards ISO 3104-1994 and 3105-1994 (Methods for the determination of the viscosity of liquids).

J. Circular Dichroism

(Ph. Eur. method 2.2.41)

The difference in absorbance of optically active substances within an absorption band for left and right circularly polarised light is referred to as circular dichroism.

Direct measurement gives a mean algebraic value:

$$\Delta A = A_L - A_R$$

ΔA = circular dichroic absorbance,
A_L = absorbance of left circularly polarised light,
A_R = absorbance of right circularly polarised light.

Circular dichroism is calculated using the equation:

$$\Delta\varepsilon = \varepsilon_L - \varepsilon_R = \frac{\Delta A}{c \times l}$$

$\Delta\varepsilon$ = molar circular dichroism or molar differential dichroic absorptivity expressed in litre·mole^{-1}·cm^{-1},
ε_L = molar absorptivity (*2.2.25*) of left circularly polarised light,
ε_R = molar absorptivity of right circularly polarised light,
c = concentration of the test solution in mole·litre^{-1},
l = optical path of the cell in centimetres.

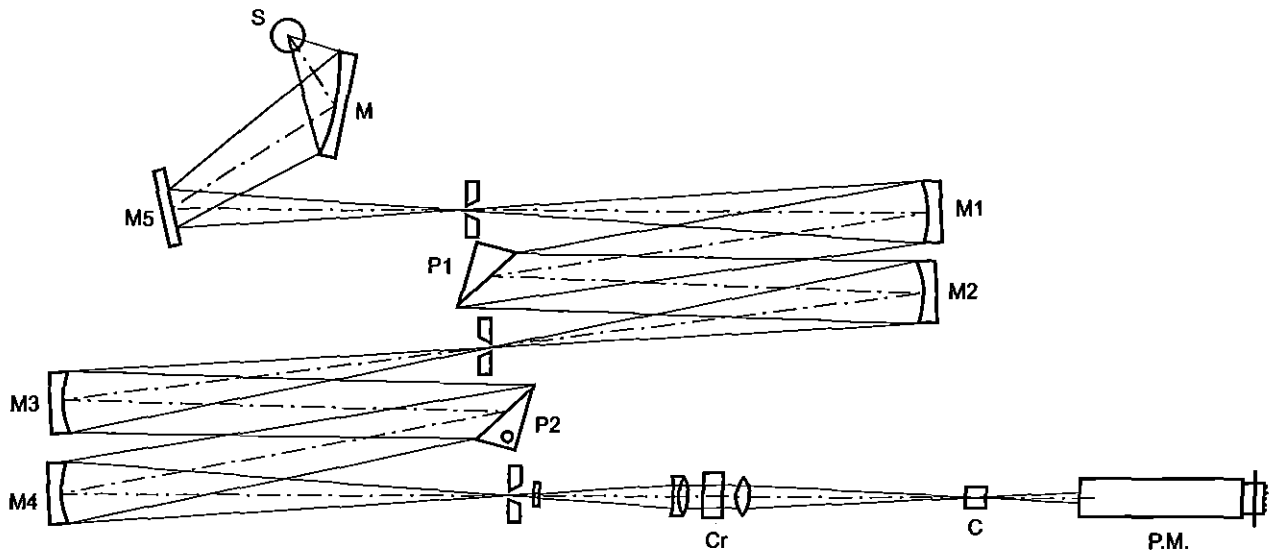


Figure 2.2.41.-1. – *Optical scheme of a dichrograph*

The following units may also be used to characterise circular dichroism:

Dissymmetry factor:

$$g = \frac{\Delta\varepsilon}{\varepsilon}$$

ε = molar absorptivity (*2.2.25*).

Molar ellipticity:

Certain types of instruments display directly the value of ellipticity Θ, expressed in degrees. When such instruments are used, the molar ellipticity [Θ] may be calculated using the following equation:

$$[\Theta] = \frac{\Theta \times M}{c \times l \times 10}$$

[Θ] = molar ellipticity, expressed in degrees·cm^2·decimole^{-1},
Θ = value of ellipticity given by the instrument,
M = relative molecular mass of the substance to be examined,
c = concentration of the solution to be examined in g/mL,
l = optical path of the cell in centimetres.

Molar ellipticity is also related to molar circular dichroism by the following equation:

$$[\Theta] = 2.303\Delta\varepsilon\frac{4500}{\pi} \approx 3300\Delta\varepsilon$$

Molar ellipticity is often used in the analysis of proteins and nucleic acids. In this case, molar concentration is expressed in terms of monomeric residue, calculated using the expression:

$$\frac{\text{molecular mass}}{\text{number of amino acids}}$$

The mean relative molecular mass of the monomeric residue is 100 to 120 (generally 115) for proteins and about 330 for nucleic acids (as the sodium salt).

Apparatus

The light source (S) is a xenon lamp (Figure 2.2.41.-1); the light passes through a double monochromator (M) equipped with quartz prisms (P1, P2).

The linear beam from the first monochromator is split into 2 components polarised at right angles in the second monochromator. The exit slit of the monochromator eliminates the extraordinary beam.

The polarised and monochromatic light passes through a birefringent modulator (Cr): the result is alternating circularly polarised light.

The beam then passes through the sample to be examined (C) and reaches a photomultiplier (PM) followed by an amplifier circuit which produces 2 electrical signals: one is a direct current V_c and the other is an alternating current at the modulation frequency V_{ac} characteristic of the sample to be examined. The phase gives the sign of the circular dichroism. The ratio V_{ac}/V_c is proportional to the differential absorption ΔA which created the signal. The region of wavelengths normally covered by a dichrograph is 170 nm to 800 nm.

Calibration of the apparatus

Accuracy of absorbance scale Dissolve 10.0 mg of *isoandrosterone R* in *dioxan R* and dilute to 10.0 mL with the same solvent. Record the circular dichroism spectrum of the solution between 280 nm and 360 nm. Measured at the maximum at 304 nm, $\Delta\varepsilon$ is + 3.3.

The solution of *(1S)-(+)-10-camphorsulfonic acid R* may also be used.

Linearity of modulation Dissolve 10.0 mg of *(1S)-(+)-10-camphorsulfonic acid R* in *water R* and dilute to 10.0 mL with the same solvent. Determine the exact concentration of camphorsulfonic acid in the solution by ultraviolet spectrophotometry (*2.2.25*), taking the specific absorbance to be 1.49 at 285 nm.

Record the circular dichroism spectrum between 185 nm and 340 nm. Measured at the maximum at 290.5 nm, $\Delta\varepsilon$ is + 2.2 to + 2.5. Measured at the maximum at 192.5 nm, $\Delta\varepsilon$ is −4.3 to −5.

(1S)-(+)- Or antipodal *(1R)-(−)-ammonium 10-camphorsulfonate R* can also be used.

K. Approximate pH of Solutions

(Ph. Eur. method 2.2.4)

Determine the approximate pH using a *pH indicator strip R*. Alternatively, pH indicators such as those described in Table 2.2.4.-1 can be used.

Table 2.2.4.-1

Reaction	pH	Indicator
Alkaline	> 8	*Red litmus paper R*
Slightly alkaline	8 – 10	*Phenolphthalein solution R*
		Thymol blue solution R
Strongly alkaline	> 10	*Phenolphthalein paper R*
		Thymol blue solution R
Neutral	6 – 8	*Methyl red solution R*
		Phenol red solution R
Acid	< 6	*Methyl red solution R*
		Bromothymol blue solution R1
Slightly acid	4 – 6	*Methyl red solution R*
		Bromocresol green solution R
Strongly acid	< 4	*Congo red paper R*

L. Determination of pH Values

(Ph. Eur. method 2.2.3)

The pH of an aqueous solution is defined as the negative logarithm of the activity of its hydrogen ions, expressed conventionally as the hydrogen ion concentration of the solution. For practical purposes, its definition is an experimental one. The pH of a solution to be examined is related to that of a reference solution (pH_s) by the following equation:

$$pH = pH_s - \frac{E - E_s}{k}$$

in which E is the potential, expressed in volts, of the cell containing the solution to be examined and E_s is the potential, expressed in volts, of the cell containing the solution of known pH (pH_s), k is the change in potential per unit change in pH, expressed in volts and calculated from the Nernst equation.

Table 2.2.3.-1. – *Values of k at different temperatures*

Temperature (°C)	k (V)
15	0.0572
20	0.0582
25	0.0592
30	0.0601
35	0.0611

The potentiometric determination of pH is made by measuring the potential difference between 2 appropriate electrodes immersed in the solution to be examined; 1 of these electrodes is sensitive to hydrogen ions (usually a glass electrode) and the other is the reference electrode (e.g. a silver-silver chloride electrode). They are often combined as 1 compact electrode, together with a temperature probe.

Apparatus The measuring apparatus is usually a voltmeter with an input resistance at least 100 times that of the electrodes used. It is normally graduated in pH units and has a sensitivity such that discrimination of at least 0.05 pH unit or at least 0.003 V may be achieved.

Recent pH meters are microprocessor-controlled and are operated using the manufacturer's firmware or software, according to given instructions.

Management of electrodes The electrodes are stored appropriately and according to the manufacturer's recommendations (e.g. in an electrolyte solution or a suitable storage solution). Before measurement, the electrodes are visually checked. Refillable electrodes are checked for the absence of air bubbles in the glass bulb and to ensure that the inner electrolyte solution level is satisfactory. The filling orifice has to remain open during the measurement. It is also recommended that the diaphragm of the reference electrode is checked. Before first use, or if the electrode has been stored out of electrolyte solution, it is usually necessary to condition it, according to the recommendations of the manufacturer. If pH stabilisation is too slow (i.e. a long response time), or a zero point shift, reduced slope or any other difficulties in calibration are observed, the electrode will probably need to be cleaned or replaced. The cleaning is performed depending on the type of sample and as prescribed in the manufacturer's manual. Regular cleaning is recommended.

Calibration and measurement conditions Unless otherwise prescribed in the monograph, all measurements are carried out at the same temperature as that used for calibration (± 2.5 °C), usually between 20 °C and 25 °C. Table 2.2.3.-2 shows the variation of pH with respect to temperature of a number of reference buffer solutions used for calibration. Follow the manufacturer's instructions for temperature correction.

The calibration consists of the determination of the slope (e.g. 95-105 per cent) and the offset of the measuring system. Most commercial pH meters offer a "self test" or "start-up test" where, for example, the slope and asymmetry potential are tested and compared to the manufacturer's specifications. The apparatus is calibrated using at least 2 buffer solutions chosen so that the expected pH value of the solution to be examined lies between the pH values of the buffer solutions. The range must be at least 2 pH units. The pH of another buffer solution of intermediate pH, read from the scale, must not differ by more than 0.05 pH units from the value corresponding to that solution.

Reference buffer solutions are preferably commercially available certified reference materials. Alternatively, buffer solutions can be prepared in-house according to Table 2.2.3.-2. These solutions must be traceable to primary standards. Calibration has to be performed on a regular basis, preferably each day of use or before each series of measurements.

Immerse the electrodes in the solution to be examined and take the reading in the same conditions as those applied for the reference buffer solutions.

If suspensions, emulsions or solutions of non-aqueous or partially non-aqueous character are measured on a system calibrated as described above, the pH reading can only be considered to be an approximation of the true value. Suitable electrodes have to be used for pH measurements of such mixtures.

PREPARATION OF REFERENCE BUFFER SOLUTIONS

Potassium tetraoxalate 0.05 M

Dissolve 12.61 g of $C_4H_3KO_8,2H_2O$ in *carbon dioxide-free water R* and dilute to 1000.0 mL with the same solvent.

Table 2.2.3.-2. – *pH of reference buffer solutions at various temperatures*

Temperature (°C)	Potassium tetraoxalate 0.05 M	Potassium hydrogen tartrate saturated at 25 °C	Potassium dihydrogen citrate 0.05 M	Potassium hydrogen phthalate 0.05 M	Potassium dihydrogen phosphate 0.025 M+ Disodium hydrogen phosphate 0.025 M	Potassium dihydrogen phosphate 0.0087 M+ Disodium hydrogen phosphate 0.0303 M	Disodium tetraborate 0.01 M	Sodium carbonate 0.025 M+ Sodium bicarbonate 0.025 M	Calcium hydroxide, saturated at 25°C
	$C_4H_3KO_8$, $2H_2O$	$C_4H_5KO_6$	$C_6H_7KO_7$	$C_8H_5KO_4$	KH_2PO_4+ Na_2HPO_4	KH_2PO_4+ Na_2HPO_4	$Na_2B_4O_7$, $10H_2O$	Na_2CO_3+ $NaHCO_3$	$Ca(OH)_2$
15	1.67		3.80	4.00	6.90	7.45	9.28	10.12	12.81
20	1.68		3.79	4.00	6.88	7.43	9.23	10.06	12.63
25	1.68	3.56	3.78	4.01	6.87	7.41	9.18	10.01	12.45
30	1.68	3.55	3.77	4.02	6.85	7.40	9.14	9.97	12.29
35	1.69	3.55	3.76	4.02	6.84	7.39	9.10	9.93	12.13
$\frac{\Delta pH^{(1)}}{\Delta t}$	+ 0.001	−0.0014	−0.0022	+ 0.0012	−0.0028	−0.0028	−0.0082	−0.0096	−0.034

(1) pH variation per degree Celsius.

Potassium hydrogen tartrate, saturated at 25 °C
Shake an excess of $C_4H_5KO_6$ vigorously with *carbon dioxide-free water R* at 25 °C. Filter or decant. Prepare immediately before use.

Potassium dihydrogen citrate 0.05 M
Dissolve 11.41 g of $C_6H_7KO_7$ in *carbon dioxide-free water R* and dilute to 1000.0 mL with the same solvent. Prepare immediately before use.

Potassium hydrogen phthalate 0.05 M
Dissolve 10.13 g of $C_8H_5KO_4$, previously dried for 1 h at 110 ± 2 °C, in *carbon dioxide-free water R* and dilute to 1000.0 mL with the same solvent.

Potassium dihydrogen phosphate 0.025 M + Disodium hydrogen phosphate 0.025 M
Dissolve 3.39 g of KH_2PO_4 and 3.53 g of Na_2HPO_4, both previously dried for 2 h at 120 ± 2 °C, in *carbon dioxide-free water R* and dilute to 1000.0 mL with the same solvent.

Potassium dihydrogen phosphate 0.0087 M + Disodium hydrogen phosphate 0.0303 M
Dissolve 1.18 g of KH_2PO_4 and 4.30 g of Na_2HPO_4, both previously dried for 2 h at 120 ± 2 °C, in *carbon dioxide-free water R* and dilute to 1000.0 mL with the same solvent.

Disodium tetraborate 0.01 M
Dissolve 3.80 g of $Na_2B_4O_7,10H_2O$ in *carbon dioxide-free water R* and dilute to 1000.0 mL with the same solvent. Store protected from atmospheric carbon dioxide.

Sodium carbonate 0.025 M + Sodium hydrogen carbonate 0.025 M
Dissolve 2.64 g of Na_2CO_3 and 2.09 g of $NaHCO_3$ in *carbon dioxide-free water R* and dilute to 1000.0 mL with the same solvent. Store protected from atmospheric carbon dioxide.

Calcium hydroxide, saturated at 25 °C
Shake an excess of *calcium hydroxide R* with *carbon dioxide-free water R* and decant at 25 °C. Store protected from atmospheric carbon dioxide.

STORAGE OF BUFFER SOLUTIONS
Store buffer solutions in suitable chemically-resistant, airtight containers, such as type I glass bottles or plastic containers suitable for aqueous solutions.

Additional points for monographs of the British Pharmacopoeia
[Suitable glass electrodes and pH meters of both the analogue and digital type are described in British Standards 2586:1979 and 3145:1978.]

M. Thermal Analysis[1]

(Ph. Eur. method 2.2.34)

Thermal analysis is a group of techniques in which the variation of a physical property of a substance is measured as a function of temperature. The most commonly used techniques are those which measure changes in the mass or energy of a sample of a substance.

These techniques have different applications:
— determination of phase changes;
— determination of changes in chemical composition;
— determination of purity.

THERMOGRAVIMETRY
Thermogravimetry (TG) or thermogravimetric analysis (TGA) is a technique in which the mass of a sample of a substance is recorded as a function of temperature according to a controlled temperature programme.

Instrument
The essential components of a thermobalance are a device for heating or cooling the substance according to a given temperature programme, a sample holder in a controlled atmosphere, an electrobalance and a means of electronic signal output to a recorder or a computer.

Temperature calibration
The temperature sensor close to or in contact with the sample is calibrated using the Curie temperature of a ferromagnetic substance such as nickel. In the case of an instrument capable of simultaneously conducting TG/TGA and differential thermal analysis (DTA) or differential scanning calorimetry (DSC), the same certified reference materials as those for DTA and DSC may be used, for example, indium, tin and/or zinc.

[1] *This chapter has undergone pharmacopoeial harmonisation. See chapter 5.8 Pharmacopoeial harmonisation.*

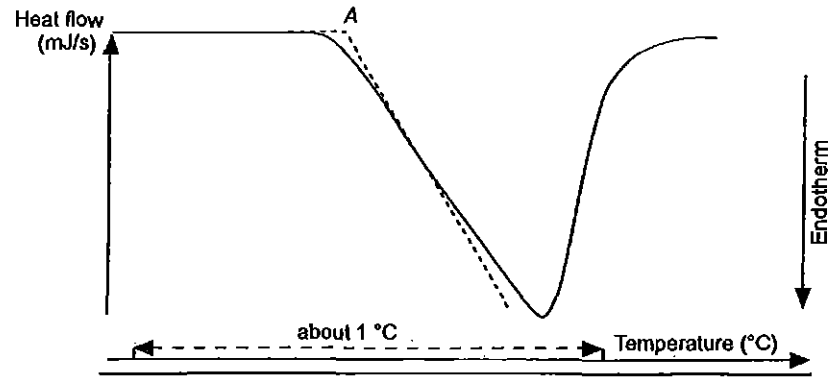


Figure 2.2.34.-1. – *Thermogram*

Calibration of the electrobalance
Place an appropriate quantity of a suitable certified reference material (e.g. *calcium oxalate monohydrate CRS*) in the sample holder and record the mass. Set the heating rate according to the manufacturer's instructions (e.g. 5 °C/min) and start the temperature increase. Record the thermogravimetric curve as a graph with temperature or time on the abscissa, increasing from left to right, and mass on the ordinate, decreasing downwards. Stop the temperature increase at about 250 °C. Measure the difference on the graph between the initial and final mass-temperature or mass-time plateaux, which corresponds to the loss of mass. The declared loss of mass for the certified reference material is stated on the label.

Method
Apply the same procedure to the substance to be examined using the conditions prescribed in the monograph. Calculate the loss of mass of the substance to be examined from the difference measured in the graph obtained. Express the loss of mass as $\Delta m/m$ (per cent).

If the instrument is in frequent use, carry out temperature calibration regularly. Otherwise, calibration is carried out before each measurement.

As the operating conditions are critical, the following parameters are noted for each measurement: pressure or flow rate, gas composition, mass of the sample, heating rate, temperature range and sample pre-treatment including any isothermal period.

DIFFERENTIAL SCANNING CALORIMETRY

Differential scanning calorimetry (DSC) is a technique that can be used to demonstrate the energy phenomena produced during heating (or cooling) of a substance (or a mixture of substances) and to determine the changes in enthalpy and specific heat and the temperatures at which these occur.

The technique is used to determine the difference in heat flow (with reference to the temperature) evolved or absorbed by the test sample compared with the reference cell, as a function of the temperature. Two types of DSC instruments are available, those using power compensation to maintain a null temperature difference between sample and reference and those that apply a constant rate of heating and detect temperature differential as a difference in heat flow between sample and reference.

Instrument
The instrument for power compensation DSC consists of a furnace containing a sample holder with a reference cell and a test cell. The instrument for heat flow DSC consists of a furnace containing a single cell with a sample holder for the reference crucible and the test crucible.

A temperature-programming device, thermal detector(s) and a recording system which can be connected to a computer are attached. The measurements are carried out under a controlled atmosphere.

Calibration of the instrument
Calibrate the instrument for temperature and enthalpy change, using suitable certified materials or reference standards.

Temperature calibration It can be performed using certified reference materials having an intrinsic thermal property, such as the melting point of pure metals or organic substances, or the phase transition point of crystalline inorganic salts or oxides. Melting points of indium, tin and/or zinc are usually employed for calibration.

Heat-quantity calibration For accurate estimation of the quantity of heat change (enthalpic change) of a test sample, caused by a certain physical change accompanying a temperature change, it is necessary to calibrate the instrument using suitable certified reference materials. Similar to temperature calibration, heat-quantity calibration may be performed using suitable certified reference materials showing a known definite enthalpic change caused by physical changes, such as the melting of pure metals and/or organic substances, or the phase transition of crystalline inorganic salts. The heats of fusion of indium, tin and/or zinc are usually employed for calibration.

Operating procedure
Weigh in a suitable crucible an appropriate quantity of the substance to be examined and place it in the sample holder. Place an empty crucible in the reference holder. Set the initial and final temperatures, and the heating rate according to the operating conditions prescribed in the monograph.

Begin the analysis and record the DSC curve with the temperature or time on the abscissa (values increasing from left to right) and the energy change on the ordinate (specify whether the change is endothermic or exothermic).

The temperature at which the phenomenon occurs (the onset temperature) corresponds to the intersection (A) of the extension of the baseline with the tangent at the point of greatest slope (inflexion point) of the curve (see Figure 2.2.34.-1). The end of the thermal phenomenon is indicated by the peak of the curve.

The enthalpy of the phenomenon is proportional to the area under the curve limited by the baseline; the proportionality factor is determined from the measurement of the heat of fusion of a known substance (e.g. indium) under the same operating conditions.

Each thermogram may be accompanied by the following data: conditions employed, record of last calibration, mass of

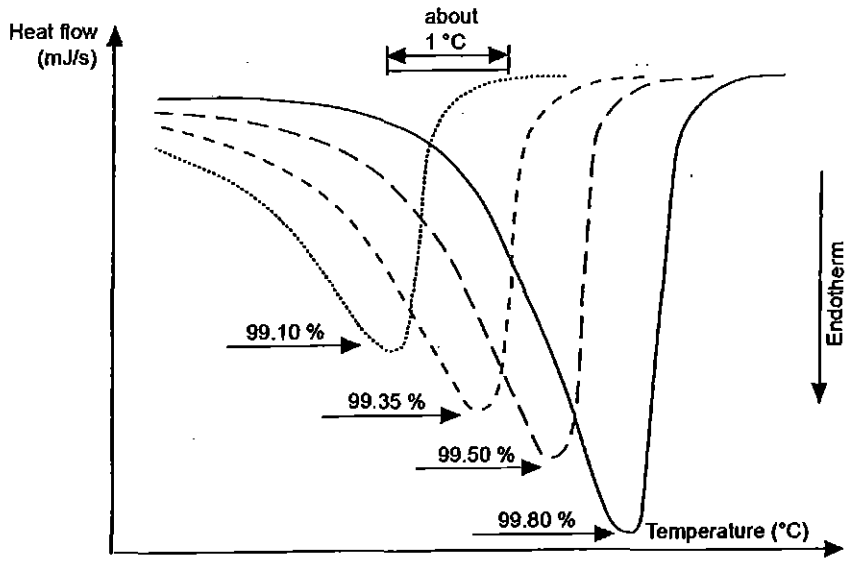


Figure 2.2.34.-2. – *Thermal diagrams according to purity*

the sample and identification (including thermal history), container, atmosphere (identity, flow rate, pressure), direction and rate of temperature change, instrument and recorder sensitivity.

Applications

Phase changes Determination of the temperature, heat capacity change and enthalpy of phase changes undergone by a substance as a function of temperature. The transitions that may be observed include those shown in Table 2.2.34.-1.

Table 2.2.34.-1.

solid - solid transition:	allotropy - polymorphism desolvation amorphous-crystalline
solid - liquid transition:	melting glass-transition
solid - gas transition:	sublimation
liquid - solid transition:	freezing recrystallisation glass-transition
liquid - gas transition:	evaporation

Changes in chemical composition Measurement of heat and temperatures of reaction under given experimental conditions, so that, for example, the kinetics of decomposition or desolvation can be determined.

Application to phase diagrams Establishment of phase diagrams for solid mixtures. The establishment of a phase diagram may be an important step in the preformulation and optimisation of the freeze-drying process.

Determination of purity The measurements of the fraction of substance melted at a given temperature and the heat of fusion by DSC enable the impurity content of a substance to be determined from a single thermal diagram, requiring the use of only a few milligrams of sample with no need for repeated accurate measurements of the true temperature.

In theory, the melting of an entirely crystalline, pure substance at constant pressure is characterised by a heat of fusion ΔH_f in an infinitely narrow range, corresponding to the melting point T_0. A broadening of this range is a sensitive indicator of impurities. Therefore, samples of the same substance, whose impurity contents vary by a few tenths of a per cent, give thermal diagrams that are visually distinct (see Figure 2.2.34.-2).

The determination of molar purity by DSC is based on the use of a mathematical approximation of the integrated form of the van't Hoff equation applied to the concentrations (not the activities) in a binary system [$\ln(1 - x_2) \approx -x_2$ and $T \times T_0 \approx T_0^2$]. For low amounts of impurities ($x_2 \ll 1$) and for temperatures close to the melting point T_0 the equation can be written as follows, in which T and x_2 are variables:

$$T = T_0 - \frac{RT_0^2}{\Delta H_f} \times x_2 \qquad (1)$$

T = temperature of the sample, in kelvins;
T_0 = melting point of the chemically pure substance, in kelvins;
R = gas constant for ideal gases, in joules·kelvin^{-1}·mole^{-1};
ΔH_f = molar heat of fusion of the pure substance, in joules·mole^{-1};
x_2 = mole fraction of the impurity, i.e., the number of molecules of the impurity divided by the total number of molecules in the liquid phase (or molten phase) at temperature T (expressed in kelvins).

Hence, the determination of purity by DSC is limited to the detection of impurities forming a eutectic mixture with the principal compound and present at a mole fraction of typically less than 2 per cent in the substance to be examined.

This method cannot be applied to:
— amorphous substances;
— solvates or polymorphic compounds that are unstable within the experimental temperature range;
— impurities forming solid solutions with the principal substance;
— impurities that are insoluble in the liquid phase or in the melt of the principal substance.

During the heating of the substance to be examined, the impurity melts completely at the eutectic temperature. Above this temperature, the solid phase contains only the pure substance. As the temperature increases progressively from the eutectic temperature to the melting point of the pure substance, the mole fraction of the impurity in the liquid phase decreases, since the quantity of liquefied pure substance increases.

For all temperatures above the eutectic point:

$$x_2 = \frac{1}{F} \times x_2^* \qquad (2)$$

F = molten fraction of the analysed sample;
x_2^* = mole fraction of the impurity in the analysed sample.

When the entire sample has melted, $F = 1$ and $x_2 = x_2^*$.

If equation (2) is combined with equation (1), the following equation is obtained:

$$T = T_0 - \frac{RT_0^2}{\Delta H_f} \times \frac{1}{F} \times x_2^*$$

The value of the heat of fusion of the pure substance is obtained by integrating the melting peak.

The melting point T_0 of the pure substance is extrapolated from the plot of temperature T (expressed in kelvins) versus $1/F$. The slope α of the curve (obtained after linearisation, if necessary) corresponding to $RT_0^2 \frac{x_2^*}{\Delta H_f}$, allows x_2^* to be evaluated.

The fraction x_2^* multiplied by 100 gives the mole fraction in per cent for total eutectic impurities.

N. Osmolality

(Ph. Eur. method 2.2.35)

PRINCIPLE

GENERAL

Osmolality is a measure of the total number of chemical entities per kilogram of solvent, and thus provides an indication of the osmotic pressure of the solution. Osmolality is dependent on the molal concentration of the solute(s) in the solution, on their dissociation and on the deviation of the solution from ideal behaviour (Raoult's law).

The unit of osmolality is the osmole per kilogram (osmol/kg), but the submultiple milliosmole per kilogram (mosmol/kg) is more commonly used.

The osmolality (ξ_m) of a solution containing i solutes is given by the expression:

$$\xi_m = \sum \nu_i m_i \Phi_{m,i}$$

ν_i = number of entities formed by the dissociation of one molecule of the i^{th} solute; if the solute is non-ionic (non-dissociating), ν_i equals 1,
m_i = molality of the i^{th} solute in the solution, in moles per kilogram of solvent,
$\Phi_{m,i}$ = molal osmotic coefficient, a dimensionless factor.

The molal osmotic coefficient is a measure of the deviation of the solution from ideal behaviour. For an ideal solution, osmolality equals molality (Φ=1).

In the case of a real, non-ideal solution, the molal osmotic coefficient is influenced by the interactions occurring amongst the components (i.e. molecules, ions, solvent) of the solution. The more complex the composition of the solution, the harder it becomes to determine Φ.

For this reason, the measurement of a colligative property such as the freezing-point depression is used as a practical means of determining osmolality by obtaining an overall measure of the contribution of the various solutes present in a solution.

PRINCIPLE OF MEASUREMENT

Unless otherwise prescribed, osmolality is determined by measuring the freezing-point depression (ΔT_f) of a solution. The relationship between osmolality and freezing-point depression is given by the expression:

$$\Delta T_f = k_f \xi_m$$

where k_f is the molal cryoscopic constant, which is solvent-dependent. For water, the value of k_f is 1.86 K/osmol (i.e. adding 1 mol of a non-dissociating solute to 1 kg of water results in a decrease in freezing-point of 1.86 K).

EQUIPMENT

An osmometer for freezing-point depression measurement typically consists of:

— an appropriate sample container;
— a means of cooling the sample;
— a temperature-sensitive resistor (thermistor), with an appropriate current or potential difference measurement device that can indicate a temperature depression or give osmolality values directly;
— a means of mixing the sample and/or inducing solidification when supercooling occurs.

PROCEDURE

CALIBRATION

Prepare reference solutions as specified in Table 2.2.35.-1, as necessary, using dried *sodium chloride R*. Commercially available certified solutions for osmometer calibration, with osmolalities equal or similar to those listed in Table 2.2.35.-1, may be used. Calibrate the equipment according to the manufacturer's instructions using *water R* to determine the zero value and at least 2 of the reference solutions listed in Table 2.2.35.-1. Confirm the calibration using at least one additional reference solution with a known osmolality (see Table 2.2.35.-1). Select a reference solution preferably with an osmolality within ± 50 mosmol/kg of the expected value for the solution to be examined or close to the centre of the expected osmolality range of the solutions to be examined. It is recommended that the reading is within ± 4 mosmol/kg of the osmolality of the chosen reference solution.

Table 2.2.35.-1. – *Reference solutions for osmometer calibration*

Mass of *sodium chloride R* in *water R* (g/kg)	Osmolality, ξ_m (mosmol/ kg)	Freezing-point depression, ΔT_f (K)
3.087	100	0.186
6.260	200	0.372
9.463	300	0.558
12.684	400	0.744
15.916	500	0.930
19.147	600	1.116
22.380	700	1.302

METHOD

Rinse the sample container with the solution to be examined before each measurement. Programme the device inducing solidification to start at a defined temperature below the expected freezing-point of the solution to be examined. Introduce an appropriate volume of the solution to be examined into the sample container according to the manufacturer's instructions, and start the cooling system. The equipment indicates when equilibrium has been reached.

Perform the test under the conditions (cooling temperature and volume) used to calibrate the equipment. Depending on the type of equipment, the osmolality can be read directly or

can be calculated from the measured freezing-point depression.

The test is not valid unless the measured osmolality of the solution to be examined lies within the calibrated osmolality range.

O. Conductivity[1]

(Ph. Eur. method 2.2.38)

INTRODUCTION

This general chapter provides information on how to carry out electrical conductivity measurements (hereafter referred to as 'conductivity') of fluids, including pure liquids. It is intended for fluid applications when conductivity is used to measure, monitor or control chemical dispensing (e.g. chemical purity or ionic concentration), and other applications where the ionic character of the fluid needs to be known or controlled.

Applications include, but are not limited to, solutions that may be used in clean-in-place, chromatography detection, ionic solution preparation, end point detection, dosing, fermentation and buffer production. In some cases, conductivity measurements can be extended to pure organic fluids such as alcohols and glycols where a weak conductivity signal exists, and the signal can be significantly increased if the organic fluids become contaminated with water or salts.

Conductivity is the measurement of the ability of a fluid to conduct electricity via its ions. The ability of any ion to electrically conduct is directly related to its ion mobility. Conductivity is directly proportional to the concentrations of ions in the fluid according to the following equation:

$$\kappa = 1000 \sum_{i}^{\text{all ions}} C_i \lambda_i$$

κ	=	conductivity, in siemens per centimetre;
C_i	=	concentration of ion i, in moles per litre;
λ_i	=	specific molar conductance of ion i, in siemens square centimetres per mole ($S \cdot cm^2 \cdot mol^{-1}$).

Although siemens per metre is the appropriate SI unit for conductivity, historically the unit siemens per centimetre has been selected by industry as the accepted unit.

Based on this equation, conductivity is not ion-selective because it responds to all ions. Furthermore, the specific molar conductance of each ion is different. As a result, unless the percentage composition of ions in the solution is limited and known, the precise concentrations of ionic species cannot be determined from conductivity measurements. However, for examples such as a solution of a single salt, acid or base (e.g. a caustic solution used in cleaning) the precise concentration can be directly determined. Despite the lack of ionic specificity, conductivity is a valuable laboratory and process tool for measurement and control of total ionic content because it is proportional to the sum of the concentrations of all ionic species (anions and cations) for diluted solutions, as described in the equation above.

At higher concentrations, conductivity measurements are not perfectly linear with concentration. Conductivity measurements cannot be applied to solids or gases, but they can be applied to the condensate of gases.

[1] *This general chapter has undergone pharmacopoeial harmonisation. See chapter 5.8. Pharmacopoeial harmonisation.*

Another variable that influences conductivity measurements is the fluid temperature. As fluid temperature increases, the ion conductance increases, making this physico-chemical phenomenon the predominant reason for the temperature-compensation requirement when testing conductive fluids.

The conductivity (κ) is proportional to the conductance (G), of a fluid between 2 electrodes:

$$\kappa = G \times \left(\frac{d}{A}\right) = G \times K$$

κ	=	conductivity, in siemens per centimetre;
G	=	conductance, in siemens;
d	=	distance between the electrodes, in centimetres;
A	=	area of the conducting electrodes, in square centimetres;
K	=	cell constant, in reciprocal centimetres, which is also equal to the ratio of d/A.

The resistivity ρ, expressed in ohm centimetres, of the fluid is, by definition, the reciprocal of the conductivity:

$$\rho = \frac{1}{\kappa} = \frac{1}{G \times K} = \frac{R}{K}$$

ρ	=	resistivity, in ohm centimetres;
κ	=	conductivity, in siemens per centimetre;
G	=	conductance, in siemens;
R	=	resistance, in ohms, which is the reciprocal of the conductance (G);
K	=	cell constant, in reciprocal centimetres.

EQUIPMENT

An electrical conductivity measurement consists of the determination of resistance of the fluid between and around the electrodes of the conductivity sensor. To achieve this measurement, the primary instrumentation is the resistance-measuring circuit and the conductivity sensor, and they are usually connected by a cable when the sensor and the user interface are separated.

The resistance measurement is made by applying an AC (alternating current, meaning the flow of electric charge periodically reverses direction) voltage (or current) to the electrodes, measuring the current (or voltage), and calculating the resistance according to Ohm's law.

The alternating source is utilised to prevent polarisation (collection of ions) at the electrodes. Depending on the equipment, the frequency of the measuring system adjusts automatically according to the measuring conditions of the instrument, and there may be multiple resistance-measuring circuits embedded in the measuring system. The resistance-measurement circuit may be embedded in the transmitter or in the sensor.

The conductivity sensor consists of at least 2 electrical conductors of a fixed size and geometry, separated by an electrical insulator. The electrodes, insulator and any other wetted components are constructed of materials that are unreactive to fluids with which they may come into contact. In addition, the sensor construction must be able to withstand the environmental conditions (process or ambient temperature, pressure, cleaning applications) that it would be subjected to.

Most conductivity sensors have temperature devices such as a platinum RTD (resistance temperature device) or NTC (negative temperature coefficient) thermistor embedded inside the sensor, although external temperature measurement is possible. The purpose of the temperature measurement is for temperature compensation of the conductivity measurement.

CELL CONSTANT DETERMINATION

The purpose of the sensor's cell constant is to normalise the conductance (or resistance) measurement for the geometrical construction of the 2 electrodes.

The cell constant is determined by immersing the conductivity sensor in a solution of known conductivity. Solutions of known conductivity can be obtained by preparation of specific recipes according to national authoritative sources, or procurement of commercially available certified and traceable standard solutions. These recipes or certified solutions can range from 5 to 200 000 $\mu S \cdot cm^{-1}$ depending on the level of accuracy desired. Alternatively, the cell constant is determined by comparison to other reference conductivity measuring systems (also available as an accredited calibration service). (*NOTE*: conductivity measurements are not perfectly linear with concentration.)

The measured cell constant of the conductivity sensor must be within 5 per cent of the nominal value indicated by the sensor certificate, unless otherwise prescribed.

Modern conductivity sensors normally do not change their cell constant over their lifetime. If a change in the cell constant is detected during calibration, cleaning of the sensor according to the manufacturer's recommendations is appropriate, followed by a repeat of the calibration procedure. Sometimes 'memory effects' appear, particularly when changing from high to low concentrations if the sensor is not well flushed.

CALIBRATION OF TEMPERATURE

In addition to verifying the sensor's cell constant, the embedded temperature device (or external temperature device) must be appropriately calibrated for the application, in order to apply the temperature compensation algorithm accurately. The temperature accuracy that is required depends on the criticality of the temperature to the application. An accuracy of ± 1 °C typically suffices.

CALIBRATION OF MEASUREMENT ELECTRONICS

The measurement circuit of the system is fundamentally an AC resistance measuring device. Appropriate verification and/or calibration of the measuring circuit is required for measurement systems with signal transfer via analog cable. This is accomplished by disconnecting the measuring circuit from the sensor's electrodes, attaching traceable resistors of known value to the measuring circuit using the same measurement system cable, and verifying that the measured resistance agrees with the resistor value to an acceptable level. A typical acceptance criterion for the resistance accuracy is below 2 per cent of the reading at resistances greater than 100 Ω, and increasing to 5 per cent at lower resistances. However, it is recommended that the application criticality ultimately determines the desired accuracy.

For conductivity systems that cannot have the resistance-measuring circuit disconnected from the electrodes (e.g. measurement circuit and electrodes in 1 mutual housing), it may be difficult to directly adjust or verify the circuit accuracy, depending on the sensor design.
An alternative method of verifying the measurement system integrity is a system calibration according to the procedures for cell constant determination for each measuring circuit that is intended to be used.

If verification/calibration of the sensor's cell constant, temperature device and measuring circuit are done at the same service interval, it is recommended to verify the measuring circuit first, the temperature device next and the cell constant last. Because all of these parameters are typically very stable due to modern electronics and stable sensor construction, frequent calibration (such as daily) is not usually required. Comparison to qualified reference systems is also a suitable means of calibration. Calibration is performed at appropriate intervals as defined in the quality management system.

TEMPERATURE COMPENSATION

As the conductivity of a fluid is temperature dependent, temperature compensation of the conductivity measurement is necessary unless otherwise prescribed (e.g. purified water, water for injection). An appropriate temperature compensation algorithm will ensure that changes in the conductivity measurements can be ascribed to concentration changes and not temperature changes. Conductivity measurements are normally referenced to 25 °C. A common form of linear temperature compensation uses a temperature coefficient according to the following equation:

$$\kappa_{25} = \frac{\kappa_T}{[1 + \alpha(T - 25)]}$$

κ_{25} = conductivity compensated to 25 °C;
κ_T = conductivity at T;
α = temperature coefficient of conductivity;
T = measured temperature.

A temperature coefficient of 2.1 per cent per degree Celsius is commonly used for many salt solutions. Most salt-based solutions have temperature coefficients of conductivity ranging from 1.9 to 2.2 per cent per degree Celsius. Depending on the fluid samples, other forms of temperature compensation may be appropriate. Non-linear temperature compensation data for a variety of solutions is widely available, e.g. as described in ISO 7888 *Water Quality – Determination of electrical conductivity*. In cases of very low conductivity (below 10 $\mu S \cdot cm^{-1}$), for example, purified water used for cleaning/rinsing purposes, two compensations need to be made. One is for the intrinsic conductivity of water, and the other is for the other ionic species in water. These compensations are normally combined and embedded in the microprocessor-controlled conductivity measurement systems. This is not supplied in all conductivity measurement technologies.

CONDUCTIVITY MEASUREMENT OF FLUIDS

For off-line or at-line batch measurements, rinse the cleaned sensor with the fluid to be measured, then perform the measurement. Ensure that the position of the sensor in the container does not impact the conductivity measurement, as the container walls can impact the measurement for some electrode designs. Record the temperature and the temperature-compensated conductivity as required.

For continuous on-line or in-line measurements, install the cleaned sensor into the pipe, tank or other containment vessel, and flush if necessary. Make sure proper installation procedures are applied to prevent bubbles or particles from collecting between the electrodes. Ensure that the position of the sensor in the pipe or tank does not impact the conductivity measurement, as the nearby surfaces can affect the measurement for some electrode designs. Record the temperature and the temperature-compensated conductivity as required.

For all batch or continuous measurements, ensure that the wetted components of the sensor are compatible with the fluid and the temperature to be measured.

P. Total Organic Carbon in Water for Pharmaceutical Use

(Ph. Eur. method 2.2.44)

Total organic carbon (TOC) determination is an indirect measure of organic substances present in water for pharmaceutical use. TOC determination can also be used to monitor the performance of various operations in the preparation of medicines.

A variety of acceptable methods is available for determining TOC. Rather than prescribing a given method to be used, this general chapter describes the procedures used to qualify the chosen method and the interpretation of results in limit tests. A standard solution is analysed at suitable intervals, depending on the frequency of measurements; the solution is prepared with a substance that is expected to be easily oxidisable (for example, sucrose) at a concentration adjusted to give an instrument response corresponding to the TOC limit to be measured. The suitability of the system is determined by analysis of a solution prepared with a substance expected to be oxidisable with difficulty (for example, 1,4-benzoquinone).

The various types of apparatus used to measure TOC in water for pharmaceutical use have in common the objective of completely oxidising the organic molecules in the sample water to produce carbon dioxide followed by measurement of the amount of carbon dioxide produced, the result being used to calculate the carbon concentration in the water.

The apparatus used must discriminate between organic and inorganic carbon, the latter being present as carbonate. The discrimination may be effected either by measuring the inorganic carbon and subtracting it from the total carbon, or by purging inorganic carbon from the sample before oxidisation. Purging may also entrain organic molecules, but such purgeable organic carbon is present in negligible quantities in water for pharmaceutical use.

Apparatus Use a calibrated instrument installed either on-line or off-line. Verify the system suitability at suitable intervals as described below. The apparatus must have a limit of detection specified by the manufacturer of 0.05 mg or less of carbon per litre.

TOC water Use highly purified water complying with the following specifications:
— conductivity: not greater than 1.0 $\mu S \cdot cm^{-1}$ at 25 °C,
— total organic carbon: not greater than 0.1 mg/L.

Depending on the type of apparatus used, the content of heavy metals and copper may be critical. The manufacturer's instructions should be followed.

Glassware preparation Use glassware that has been scrupulously cleaned by a method that will remove organic matter. Use *TOC water* for the final rinse of glassware.

Standard solution Dissolve *sucrose R*, dried at 105 °C for 3 h in *TOC water* to obtain a solution containing 1.19 mg of sucrose per litre (0.50 mg of carbon per litre).

Test solution Using all due care to avoid contamination, collect water to be tested in an airtight container leaving minimal head-space. Examine the water with minimum delay to reduce contamination from the container and its closure.

System suitability solution Dissolve *1,4-benzoquinone R* in *TOC water* to obtain a solution having a concentration of 0.75 mg of 1,4-benzoquinone per litre (0.50 mg of carbon per litre).

TOC water control Use *TOC water* obtained at the same time as that used to prepare the standard solution and the system suitability solution.

Control solutions In addition to the *TOC water control*, prepare suitable blank solutions or other solutions needed for establishing the baseline or for calibration adjustments following the manufacturer's instructions; run the appropriate blanks to zero the instrument.

System suitability Run the following solutions and record the responses: *TOC water* (r_w); *standard solution* (r_s); *system suitability solution* (r_{ss}). Calculate the percentage response efficiency using the expression:

$$\frac{r_{ss} - r_w}{r_s - r_w} \times 100$$

The system is suitable if the response efficiency is not less than 85 per cent and not more than 115 per cent of the theoretical response.

Procedure Run the test solution and record the response (r_u). The test solution complies with the test if r_u is not greater than $r_s - r_w$.

The method can also be applied using on-line instrumentation that has been adequately calibrated and shown to have acceptable system suitability. The location of instrumentation must be chosen to ensure that the responses are representative of the water used.

Q. Density of Solids

(Ph. Eur. method 2.2.42)

The density of solids corresponds to their average mass per unit volume and typically is expressed in grams per cubic centimetre (g/cm^3) although the International Unit is the kilogram per cubic metre (1 g/cm^3 = 1000 kg/m^3).

Unlike gases and liquids whose density depends only on temperature and pressure, the density of a solid also depends on its assembly and therefore varies with the crystal structure and degree of crystallinity.

When a solid is amorphous or partially amorphous, its density may further depend upon the history of preparation, treatment and storage.

Therefore, unlike fluids, the densities of 2 chemically equivalent solids may be different, and this difference reflects a difference in solid-state structure. The density of constituent particles is an important physical characteristic of pharmaceutical powders.

The density of a solid particle can assume different values depending on the method used to measure the volume of the particle. It is useful to distinguish 3 levels of expression of density:
— the *true density*, which only includes the solid fraction of the material; in case of crystalline material, the true density is also called *crystal density*;
— the *particle density*, which also includes the volume due to intraparticulate pores;
— the *bulk density*, which further includes the interparticulate void volume formed in the powder bed.

TRUE DENSITY

The true density of a substance is the ratio of the mass to the volume of the unit cell, exclusive of all voids that are not a fundamental part of the molecular packing arrangement. It is an intrinsic property of the specified crystal structure of

substance, and hence should be independent of the method of determination. The true density is determined by calculation.

It is obtained using crystallographic data (volume and composition of the unit cell) from, for example, X-ray diffraction data, either on a single crystal or by refinement of the crystalline structure from X-ray powder diffraction data.

PARTICLE DENSITY

The particle density takes into account both the true density and the intraparticulate porosity (sealed and/or experimentally non-accessible open pores). Thus, particle density depends on the value of the volume determined, which in turn depends on the method of measurement. The particle density can be determined using one of the 2 following methods.

The gas *pycnometric density* is determined by measuring the volume occupied by a known mass of powder, which is equivalent to the volume of gas displaced by the powder using a gas displacement pycnometer (*2.9.23*). In gas pycnometric density measurements, the volume determined excludes the volume occupied by open pores; however, it includes the volume occupied by sealed pores or pores inaccessible to the gas. Due to the high diffusivity of helium, which is the preferred choice of gas, most open pores are accessible to the gas. Therefore, the gas pycnometric density of a finely milled powder is generally not very different from the true density. Hence, this density is the best estimate of the true density of an amorphous or partially crystalline sample and is therefore widely applicable for processed pharmaceutical powder samples.

The *mercury porosimeter density* is also called *granular density*. With this method the volume determined includes the volume occupied by sealed pores or pores inaccessible to mercury; however, it includes the volume only from open pores smaller than some size limit. This pore-size limit or minimal access diameter depends on the maximal mercury intrusion pressure applied during the measurement, and under normal operating pressures the mercury does not penetrate the finest pores accessible to helium. Various granular densities can be obtained from one sample since, for each applied mercury intrusion pressure, a density can be determined that corresponds to the pore-size limit at that pressure.

BULK AND TAPPED DENSITY

The bulk density of a powder includes the contribution of interparticulate void volume. Hence, the bulk density depends on both the density of powder particles and the spatial arrangement of particles in the powder bed.

The bulk density of a powder is often very difficult to measure with good reproducibility since the slightest disturbance of the bed may result in a new density. Thus, it is essential in reporting bulk density to specify how the determination was made.

The bulk density and the tapped density are determined as mentioned in chapter *2.9.34. Bulk density and tapped density*.

R. Detection and Measurement of Radioactivity

(Ph. Eur. method 2.2.66)

INTRODUCTION

Within the context of the European Pharmacopoeia, the term 'radioactivity' is used both to describe the phenomenon of radioactive decay and to express the physical quantity of this phenomenon. In the monographs on radiopharmaceutical preparations, the detection and measurement of radioactivity are performed for different purposes: verification of the characters, identification, determination of radionuclidic and radiochemical purity, as well as determination of the radioactivity in a substance (assay).

Under these assumptions, the measurement can be qualitative, quantitative or both, depending whether it is directed to the identification of the radionuclide or the determination of its activity (rate of decay) or both of them.

Radioactive sources can produce various types of emissions, such as alpha particles, electrons, positrons, gamma- and X-rays, according to the radionuclidic composition.

Each radionuclide yields characteristic emissions, with specific energies and relative intensities. Such radiations can be detected as a result of their ionising properties in an ionisation chamber but without further characterisation; when they are detected and analysed using a spectrometer, an energy spectrum is obtained. A detailed spectrum analysis is typically used to identify radionuclides present in a sample. Spectrometry can also be used for quantitative determination of the radioactivity in sources made of a single radionuclide or radionuclide mixtures or of the individual radionuclides present.

A measurement of radioactivity is generally performed by counting the number of detected decay events (emissions). Therefore, the geometry of the sample during the measurement of radioactivity and the acquisition time strongly influence the result. In general, the measurement geometry must correspond to a calibrated geometry and the acquisition time must be long enough to reach sufficient counting statistics.

A measurement of radioactivity can be done in a stand-alone mode (e.g. using an ionisation chamber or a spectrometer) or in combination with a separation technique (e.g. radiochromatography) to account for relative contributions from different radioactive chemical species that may be present in a mixture.

MEASUREMENT OF RADIOACTIVITY

A direct determination of the radioactivity of a given sample, in becquerel (Bq), may be carried out if the decay scheme of the radionuclide is known, but in practice many corrections are required to obtain accurate results. For this reason, it is possible to carry out the measurement with the aid of a primary standard source or by using measuring instruments such as an ionisation chamber or a spectrometer calibrated using suitable standards for the particular radionuclides.

A spectrometer is used when measuring the radioactivity of radionuclides in a mixture, each radionuclide being identified by its emissions and their characteristic energies.

All measurements of radioactivity must be corrected for dead-time losses and by subtracting the background signal due to radiation in the environment and to spurious signals generated in the equipment itself.

The radioactivity of a preparation is stated at a given date. If the half-life of the radionuclide is less than 70 days, the time is also indicated. This statement of the radioactive content must be made with reference to a specified time zone. The radioactivity at other times may be calculated from the exponential decay equation or from tables.

In general, a correct measurement of radioactivity requires that consideration is given to some or all of the following:

Dead-time losses

Due to the finite resolving time (dead time) of the detector and its associated electronic equipment, it may be necessary to correct for losses by coincidence. The resolving time of a counter is the minimum time interval required by the counter to resolve 2 single pulses. Incident radiation events at shorter intervals may not be detected or may be detected as a single event with the summed energy. These losses are sometimes referred to as 'dead-time losses'. For a counting system with a fixed dead time τ following each count, the true count rate, per second, is calculated using the following expression:

$$\frac{N_1}{1 - N_1\tau}$$

N_1 = the observed count rate, per second;
τ = the dead time, in seconds.

With some equipment this correction is made automatically. Corrections for losses by coincidence must be made before the correction for background radiation.

Correction for decay during measurement

If the time period of an individual measurement, t_m, is not negligibly short compared with the half-life of the radionuclide, $T_{1/2}$, the decay during this measurement time must be taken into account. For example, there is a 5 per cent cumulative loss of counts due to decay during a counting period that is 15 per cent of the half-life of the radionuclide.

After having corrected the instrument reading (count rate, ionisation current, etc.) for background signals and, if necessary, for losses due to electronic effects, the instrument reading corrected to the beginning of the individual measurement is calculated using the following expression:

$$\frac{R(\lambda t_m)}{1 - (e^{-\lambda t_m})}$$

R = instrument reading before decay correction, but already corrected for background signal, etc.;
λ = radionuclide decay constant ($\ln 2/T_{1/2}$);
e = base of natural logarithm;
t_m = measurement duration.

Statistics of radioactivity measurement

The results of determinations of radioactivity show variations that derive mainly from the random nature of nuclear transformations. Counting for any finite time can yield only an estimate of the true rate of nuclear transformations. A sufficient number of counts must be registered in order to compensate for variations in the number of transformations per time. In the case of measurement of radioactivity, the standard deviation of the recorded counts is the square root of the counts, so at least 10 000 counts are necessary to obtain a relative standard deviation of not more than 1 per cent.

Linearity

The linearity of an instrument is the range of radioactivity for a particular radionuclide over which its efficiency remains constant.

The linear range of a radioactivity measurement assembly can be determined by repeatedly counting a radioactive sample in a fixed geometry as it decays from an activity level that is above the linear range. After correction for the background signal, the natural logarithm of the count rate data is plotted against the elapsed time after the first measurement (Figure 2.2.66.-1).

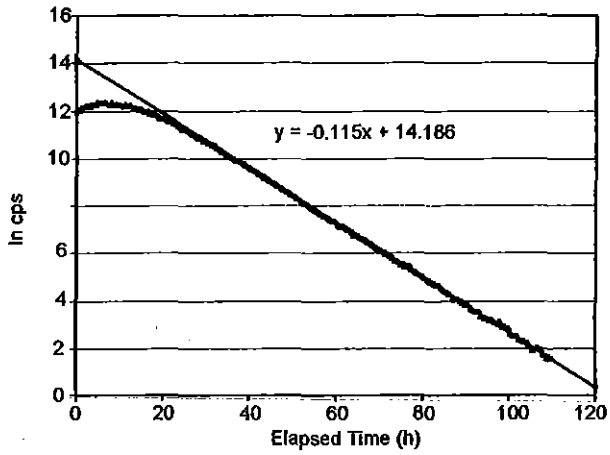


Figure 2.2.66.-1. – *Plot showing the measured and extrapolated count rate (natural logarithm of counts per second (cps)) from a technetium-99m source as a function of time, starting with a level of radioactivity above the linear range of the measuring equipment*

Linear regression analysis of the central, linear portion of the data set yields a slope which is the decay constant λ, which has a characteristic value for each radionuclide:

$$\ln \text{cps} = -\lambda t + c$$

c represents the natural logarithm of the count rate at $t = 0$ of a perfectly linear instrument.

The resulting regression equation is used to calculate the theoretical count rate at each time that the actual data were recorded. Where the deviation of the measured count rate from the theoretical count rate is unacceptably high, the linear range of the measuring equipment has been exceeded.

Alternatively, a series of dilutions can be made of a radioactive solution of known radioactivity concentration. Equal volumes of each of the dilutions are then counted using standardised geometry and counter settings. The ratio of the count rate for each sample (after correction for background signals and decay) to the calculated radioactivity of the respective sample in Bq is the counting efficiency. The range over which this ratio is constant is the useable range of the measuring equipment for the radionuclide concerned.

The limit of detection and the limit of quantification for equipment and procedures used for radioactivity measurement must be established before their routine use.

Limit of detection

The limit of detection (LOD) of an individual procedure is the lowest amount of radioactivity in a sample that can be detected but not necessarily quantified as an exact value. In practical terms this requires an estimate of the background signal and its standard deviation. The LOD is usually considered to be 3 times the standard deviation of the background signal.

Limit of quantification
The limit of quantification (LOQ) of an individual procedure is the lowest amount of radioactivity in a sample that can be quantitatively determined with suitable precision and accuracy. The LOQ is used particularly for the determination of impurities and/or degradation products. In practical terms the LOQ is usually considered to be 10 times the standard deviation of the background signal.

MEASUREMENT OF RADIOACTIVITY USING IONISATION CHAMBERS

Apparatus
Ionisation chambers (including dose calibrators) are the most common equipment for the measurement of radioactivity in the practice of radiopharmacy. It generally can measure activities from a few tens of kBq to hundreds of GBq.
It usually comprises a sealed well-type ionisation chamber and built-in electronics to convert the detector signal to a measure of radioactivity.

The chamber is filled with a gas across which an electrical field is applied. When the gas is ionised by the radiation emitted by the source, the resulting ionisation current is measured and related to the radioactivity present in the ionisation chamber. The ionisation current is influenced by the applied voltage, the energy and the intensity of the radiation and the nature and pressure of the gas.
The instrument settings (calibration factor) may be adjusted to keep a direct relationship between the ionisation produced by the radiation of a specific radionuclide and the radioactivity value obtained for each measurement geometry.

As an ionisation chamber measures only the current resulting from the overall ionisation produced within the chamber, it cannot discriminate between the emissions of different radionuclides.

For an accurate measurement of the radioactivity of a specific radionuclide, the measurement must be corrected for the contributions to the ionisation current caused by radionuclidic impurities present in the preparation.

The activity levels to be measured are limited by saturation considerations, the range of the amplifier and the design of the chamber itself. The linearity range of the ionisation chamber is established as described above under Linearity.

The ionisation chamber must be shielded to minimise background signals to an acceptable level.

Method
The sample is positioned inside the well of the ionisation chamber at a given position, using a holder. After putting the sample in the ionisation chamber, the activity reading is made once the response is stable. Measuring the sample under exactly the same geometrical conditions as the calibration source will yield the most accurate results.
If necessary, dilute the preparation to be measured to the same volume as that of the calibration source.

Calibration The ionisation chamber is calibrated taking into account the shape, dimensions, material of the container, volume and composition of the solution, the position within the chamber and the radionuclide being measured. Limits for uncertainty in calibration can be found in national and international regulations.

Calibrate the ionisation chamber at least once a year, by using sources of radionuclides traceable to national or international standards in the appropriate containers (vial, syringe) with regard to geometry. Establish and implement subsidiary correction factors to take account of the differing configurations of the radionuclides to be measured. Perform a linearity check of the instrument's response over the complete range of energies and activities for which the equipment is used.

For each setting and before each use (minimum once on each day of use) perform a constancy check of the ionisation chamber using standard sources of radionuclides with long half-lives to verify its calibrated state. A check with a reference source, such as caesium-137, must be performed on each day of use to verify that the ionisation chamber is still in its calibrated state.

MEASUREMENT OF RADIOACTIVITY USING SOLID-STATE DETECTORS

Solid-state detectors include scintillating plastic fluors and crystals, and semiconductors. Further to their application in spectrometry (see section Spectrometry), solid-state detectors can be used for the measurement of radioactivity.
In particular, due to their high sensitivity, plastic and crystal scintillation detectors are used in counting low levels of radioactivity. Dead-time losses must be carefully considered with these types of detectors. Semiconductor detectors are used when a higher energy discrimination is required, for example in mixtures of radionuclides or when there are potential radionuclidic impurities with emissions of similar energy.

Apparatus
The equipment consists of a shielded detector comprising a plastic or crystal scintillator coupled to a photomultiplier, or a semiconductor, which are connected to an amplifier and counting electronics. The system may have an adjustable energy window, used for selecting a counting region of the radionuclide energy spectrum that may be adjusted by the operator.

Instruments have different properties of energy resolution and detection efficiency depending on the type of detector and its volume and geometry. Lower efficiency requires a longer counting time.

Samples to be measured may be placed in front of the detector or into the well of a well-type detector. Measuring chambers may be enclosed in the detector shielding and single samples may be introduced using lids or other positioning systems to ensure correct measurement geometry.

A scintillation detector can be used for dynamic radioactivity measurement when, for example, the eluate of a liquid chromatograph is directed over or through a detector, see section on Detection and measurement of radioactivity in combination with a separation technique.

Method
Ensure that the sample radioactivity gives a counting rate in the linearity range of the equipment. The measurement is started after any shielding is in place or the well cover is replaced and the counting time is selected to reach sufficient counts for a statistically significant value.

Calibration The detector has to be calibrated by measuring its efficiency using a source of the radionuclide in question traceable to national or international standards. Calibration in terms of efficiency uses sources such as caesium-137, cobalt-60, barium-133 and others covering the desired energy range.

MEASUREMENT OF RADIOACTIVITY USING LIQUID SCINTILLATION DETECTORS

Liquid scintillation counting is commonly used for beta-particle emitting samples, but is also used for alpha-particle emitting samples. For the principles of the detection of

radioactivity using liquid scintillation detectors see under Beta-particle spectrometry below.

Calibration In order to take into account the loss of counting efficiency due to quenching, the liquid scintillation counter may make use of an external source, typically barium-133 or europium-152, which is brought close to the sample vial to release Compton electrons. The shape of the resulting spectrum is analysed automatically to compute a quench-indicating parameter. This parameter can then be related to the counting efficiency measuring sources of known activity at a determined level of quenching agent. The obtained quench curve allows the determination of the activity of an unknown sample knowing the count rate and the value of the quenching parameter.

DETERMINATION OF HALF-LIFE

The half-life is a characteristic of the radionuclide that may be used for its identification. The half-life is calculated by measuring the variation of radioactivity of a sample to be tested as a function of time. Perform the measurements in the linearity range of a calibrated instrument.

Apparatus

Half-life can be measured by using any type of quantitative radioactivity detector provided it is used within a linearity range throughout the range of activities that are present during the measurement and the geometry is not changed during the measurement.

For preparations containing a radionuclide with a short half-life and when stated in a monograph, determination of the approximate half-life contributes to the identification.

Method

Half-life The preparation to be examined is used as such or diluted or dried in a capsule after appropriate dilution. The radioactive sample is prepared in a manner that will avoid loss of material during handling. If it is a liquid (solution), it is contained in a closed flask or a sealed tube. If it is a residue from drying in a capsule, it is protected by a cover consisting of a sheet of adhesive cellulose acetate or of some other material.

The radioactivity of the sample must be high enough to allow measurements over a period corresponding to 3 estimated half-lives but must be, for each measurement, within the linearity range of the equipment. Correction for dead-time losses is applied if necessary.

The same source is measured in the same geometrical conditions and at intervals usually corresponding to at least half of the estimated half-life. Each value is tabulated against the time interval from the initial measurement. To avoid influence of decay during measurement, the counting time is the same for all measurements.

A graph can be drawn with time as the abscissa and the logarithm of the relative instrument reading (e.g. count rate) as the ordinate. The half-life is calculated from the slope of the best linear fit of the measured values against the time corresponding to each measurement.

Approximate half-life For this purpose, not fewer than 3 measurements are made over a period of not less than 1/4 of the estimated half-life.

The sample to be examined and the instrument to be used comply with the indications given above. The data are processed in the same way as above.

SPECTROMETRY

Radionuclides can be identified by their emission spectrum. Each type of emission (i.e. alpha particles, beta particles and electrons, gamma- and X-rays) requires specific equipment to acquire an emission spectrum. Spectrometers must be calibrated in order to work properly and the following sections describe the different equipment and detail the general procedures for a reliable measurement.

GAMMA-RAY SPECTROMETRY

General principles

In gamma-ray spectrometry using a scintillation detector, absorption of gamma- and X-rays results in production of light, which is converted into an electrical pulse by a photomultiplier. In gamma-ray spectrometry using a semiconductor detector, absorption of gamma- and X-rays results in the immediate production of an electrical pulse.

In both cases the pulse amplitude is proportional to the energy of the absorbed radiation. The most common detectors for gamma- and X-ray spectrometry are thallium-activated sodium iodide (NaI(Tl)) scintillation counters and high-purity germanium (HPGe) semiconductor detectors.

A gamma-ray spectrum can be produced by collecting and analysing a sufficient number of pulses.

Apparatus

A gamma-ray spectrometer usually comprises a shielded measuring chamber where the sample is positioned, a detector, an electronic chain and a multichannel analyser.

The shielding of the chamber must be able to reduce the background signal to a level that allows the registration of a correct gamma-ray spectrum.

The measurement chamber has a movable cover or a drawer to allow the positioning of the sample. A sample holder may be present to ensure reproducible geometry between measurements.

The duration of measurement is related to the radioactivity of the target radionuclide and a long period of acquisition may be required to achieve the necessary counting statistics. Dead-time losses must be carefully considered with this type of detector.

The sensitivity of a NaI(Tl) detector is higher than that of a germanium detector of the same size. In general, peaks in an energy spectrum are identified with an uncertainty depending upon the full width of the peak at its half-maximum height (FWHM). The energy resolution of a solid-state scintillation detector is much poorer than that of a semiconductor detector and hence peaks obtained with a semiconductor detector are much narrower than those obtained with a scintillation detector. Figure 2.2.66.-2 shows a comparison of the spectra obtained from the same source with the 2 types of detector.

The different performances of NaI(Tl) and HPGe detectors may limit their use in some spectrometric analyses.

For the identification of the radionuclide(s) in a preparation and determination of radionuclidic purity, a risk assessment on the process of radionuclide production must assess the potential presence of other radionuclides with photon energies in the same range (± 10 per cent) as that of the radionuclide(s) present in the radiopharmaceutical.

In case radionuclidic impurities can be present that emit gamma- or X-rays with an energy in the same range as that of the photons emitted by the radionuclide in the preparation, a measured peak energy within a maximum interval of ± 2 keV or ± 2 per cent (whichever is the larger) with respect to the nominal peak energy (see *5.7. Table of physical characteristics of radionuclides)* is sufficient for peak identification.

In the case where such impurities are not expected to be present, a maximum interval of ± 10 keV or ± 6 per cent

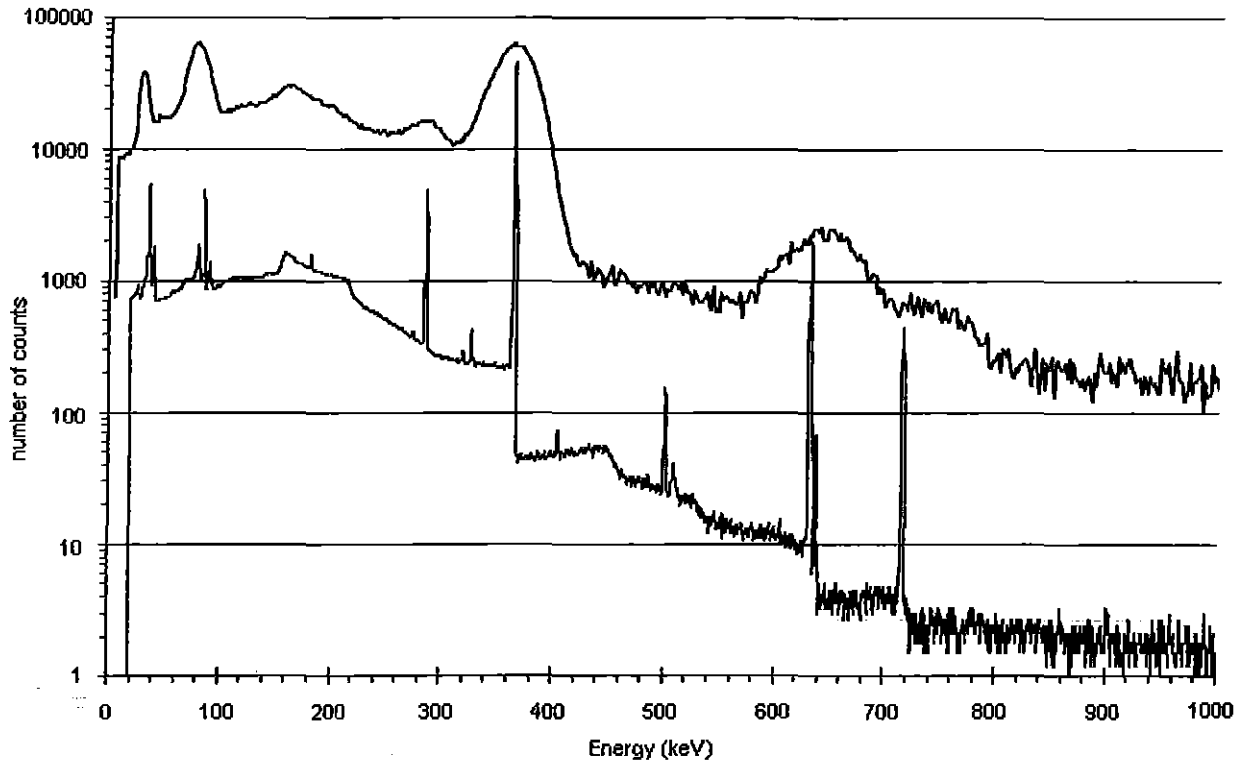


Figure 2.2.66.-2. – *Comparative pulse-height spectra recorded using a thallium-activated sodium iodide scintillator (upper curve) and a high-purity germanium semiconductor detector (lower curve). The source was gamma- and X-ray radiation from the decay of iodine-131.*

(whichever is the larger) with respect to the nominal peak energy is acceptable for peak identification.

Method

Ensure that the counting rate of the sample falls within the linearity range of the equipment. For liquid samples this may be achieved by appropriate dilution; for solid samples, by increasing the source-to-detector distance or by using an attenuating material. Introduce the preparation to be examined in a container into the instrument chamber and record the spectrum after closing the shielding.

Ensure that the container used for quantitative measurements is of the same shape, dimensions, volume and material as that of the calibration standard.

Ensure that the composition of the solution and the position of the container in the measuring chamber is the same for the container for the quantitative measurement as for the calibration standard.

Radionuclide identification Calibrate the spectrometer in relation to energy. Determination of the correspondence of the energy of the peaks detected from the sample to the energies prescribed by a monograph is a valid identification test.

Radionuclidic purity Calibrate the spectrometer in relation to efficiency and energy. Determine the LOQ and resolution of the equipment and ensure that they are in line with the limits of the radionuclides to be determined. Record the spectrum of the preparation.

Identify the radionuclides present in the preparation to be examined and determine their radioactivity with the aid of chapter *5.7. Table of physical characteristics of radionuclides.* Because the level of radionuclidic impurities, expressed as a percentage of the total radioactivity, may increase or decrease with time, the measured activity of each impurity must be recalculated to the activity during the period of validity of the preparation. The activities of all radionuclidic impurities need to be summed (taking into account the limit of quantification) and related to the total radioactivity of the preparation.

The sample is placed close to the detector or within a well-type detector. All the events within a pre-set energy range are collected and displayed on a ratemeter as counts per second or accumulated over a pre-set period of time. If there is sufficient difference in photon energies emitted by the radionuclide(s), a sodium iodide detector can be suitable, given its high sensitivity. However, if there is a need to discriminate emissions of similar energy, a HPGe detector or another semiconductor detector is needed.

Calibration Calibration in relation to energy is done by using the peaks of known sources traceable to national or international standards, such as cobalt-57, caesium-137, cobalt-60 and others covering the desired energy range. A calibration in relation to efficiency can be simultaneously obtained, so that not only the energy spectrum but also the activity of the sample and the radionuclide impurities can be further determined. The calibration of efficiency can be performed with a traceable radionuclide source with energy peaks covering the desired range or with the aid of a mixed, traceable radionuclide standard with gamma-ray energies covering the desired range.

To obtain the efficiency curve, the detector response as a function of the energy has to be measured using each separate sample/detector geometry. For this reason, it is possible to carry out the measurement with the aid of a primary standard source. Primary standards may not be available for radionuclides with a short half-life, e.g. some positron emitters. When measuring, the sample will mostly have to be in a container and set at a defined position in relation to the detector. The sample/detector geometry is

then defined by the position of the sample relative to the detector and the characteristics of the container and sample, e.g. shape, volume and density. Figure 2.2.66.-3 shows a typical HPGe detector efficiency curve obtained for a cylindrical container placed on top of the detector.

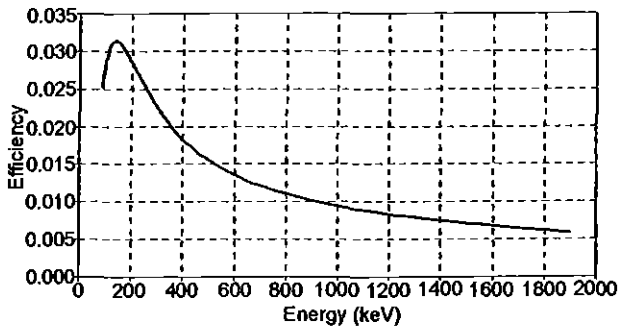


Figure 2.2.66.-3. – *Typical HPGe efficiency curve measured using a dedicated container set on top of the detector*

BETA-PARTICLE SPECTROMETRY

In the case of a beta-particle emitter, a beta-particle spectrometer is necessary to determine the energy distribution of the emitted beta particles. It is analogous to a gamma-ray spectrometer, but frequently uses liquid scintillators to convert the energy of the beta particles into detectable light, which can then be analysed. Beta-particle spectrometry is mostly achieved by dissolving or suspending the sample in a liquid scintillation cocktail in transparent or translucent (glass or plastic) containers and subsequent counting of the electrical pulses generated by a photomultiplier from the emitted light. The pulse amplitude is related to the energy of the absorbed radiation. A beta-particle spectrum can be produced by collecting a sufficient number of pulses. The liquid scintillation cocktail is chosen in such a way that counting errors due to quenching, chemoluminescence, phosphorescence, etc., are minimised. Coincidence counting with 2 or more photomultipliers is also used to minimise counts from background radiation, electronics, etc.

To differentiate between alpha- and beta-particle emissions, pulse-shape discrimination is commonly used.

Radionuclide identification Determination of the correspondence of the mean and/or maximum energies in the energy spectrum from the sample to the energies prescribed by a monograph is a valid identification test.

Calibration A common method of energy calibration is to use an unquenched reference sample to determine the maximum energy of the beta particles emitted by the radionuclide of interest.

ALPHA-PARTICLE SPECTROMETRY

For the identification and assay of alpha-particle emitters, spectrometry using liquid scintillation is mostly used. The principle is explained in the previous section on beta-particle spectrometry.

For the identification and determination of radionuclidic purity of alpha-particle emitters, spectrometry using a silicon-diode semiconductor detector can be used. Using this detector, the absorption of alpha particles results in the immediate production of an electrical pulse. The movement of electron-hole pairs created by the interaction of radiation induces an electrical charge, which is amplified and measured.

The sample preparation is of crucial importance. After a chemical separation of the radionuclide of interest, the sample is electro-deposited on a stainless steel disk in the form of a very thin layer of material to minimise self-absorption. The yield of the whole procedure can be determined experimentally by adding a known amount of a tracer, which will take into account the chemical separation efficiency, the electro-deposition efficiency and the counting efficiency.

For both types of detectors, the pulse amplitude is related to the energy of the absorbed radiation. An alpha-particle spectrum can be produced by collecting a sufficient number of pulses.

Radionuclide identification Determination of the correspondence of the energy of the peaks detected from the sample to the energies prescribed by a monograph is a valid identification test.

Calibration An alpha-particle spectrometer has to be calibrated in relation to energy and efficiency. This is done by using the peaks from known sources covering the desired energy range, such as americium-241 and plutonium-242. Not all alpha particles emitted by the source will produce a count in the system. The probability that an emitted alpha particle will interact with the detector material and produce a count is the efficiency of the detector, which depends on the geometry.

DETECTION AND MEASUREMENT OF RADIOACTIVITY IN COMBINATION WITH A SEPARATION TECHNIQUE

A radioactive preparation may contain the radionuclide in different chemical forms other than the intended one. Therefore it is necessary to separate the different substances containing the radionuclide and determine the percentage of radioactivity due to the radionuclide concerned associated with the stated chemical form and the contribution to the total radioactivity due to the radionuclide concerned coming from other substances. For this purpose, instruments for the detection and measurement of radioactivity are used in combination with a physico-chemical separation technique. In principle, any method of separation may be used.

Monographs for radiopharmaceutical preparations may include the combined use of radioactivity measurement with paper chromatography (*2.2.26*), thin-layer chromatography (*2.2.27*), gas chromatography (*2.2.28*), liquid chromatography (*2.2.29*), size-exclusion chromatography (*2.2.30*) or electrophoresis (*2.2.31*).

In all cases the radioactivity of each analyte is measured after the separation has been achieved using the stated method.

Radioactivity measurement may be performed using detectors mounted in series with other detectors in analytical instruments, such as liquid chromatographs, making in-line detection of analytes, or performed off-line, i.e. after the analytical separation has been completed, by measuring the radioactivity of eluate fractions obtained after liquid chromatographic separation of equal volume or as the distribution of radioactivity on paper chromatography or thin-layer chromatography supports.

IN-LINE DETECTION AND MEASUREMENT OF RADIOACTIVITY IN COMBINATION WITH LIQUID CHROMATOGRAPHY

Apparatus

Liquid chromatography (see *2.2.29*) may be used to separate the principal radioactive substance of a radiopharmaceutical preparation from radiochemical impurities or degradation

products. In-line detection is usually obtained by using a scintillation detector connected to a ratemeter and recording device. The scintillating material of the detector is selected on the basis of the emission to be detected, e.g. plastic scintillator for beta-particle emissions or scintillation crystals for gamma- and X-ray radiations. The addition of a liquid scintillation cocktail before the eluate reaches the in-line radioactivity detector may also be used in the case of beta-particle-emitting radionuclides.

The simultaneous use of a radioactivity detector and other detectors (UV, refractive index, conductimetric, etc.) connected in series may be used to identify the substance, e.g. in relation to the retention time of a known standard, to determine the amount of the substance using a suitable reference standard and to measure the radioactivity associated with such a substance. When different detectors are coupled in series, correct the experimentally obtained retention times for the delay in time between the detectors.

In liquid chromatography some radiochemical impurities, such as colloidal impurities, may be retained on the column. In such cases a separate method is required for the determination of the content of the retained radiochemical impurities and the calculation formula for the expression of the total radiochemical purity takes into account the relative amount of the retained radiochemical impurities.

One possibility to evaluate such retention problems during method validation is to evaluate the radioactivity recovery from the column by measuring the total radioactivity recovered from the chromatographic equipment with and without the column.

Method

The sample is diluted if necessary and then applied to the column in the prescribed volume and conditions. In this respect it is important to demonstrate the LOD and LOQ, and the linearity of the detector throughout the range of activities to be measured.

Flow-through detector A portion of the tubing where the eluate containing the radioactive species is flowing is placed in front of or within the detector. Counting efficiency may be increased using a longer portion of the tube (e.g. making multiple turns in front of or within the detector); however, this will reduce the ability of the system to separate 2 closely eluting peaks of radioactivity.

When the radiochemical purity test prescribes determination of the total radiochemical impurities or there is a quantitative determination of an individual impurity, it is important to choose an appropriate threshold setting and appropriate conditions for integration of the peak areas. In such tests the disregard limit, i.e. the limit at or below which a peak is disregarded, is dependent on the method and is related to the limit of detection and limit of quantification. Thus, the threshold setting of the data collection system corresponds to at least half of the disregard limit.

Record the signal of the detectors as a function of time.

Identification of peaks in the radiometric signal (radiochromatogram) is made on the basis of the retention time of the analytes. The profile from other detectors may be used for this purpose.

Quantification of the different components of chromatogram and radiochromatogram profiles is made on the basis of peak areas. Peak areas are usually obtained by direct integration of the detector signal using commercially available software.

OFF-LINE DETECTION AND MEASUREMENT OF RADIOACTIVITY

Liquid chromatography (*2.2.29*). Provided the retention times of the various radiochemical species are reproducibly consistent, an alternative method of radioactivity quantification is to collect the liquid chromatography effluent in a series of timed samples (fractions) for off-line analysis for radioactivity content. The radioactivity in the fractions corresponding to the peaks can be expressed as a percentage of the total of the radioactivity in all fractions, taking into account the limit of quantification.

Method

The sample is applied on the column in the prescribed volume and conditions. Fractions are collected at the end of the chromatographic line.

The volume between the detector used to identify the retention time of the peaks and the collection point is measured and a delay factor is calculated on the basis of the effluent flow rate and applied to each peak to estimate the time of elution of the peak at the point of collection. The fractions are collected on the basis of a fixed time interval or at the time of appearance estimated from the delay time so that any relevant peak is collected in one or more fractions.

The radioactivity of each fraction is counted using a calibrated instrument such as a dose calibrator or a scintillation detector, taking into account the limit of quantification and the linearity.

An elution profile is obtained tabulating the counts per fraction against the elution time or volume. The activity of fractions belonging to the same peak may be summed and the relative percentage calculated to define radiochemical purity.

Thin-layer chromatography (*2.2.27*) and paper chromatography (*2.2.26*). Provided a thin-layer chromatography or a paper chromatography analytical method has been validated for the separation of components of a radioactive preparation, the number and relative intensities of the separated spots can be detected and measured using a radioactivity detector that can relate the radioactivity to a specific position on the chromatographic support.

The positions of the spots (peaks) may permit chemical identification by comparison with solutions of the same chemical substances (non-radioactive), using a suitable detection method.

Apparatus

Scanning device The apparatus generally comprises a radioactivity detector, such as a position-sensitive proportional counter or a collimated scintillation detector placed at a fixed distance from a scanning platform where the chromatographic support to be scanned is positioned.

The radioactivity of the sample applied to the chromatography support must result in a counting rate in the linearity range of the equipment and the sample may be diluted if necessary. The area to be scanned is positioned at the reference position so that the desired lane is aligned with the detector scanning trip. Adjust the scanning time to allow enough counting time during the run.

The detector or the platform may be moved in-plane, along the *x*-axis or the *y*-axis, so that the entire surface can be scanned during a single run.

The detector is connected to a suitable counting device, so that the radioactivity revealed can be measured quantitatively and the count rate related spatially to the surface scanned.

The radioactivity is automatically reported against the development distance and the profile describes peaks having an area proportional to the number of counts per unit of distance.

Radioactivity counter In the case where a maximum of only 3 radiochemical components needs to be identified and they are fully separated, the support can be cut into equal strips, each having a size not more than half the length of the support corresponding to the difference between the retardation factors of the 2 closest spots. Each single strip is numbered starting from the origin side and counted separately. Alternatively, for well-characterised systems the support may be cut into 2 or more unequal portions, folded if necessary to approximately equal geometry before counting. An ionisation chamber or a scintillation counter can be used for this purpose, provided they are used within the instrument's linearity range and above its LOQ.

Autoradiography This may also be used to acquire an image of the radioactivity distribution on the chromatographic support. In this case, the response of the system used for the acquisition of the image, such as a phosphor imager or a photographic film, must be shown to be linear with respect to the radioactivity in the chromatogram. Otherwise the system must be pre-calibrated or exposed at the same time to a series of reference radioactive sources, obtained by dilution from a calibrated standard solution, covering the expected radioactivity range that may be present on the support.

Method

Deposit the required amount of sample at the origin of the chromatographic support, with drying if necessary to avoid spreading of the spot. Develop the chromatogram according to the prescribed method. A carrier may be added when prescribed in a particular monograph.

In paper and thin-layer chromatography, it is preferable not to dilute the preparation to be examined but it is important to avoid depositing such a quantity of radioactivity that counting losses by coincidence (dead-time losses) occur during measurement of the radioactivity.

After development, the support is dried and the positions of the radioactive areas are detected by measurement of radioactivity over the length of the chromatogram, using a suitable collimated counter, by autoradiography, or by cutting the strips into portions and counting each portion.

Radioactivity may be measured by integration using an automatic-plotting instrument or a digital counter.

The ratios of the areas under the peaks give the ratios of the percentages of radioactivity due to the respective radiochemical substances.

When the strips are cut into portions, the ratios of the quantities of radioactivity measured give the ratio of percentages of radioactivity due to the respective radiochemical species.

Calibration It is important to demonstrate the limits of detection and quantification, and the linearity of the detector throughout the range of activities to be measured and in all positions on the support of the chromatographic system. This may be done by applying samples covering a range of activities from 0.1 per cent to 100 per cent of the expected range. Prepare the samples by dilution and apply equal volumes of each, with drying if necessary. After examining the radioactivity profile using the equipment's standard settings, the peak areas are integrated for comparison with the calculated amount of radioactivity applied to each spot. Verify that the response of the detector over the complete length and width of the detector path is the same, as the response may vary with the detector position.

The peak-resolving power is influenced by the size of the spot, the total radioactivity of the radionuclide and the detector equipment. It can be checked by applying 5 µL spots separated by distances increasing from 4 mm to 20 mm in 2 mm increments. The approximate resolution of the detection system can be determined from the radioactivity profile as the distance between the 2 spots where the baseline is only just clearly separated.

Appendix VI

Qualitative Reactions and Tests

(Ph. Eur. method 2.3.1)

Identification reactions of ions and functional groups

ACETATES

a) Heat the substance to be examined with an equal quantity of *oxalic acid R*. Acid vapours with the characteristic odour of acetic acid are liberated, showing an acid reaction (*2.2.4*).

b) Dissolve about 30 mg of the substance to be examined in 3 mL of *water R* or use 3 mL of the prescribed solution. Add successively 0.25 mL of *lanthanum nitrate solution R*, 0.1 mL of *0.05 M iodine* and 0.05 mL of *dilute ammonia R2*. Heat carefully to boiling. Within a few minutes a blue precipitate is formed or a dark blue colour develops.

ACETYL GROUPS

In a test-tube about 180 mm long and 18 mm in external diameter, place about 15 mg of the substance to be examined, or the prescribed quantity, and 0.15 mL of *phosphoric acid R*. Close the tube with a stopper through which passes a small test-tube about 100 mm long and 10 mm in external diameter containing *water R* to act as a condenser. On the outside of the smaller tube, hang a drop of *lanthanum nitrate solution R*. Except for substances hydrolysable only with difficulty, place the apparatus in a water-bath for 5 min, then take out the smaller tube. Remove the drop and mix it with 0.05 mL of *0.01 M iodine* on a tile. Add at the edge 0.05 mL of *dilute ammonia R2*. After 1 min to 2 min, a blue colour develops at the junction of the two drops; the colour intensifies and persists for a short time.

For *substances hydrolysable only with difficulty* heat the mixture slowly to boiling over an open flame and then proceed as prescribed above.

ALKALOIDS

Dissolve a few milligrams of the substance to be examined, or the prescribed quantity, in 5 mL of *water R*, add *dilute hydrochloric acid R* until an acid reaction occurs (*2.2.4*), then 1 mL of *potassium iodobismuthate solution R*. An orange or orange-red precipitate is formed immediately.

ALUMINIUM SALTS

Dissolve about 15 mg of the substance to be examined in 2 mL of *water R* or use 2 mL of the prescribed solution. Add about 0.5 mL of *dilute hydrochloric acid R* and about 0.5 mL of *thioacetamide reagent R*. No precipitate is formed. Add dropwise *dilute sodium hydroxide solution R*. A gelatinous white precipitate is formed which dissolves on further addition of *dilute sodium hydroxide solution R*. Gradually add *ammonium chloride solution R*. The gelatinous white precipitate is re-formed.

AMINES, PRIMARY AROMATIC

Acidify the prescribed solution with *dilute hydrochloric acid R* and add 0.2 mL of *sodium nitrite solution R*. After 1 min to 2 min, add 1 mL of *β-naphthol solution R*. An intense orange or red colour and usually a precipitate of the same colour are produced.

AMMONIUM SALTS

To the prescribed solution add 0.2 g of *magnesium oxide R*. Pass a current of air through the mixture and direct the gas that escapes just beneath the surface of a mixture of 1 mL of *0.1 M hydrochloric acid* and 0.05 mL of *methyl red solution R*. The colour of the indicator changes to yellow. On addition of 1 mL of a freshly prepared 100 g/L solution of *sodium cobaltinitrite R* a yellow precipitate is formed.

AMMONIUM SALTS AND SALTS OF VOLATILE BASES

Dissolve about 20 mg of the substance to be examined in 2 mL of *water R* or use 2 mL of the prescribed solution. Add 2 mL of *dilute sodium hydroxide solution R*. On heating, the solution gives off vapour that can be identified by its odour and by its alkaline reaction (*2.2.4*).

ANTIMONY COMPOUNDS

Dissolve with gentle heating about 10 mg of the substance to be examined in a solution of 0.5 g of *sodium potassium tartrate R* in 10 mL of *water R* and allow to cool: to 2 mL of this solution, or to 2 mL of the prescribed solution, add *sodium sulfide solution R* dropwise; an orange-red precipitate is formed which dissolves on addition of *dilute sodium hydroxide solution R*.

ARSENIC COMPOUNDS

Heat 5 mL of the prescribed solution on a water-bath with an equal volume of *hypophosphorous reagent R*. A brown precipitate is formed.

BARBITURATES, NON-NITROGEN SUBSTITUTED

Dissolve about 5 mg of the substance to be examined in 3 mL of *methanol R*, add 0.1 mL of a solution containing 100 g/L of *cobalt nitrate R* and 100 g/L of *calcium chloride R*. Mix and add, with shaking, 0.1 mL of *dilute sodium hydroxide solution R*. A violet-blue colour and precipitate are formed.

BENZOATES

a) To 1 mL of the prescribed solution add 0.5 mL of *ferric chloride solution R1*. A dull-yellow precipitate, soluble in *ether R*, is formed.

b) Place 0.2 g of the substance to be examined, treated if necessary as prescribed, in a test-tube. Moisten with 0.2 mL to 0.3 mL of *sulfuric acid R*. Gently warm the bottom of the tube. A white sublimate is deposited on the inner wall of the tube.

c) Dissolve 0.5 g of the substance to be examined in 10 mL of *water R* or use 10 mL of the prescribed solution. Add 0.5 mL of *hydrochloric acid R*. The precipitate obtained, after crystallisation from warm *water R* and drying *in vacuo*, has a melting point (*2.2.14*) of 120 °C to 124 °C.

BICARBONATES

a) Introduce into a test tube 0.1 g of the substance being examined suspended in 2 mL of *water* or use 2 mL of the prescribed solution. Add 3 mL of 2M *acetic acid*, close the tube immediately using a stopper fitted with a glass tube bent at two right angles. The solution or suspension effervesces. Heat gently and collect the gas in 5 mL of a 4.73% w/v solution of *barium hydroxide*. A white precipitate is produced which dissolves on addition of an excess of 7M *hydrochloric acid*.

b) Treat a solution of the substance being examined with a solution of *magnesium sulfate*; no precipitate is produced (distinction from carbonates). Boil; a white precipitate is produced.

c) A solution liberates carbon dioxide when boiled.

BISMUTH AND BISMUTH COMPOUNDS

a) To 0.5 g of the substance to be examined add 10 mL of *dilute hydrochloric acid R* or use 10 mL of the prescribed solution. Heat to boiling for 1 min. Cool and filter if necessary. To 1 mL of the solution obtained add 20 mL of *water R*. A white or slightly yellow precipitate is formed

which on addition of 0.05 mL to 0.1 mL of *sodium sulfide solution R* turns brown.

b) To about 45 mg of the substance to be examined add 10 mL of *dilute nitric acid R* or use 10 mL of the prescribed solution. Boil for 1 min. Allow to cool and filter if necessary. To 5 mL of the solution obtained add 2 mL of a 100 g/L solution of *thiourea R*. A yellowish-orange colour or an orange precipitate is formed. Add 4 mL of a 25 g/L solution of *sodium fluoride R*. The solution is not decolorised within 30 min.

BROMIDES

a) Dissolve in 2 mL of *water R* a quantity of the substance to be examined equivalent to about 3 mg of bromide (Br^-) or use 2 mL of the prescribed solution. Acidify with *dilute nitric acid R* and add 0.4 mL of *silver nitrate solution R1*. Shake and allow to stand. A curdled, pale yellow precipitate is formed. Centrifuge and wash the precipitate with three quantities, each of 1 mL, of *water R*. Carry out this operation rapidly in subdued light disregarding the fact that the supernatant solution may not become perfectly clear. Suspend the precipitate obtained in 2 mL of *water R* and add 1.5 mL of *ammonia R*. The precipitate dissolves with difficulty.

b) Introduce into a small test-tube a quantity of the substance to be examined equivalent to about 5 mg of bromide (Br^-) or the prescribed quantity. Add 0.25 mL of *water R*, about 75 mg of *lead dioxide R*, 0.25 mL of *acetic acid R* and shake gently. Dry the inside of the upper part of the test-tube with a piece of filter paper and allow to stand for 5 min. Prepare a strip of suitable filter paper of appropriate size. Impregnate it by capillarity, by dipping the tip in a drop of *decolorised fuchsin solution R* and introduce the impregnated part immediately into the tube. Starting from the tip, a violet colour appears within 10 s that is clearly distinguishable from the red colour of fuchsin, which may be visible on a small area at the top of the impregnated part of the paper strip.

CALCIUM AND CALCIUM SALTS

For monographs from the European Pharmacopoeia, use tests A and B only.

a) To 0.2 mL of a neutral solution containing a quantity of the substance to be examined equivalent to about 0.2 mg of calcium (Ca^{2+}) per millilitre or to 0.2 mL of the prescribed solution add 0.5 mL of a 2 g/L solution of glyoxalhydroxyanil R in *ethanol (96 per cent) R*, 0.2 mL of *dilute sodium hydroxide solution R* and 0.2 mL of *sodium carbonate solution R*. Shake with 1 mL to 2 mL of *chloroform R* and add 1 mL to 2 mL of *water R*. The chloroform layer is coloured red.

b) Dissolve about 20 mg of the substance to be examined or the prescribed quantity in 5 mL of *acetic acid R*. Add 0.5 mL of *potassium ferrocyanide solution R*. The solution remains clear. Add about 50 mg of *ammonium chloride R*. A white, crystalline precipitate is formed.

c) To 5 mL of 0.4% w/v solution of the substance being examined add 0.2 mL of a 2% w/v solution of *ammonium oxalate*. A white precipitate is produced which is only sparingly soluble in 6M *acetic acid* but is soluble in *hydrochloric acid*.

CARBONATES

a) Introduce into a test tube 0.1 g of the substance being examined suspended in 2 mL of *water* or use 2 mL of the prescribed solution. Add 3 mL of 2M *acetic acid*, close the tube immediately using a stopper fitted with a glass tube bent at two right angles. The solution or suspension effervesces evolving a colourless and odourless gas. Heat gently and collect the gas in 5 mL of 0.1M *barium hydroxide*. A white precipitate is produced which dissolves on addition of an excess of 7M *hydrochloric acid*.

b) Treat a solution of the substance being examined with a solution of *magnesium sulfate*. A white precipitate is produced (distinction from bicarbonates).

CARBONATES AND BICARBONATES

Introduce into a test-tube 0.1 g of the substance to be examined and suspend in 2 mL of *water R* or use 2 mL of the prescribed solution. Add 3 mL of *dilute acetic acid R*. Close the tube immediately using a stopper fitted with a glass tube bent twice at right angles. The solution or the suspension becomes effervescent and gives off a colourless and odourless gas. Heat gently and collect the gas in 5 mL of *barium hydroxide solution R*. A white precipitate is formed that dissolves on addition of an excess of *hydrochloric acid R1*.

CHLORIDES

a) Dissolve in 2 mL of *water R* a quantity of the substance to be examined equivalent to about 2 mg of chloride (Cl^-) or use 2 mL of the prescribed solution. Acidify with *dilute nitric acid R* and add 0.4 mL of *silver nitrate solution R1*. Shake and allow to stand. A curdled, white precipitate is formed. Centrifuge and wash the precipitate with three quantities, each of 1 mL, of *water R*. Carry out this operation rapidly in subdued light, disregarding the fact that the supernatant solution may not become perfectly clear. Suspend the precipitate in 2 mL of *water R* and add 1.5 mL of *ammonia R*. The precipitate dissolves easily with the possible exception of a few large particles which dissolve slowly.

b) Introduce into a test-tube a quantity of the substance to be examined equivalent to about 15 mg of chloride (Cl^-) or the prescribed quantity. Add 0.2 g of *potassium dichromate R* and 1 mL of *sulfuric acid R*. Place a filter-paper strip impregnated with 0.1 mL of *diphenylcarbazide solution R* over the opening of the test-tube. The paper turns violet-red. The impregnated paper must not come into contact with the potassium dichromate.

CITRATES

For monographs from the European Pharmacopoeia, use test A only.

a) Dissolve in 5 mL of *water R* a quantity of the substance to be examined equivalent to about 50 mg of citric acid or use 5 mL of the prescribed solution. Add 0.5 mL of *sulfuric acid R* and 1 mL of *potassium permanganate solution R*. Warm until the colour of the permanganate is discharged. Add 0.5 mL of a 100 g/L solution of *sodium nitroprusside R* in *dilute sulfuric acid R* and 4 g of *sulfamic acid R*. Make alkaline with *concentrated ammonia R*, added dropwise until all the sulfamic acid has dissolved. Addition of an excess of *concentrated ammonia R* produces a violet colour, turning to violet-blue.

b) To a neutral solution of the substance being examined add a solution of *calcium chloride*; no precipitate is produced. Boil the solution; a white precipitate is produced which is soluble in 6M *acetic acid*.

ESTERS

To about 30 mg of the substance to be examined or the prescribed quantity add 0.5 mL of a 70 g/L solution of *hydroxylamine hydrochloride R* in *methanol R* and 0.5 mL of a 100 g/L solution of *potassium hydroxide R* in *ethanol (96 per cent) R*. Heat to boiling, cool, acidify with *dilute hydrochloric acid R* and add 0.2 mL of *ferric chloride*

solution R1 diluted ten times. A bluish-red or red colour is produced.

IODIDES

a) Dissolve a quantity of the substance to be examined equivalent to about 4 mg of iodide (I^-) in 2 mL of *water R* or use 2 mL of the prescribed solution. Acidify with *dilute nitric acid R* and add 0.4 mL of *silver nitrate solution R1*. Shake and allow to stand. A curdled, pale-yellow precipitate is formed. Centrifuge and wash with three quantities, each of 1 mL, of *water R*. Carry out this operation rapidly in subdued light disregarding the fact that the supernatant solution may not become perfectly clear. Suspend the precipitate in 2 mL of *water R* and add 1.5 mL of *ammonia R*. The precipitate does not dissolve.

b) To 0.2 mL of a solution of the substance to be examined containing about 5 mg of iodide (I^-) per millilitre, or to 0.2 mL of the prescribed solution, add 0.5 mL of *dilute sulfuric acid R*, 0.1 mL of *potassium dichromate solution R*, 2 mL of *water R* and 2 mL of *chloroform R*. Shake for a few seconds and allow to stand. The chloroform layer is coloured violet or violet-red.

IRON AND IRON SALTS

a) Dissolve a quantity of the substance to be examined equivalent to about 10 mg of iron (Fe^{2+}) in 1 mL of *water R* or use 1 mL of the prescribed solution. Add 1 mL of *potassium ferricyanide solution R*. A blue precipitate is formed that does not dissolve on addition of 5 mL of *dilute hydrochloric acid R*.

b) Dissolve a quantity of the substance to be examined equivalent to about 1 mg of iron (Fe^{3+}) in 30 mL of *water R*. To 3 mL of this solution or to 3 mL of the prescribed solution, add 1 mL of *dilute hydrochloric acid R* and 1 mL of *potassium thiocyanate solution R*. The solution is coloured red. Take two portions, each of 1 mL, of the mixture. To one portion add 5 mL of *isoamyl alcohol R* or 5 mL of *ether R*. Shake and allow to stand. The organic layer is coloured pink. To the other portion add 2 mL of *mercuric chloride solution R*. The red colour disappears.

c) Dissolve a quantity of the substance to be examined equivalent to not less than 1 mg of iron (Fe^{3+}) in 1 mL of *water R* or use 1 mL of the prescribed solution. Add 1 mL of *potassium ferrocyanide solution R*. A blue precipitate is formed that does not dissolve on addition of 5 mL of *dilute hydrochloric acid R*.

LACTATES

Dissolve a quantity of the substance to be examined equivalent to about 5 mg of lactic acid in 5 mL of *water R* or use 5 mL of the prescribed solution. Add 1 mL of *bromine water R* and 0.5 mL of *dilute sulfuric acid R*. Heat on a water-bath until the colour is discharged, stirring occasionally with a glass rod. Add 4 g of *ammonium sulfate R* and mix. Add dropwise and without mixing 0.2 mL of a 100 g/L solution of *sodium nitroprusside R* in *dilute sulfuric acid R*. Still without mixing add 1 mL of *concentrated ammonia R*. Allow to stand for 30 min. A dark green ring appears at the junction of the two liquids.

LEAD AND LEAD COMPOUNDS

a) Dissolve 0.1 g of the substance to be examined in 1 mL of *acetic acid R* or use 1 mL of the prescribed solution. Add 2 mL of *potassium chromate solution R*. A yellow precipitate is formed that dissolves on addition of 2 mL of *strong sodium hydroxide solution R*.

b) Dissolve 50 mg of the substance to be examined in 1 mL of *acetic acid R* or use 1 mL of the prescribed solution. Add 10 mL of *water R* and 0.2 mL of *potassium iodide solution R*. A yellow precipitate is formed. Heat to boiling for 1 min to 2 min. The precipitate dissolves. Allow to cool. The precipitate is re-formed as glistening, yellow plates.

MAGNESIUM AND MAGNESIUM SALTS

For monographs from the European Pharmacopoeia, use test A only.

a) Dissolve about 15 mg of the substance to be examined in 2 mL of *water R* or use 2 mL of the prescribed solution. Add 1 mL of *dilute ammonia R1*. A white precipitate is formed that dissolves on addition of 1 mL of *ammonium chloride solution R*. Add 1 mL of *disodium hydrogen phosphate solution R*. A white crystalline precipitate is formed.

b) To 0.5 mL of a neutral or slightly acidic solution of the substance being examined add 0.2 mL of a 0.1% w/v solution of titan yellow and 0.5 mL of 0.1M *sodium hydroxide*. A bright red turbidity is produced which gradually settles to give a bright red precipitate.

MERCURY AND MERCURY COMPOUNDS

a) Place about 0.1 mL of a solution of the substance to be examined on well-scraped copper foil. A dark-grey stain that becomes shiny on rubbing is formed. Dry the foil and heat in a test-tube. The spot disappears.

b) To the prescribed solution add *dilute sodium hydroxide solution R* until strongly alkaline (*2.2.4*). A dense yellow precipitate is formed (mercuric salts).

NITRATES

To a mixture of 0.1 mL of *nitrobenzene R* and 0.2 mL of *sulfuric acid R*, add a quantity of the powdered substance equivalent to about 1 mg of nitrate (NO_3^-) or the prescribed quantity. Allow to stand for 5 min. Cool in iced water and add slowly and with mixing 5 mL of *water R*, then 5 mL of *strong sodium hydroxide solution R*. Add 5 mL of *acetone R*. Shake and allow to stand. The upper layer is coloured deep violet.

PHOSPHATES (ORTHOPHOSPHATES)

a) To 5 mL of the prescribed solution, neutralised if necessary, add 5 mL of *silver nitrate solution R1*. A yellow precipitate is formed whose colour is not changed by boiling and which dissolves on addition of *ammonia R*.

b) Mix 1 mL of the prescribed solution with 2 mL of *molybdovanadic reagent R*. A yellow colour develops.

POTASSIUM AND POTASSIUM SALTS

a) Dissolve 0.1 g of the substance to be examined in 2 mL of *water R* or use 2 mL of the prescribed solution. Add 1 mL of *sodium carbonate solution R* and heat. No precipitate is formed. Add to the hot solution 0.05 mL of *sodium sulfide solution R*. No precipitate is formed. Cool in iced water and add 2 mL of a 150 g/L solution of *tartaric acid R*. Allow to stand. A white crystalline precipitate is formed.

b) Dissolve about 40 mg of the substance to be examined in 1 mL of *water R* or use 1 mL of the prescribed solution. Add 1 mL of *dilute acetic acid R* and 1 mL of a freshly prepared 100 g/L solution of *sodium cobaltinitrite R*. A yellow or orange-yellow precipitate is formed immediately.

SALICYLATES

a) To 1 mL of the prescribed solution add 0.5 mL of *ferric chloride solution R1*. A violet colour is produced that persists after the addition of 0.1 mL of *acetic acid R*.

b) Dissolve 0.5 g of the substance to be examined in 10 mL of *water R* or use 10 mL of the prescribed solution. Add 0.5 mL of *hydrochloric acid R*. The precipitate obtained,

after recrystallisation from hot *water R* and drying *in vacuo*, has a melting point (*2.2.14*) of 156 °C to 161 °C.

SILICATES

Mix the prescribed quantity of the substance to be examined in a lead or platinum crucible by means of a copper wire with about 10 mg of *sodium fluoride R* and a few drops of *sulfuric acid R* to give a thin slurry. Cover the crucible with a thin, transparent plate of plastic under which a drop of *water R* is suspended and warm gently. Within a short time a white ring is rapidly formed around the drop of water.

SILVER AND SILVER COMPOUNDS

Dissolve about 10 mg of the substance to be examined in 10 mL of *water R* or use 10 mL of the prescribed solution. Add 0.3 mL of *hydrochloric acid R1*. A curdled, white precipitate is formed that dissolves on addition of 3 mL of *dilute ammonia R1*.

SODIUM AND SODIUM SALTS

a) Dissolve 0.1 g of the substance to be examined in 2 mL of *water R* or use 2 mL of the prescribed solution. Add 2 mL of a 150 g/L solution of *potassium carbonate R* and heat to boiling. No precipitate is formed. Add 4 mL of *potassium pyroantimonate solution R* and heat to boiling. Allow to cool in iced water and if necessary rub the inside of the test-tube with a glass rod. A dense white precipitate is formed.

b) Dissolve a quantity of the substance to be examined equivalent to about 2 mg of sodium (Na^+) in 0.5 mL of *water R* or use 0.5 mL of the prescribed solution. Add 1.5 mL of *methoxyphenylacetic reagent R* and cool in ice-water for 30 min. A voluminous, white, crystalline precipitate is formed. Place in water at 20 °C and stir for 5 min. The precipitate does not disappear. Add 1 mL of *dilute ammonia R1*. The precipitate dissolves completely. Add 1 mL of *ammonium carbonate solution R*. No precipitate is formed.

SULFATES

a) Dissolve about 45 mg of the substance to be examined in 5 mL of *water R* or use 5 mL of the prescribed solution. Add 1 mL of *dilute hydrochloric acid R* and 1 mL of *barium chloride solution R1*. A white precipitate is formed.

b) To the suspension obtained during reaction (a), add 0.1 mL of *0.05 M iodine*. The suspension remains yellow (distinction from sulfites and dithionites), but is decolorised by adding dropwise *stannous chloride solution R* (distinction from iodates). Boil the mixture. No coloured precipitate is formed (distinction from selenates and tungstates).

TARTRATES

a) Dissolve about 15 mg of the substance to be examined in 5 mL of *water R* or use 5 mL of the prescribed solution. Add 0.05 mL of a 10 g/L solution of *ferrous sulfate R* and 0.05 mL of *dilute hydrogen peroxide solution R*. A transient yellow colour is produced. After the colour has disappeared add *dilute sodium hydroxide solution R* dropwise. A violet or purple colour is produced.

b) To 0.1 mL of a solution of the substance to be examined containing the equivalent of about 15 mg of tartaric acid per millilitre or to 0.1 mL of the prescribed solution add 0.1 mL of a 100 g/L solution of *potassium bromide R*, 0.1 mL of a 20 g/L solution of *resorcinol R* and 3 mL of *sulfuric acid R*. Heat on a water-bath for 5 min to 10 min. A dark-blue colour develops. Allow to cool and pour the solution into *water R*. The colour changes to red.

XANTHINES

To a few milligrams of the substance to be examined or the prescribed quantity add 0.1 mL of *strong hydrogen peroxide solution R* and 0.3 mL of *dilute hydrochloric acid R*. Heat to dryness on a water-bath until a yellowish-red residue is obtained. Add 0.1 mL of *dilute ammonia R2*. The colour of the residue changes to violet-red.

ZINC AND ZINC SALTS

Dissolve 0.1 g of the substance to be examined in 5 mL of *water R* or use 5 mL of the prescribed solution. Add 0.2 mL of *strong sodium hydroxide solution R*. A white precipitate is formed. Add a further 2 mL of *strong sodium hydroxide solution R*. The precipitate dissolves. Add 10 mL of *ammonium chloride solution R*. The solution remains clear. Add 0.1 mL of *sodium sulfide solution R*. A flocculent white precipitate is formed.

ODOUR

(Ph. Eur. method 2.3.4)

On a watch-glass 6 cm to 8 cm in diameter, spread in a thin layer 0.5 g to 2.0 g of the substance to be examined. After 15 min, determine the odour or verify the absence of odour.

Appendix VII

Limit Tests

Nessler Cylinders

(No Ph. Eur. method)

Where the use of *Nessler cylinders* is prescribed in a test of the Pharmacopoeia, Nessler cylinders complying with the following requirements should be used.

Nessler cylinders comply with British Standard 612:1966 (Specification for Nessler cylinders). They are of clear glass with a nominal capacity of 50 mL; the overall height is about 15 cm, the external height to the 50-mL mark 11.0 to 12.4 cm, the thickness of the wall 1.0 to 1.5 mm and the thickness of the base 1.0 to 3.0 mm. The external heights to the 50-mL mark of cylinders used for a test must not differ by more than 1 mm.

Tubes for Comparative Tests

(Ph. Eur. method 2.1.5)

Tubes used for comparative tests are matched tubes of colourless glass with a uniform internal diameter. The base is transparent and flat.

A column of the liquid is examined down the vertical axis of the tube against a white background, or if necessary, against a black background. The examination is carried out in diffused light.

It is assumed that tubes with an internal diameter of 16 mm will be used. Tubes with a larger internal diameter may be used instead but the volume of liquid examined must then be increased so that the depth of liquid in the tubes is not less than where the prescribed volume of liquid and tubes 16 mm in internal diameter are used.

Limit Test for Aluminium

(Ph. Eur. method 2.4.17)

Place the prescribed solution in a separating funnel and shake with 2 quantities, each of 20 mL, and then with one 10 mL quantity of a 5 g/L solution of hydroxyquinoline R in *chloroform R*. Dilute the combined chloroform solutions to 50.0 mL with *chloroform R* (test solution).

Prepare a standard in the same manner using the prescribed reference solution.

Prepare a blank in the same manner using the prescribed blank solution.

Measure the intensity of the fluorescence (*2.2.21*) of the test solution (I_1), of the standard (I_2) and of the blank (I_3) using an excitant beam at 392 nm and a secondary filter with a transmission band centred on 518 nm or a monochromator set to transmit at this wavelength.

The fluorescence (I_1–I_3) of the test solution is not greater than that of the standard (I_2–I_3).

Limit Test for Ammonium

(Ph. Eur. method 2.4.1)

Unless otherwise prescribed, use method A.

METHOD A

Introduce the prescribed solution into a test-tube or dissolve the prescribed quantity of the substance to be examined in 14 mL of *water R* in a test-tube. Make the solution alkaline if necessary by the addition of *dilute sodium hydroxide solution R*, dilute to 15 mL with *water R* and add 0.3 mL of *alkaline potassium tetraiodomercurate solution R*. Prepare a standard by mixing 10 mL of *ammonium standard solution (1 ppm NH_4) R*, 5 mL of *water R* and 0.3 mL of *alkaline potassium tetraiodomercurate solution R*. Stopper the test-tubes.

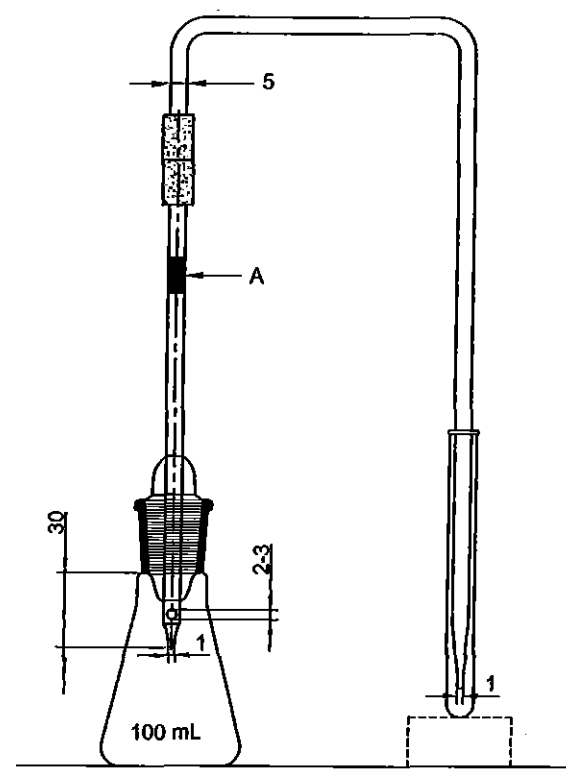
After 5 min, any yellow colour in the test solution is not more intense than that in the standard.

METHOD B

In a 25 mL jar fitted with a cap, place the prescribed quantity of the finely powdered substance to be examined and dissolve or suspend in 1 mL of *water R*. Add 0.30 g of *heavy magnesium oxide R*. Close immediately after placing a piece of *silver manganese paper R* 5 mm square, wetted with a few drops of *water R*, under the polyethylene cap. Swirl, avoiding projections of liquid, and allow to stand at 40 °C for 30 min. If the silver manganese paper shows a grey colour, it is not more intense than that of a standard prepared at the same time and in the same manner using the prescribed volume of *ammonium standard solution (1 ppm NH_4) R*, 1 mL of *water R* and 0.30 g of *heavy magnesium oxide R*.

Limit Test for Arsenic

(Ph. Eur. method 2.4.2)



A. Lead acetate paper/cotton

Figure 2.4.2.-1. – *Apparatus for the limit test for arsenic (method A)*

Dimensions in millimetres

METHOD A

The apparatus (see Figure 2.4.2.-1) consists of a 100 mL conical flask closed with a ground-glass stopper through which passes a glass tube about 200 mm long and about 5 mm in internal diameter. The lower part of the tube tapers to an internal diameter of 1 mm, and about 20 mm from its tip is a lateral orifice 2-3 mm in diameter. When the tube is in position in the stopper, the lateral orifice is at least 3 mm below the lower surface of the stopper. A second glass tube of the same internal diameter is connected to the first tube.

The second tube is bent twice at right angles and the free end of the tube tapers to an internal diameter of 1 mm. This end is immersed in a test-tube containing 3.0 mL of *silver diethyldithiocarbamate solution R*. Other suitable equipment may be used. Into the first tube insert 50-60 mg of *lead acetate cotton R*, loosely packed, or a small plug of cotton and a rolled piece of *lead acetate paper R* weighing 50-60 mg.

In the conical flask, dissolve the prescribed quantity of the substance to be examined in 25 mL of *water R*, or in the case of a solution adjust the prescribed volume to 25 mL with *water R*. Add 15 mL of *hydrochloric acid R*, 0.1 mL of *stannous chloride solution R* and 5 mL of *potassium iodide solution R*, allow to stand for 15 min and introduce 5 g of *activated zinc R*. Assemble the 2 parts of the apparatus immediately and immerse the flask in a water-bath at a temperature such that a uniform evolution of gas is maintained. Prepare a standard in the same manner, using 1 mL of *arsenic standard solution (1 ppm As) R*, diluted to 25 mL with *water R*. If foaming occurs, 1 mL of *2-propanol R* may be added to the flask.

After at least 2 h, the colour obtained in the test-tube with the test solution is not more intense than that obtained with the standard.

Suitability test The colour obtained in the test-tube with the standard is at least as intensely coloured as 3 mL of a mixture of 3.0 mL of yellow primary solution, 0.6 mL of red primary solution and 11.40 mL of a solution of *hydrochloric acid R* (10 g/L HCl) (*2.2.2, Method I*).

METHOD B

Introduce the prescribed quantity of the substance to be examined into a test-tube containing 4 mL of *hydrochloric acid R* and about 5 mg of *potassium iodide R* and add 3 mL of *hypophosphorous reagent R*. Heat the mixture on a water-bath for 15 min, shaking occasionally. Prepare a standard in the same manner, using 0.5 mL of *arsenic standard solution (10 ppm As) R*.

After heating on the water-bath, the colour obtained in the test-tube with the test solution is not more intense than that obtained with the standard.

Limit Test for Calcium

(Ph. Eur. method 2.4.3)

All solutions used for this test are prepared with distilled water R.

To 0.2 mL of *alcoholic calcium standard solution (100 ppm Ca) R* add 1 mL of *ammonium oxalate solution R*. After 1 min add a mixture of 1 mL of *dilute acetic acid R* and 15 mL of the prescribed solution or of a solution containing the prescribed quantity of the substance to be examined, and shake. Prepare a standard in the same manner using a mixture of 10 mL of aqueous *calcium standard solution (10 ppm Ca) R*, 1 mL of *dilute acetic acid R* and 5 mL of *distilled water R*.

After 15 min, any opalescence in the test solution is not more intense than that in the standard.

Limit Test for Chlorides

(Ph. Eur. method 2.4.4)

To 15 mL of the prescribed solution add 1 mL of *dilute nitric acid R* and pour the mixture as a single addition into a test-tube containing 1 mL of *silver nitrate solution R2*. Prepare a standard in the same manner using 10 mL of *chloride standard solution (5 ppm Cl) R* and 5 mL of *water R*. Examine the tubes laterally against a black background.

After standing for 5 min protected from light, any opalescence in the test solution is not more intense than that in the standard.

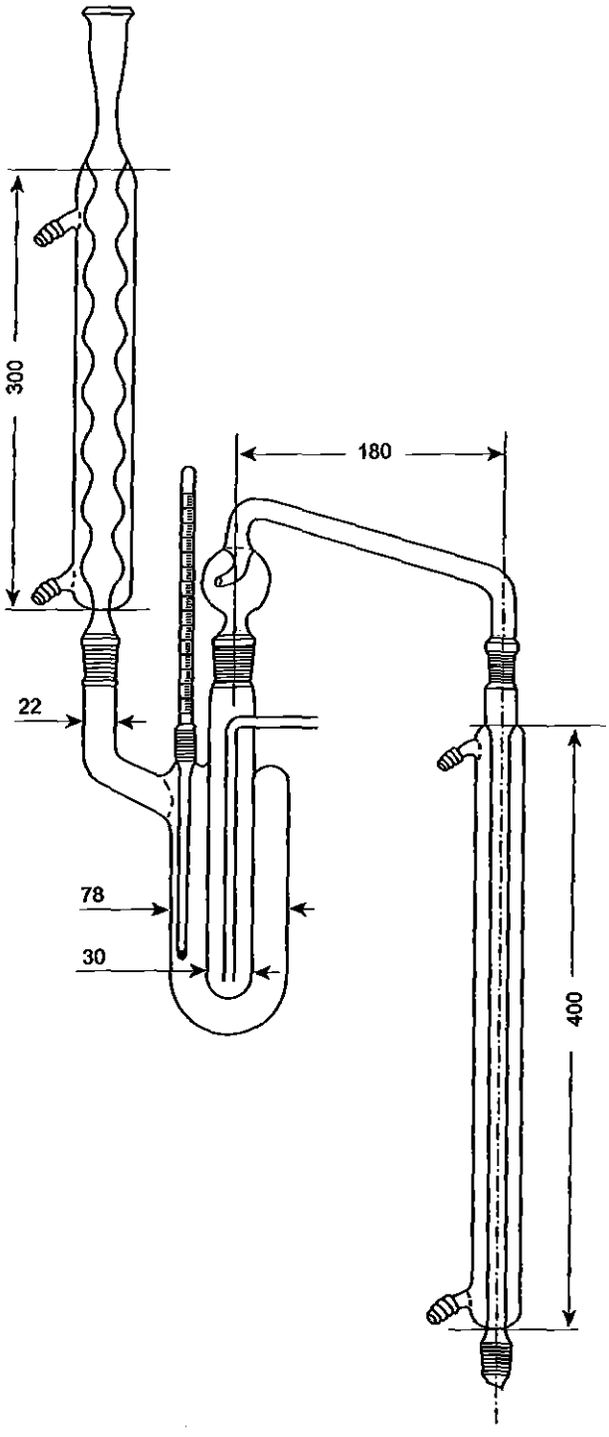


Figure 2.4.5.-1. – *Apparatus for limit test for fluorides*
Dimensions in millimetres

Limit Test for Fluorides

(Ph. Eur. method 2.4.5)

Introduce into the inner tube of the apparatus (see Figure 2.4.5.-1) the prescribed quantity of the substance to be examined, 0.1 g of acid-washed *sand R* and 20 mL of a mixture of equal volumes of *sulfuric acid R* and *water R*. Heat the jacket containing *tetrachloroethane R* maintained at its boiling point (146 °C). Heat the steam generator and distil, collecting the distillate in a 100 mL volumetric flask containing 0.3 mL of *0.1 M sodium hydroxide* and 0.1 mL of *phenolphthalein solution R*. Maintain a constant volume

(20 mL) in the tube during distillation and ensure that the distillate remains alkaline, adding *0.1 M sodium hydroxide* if necessary. Dilute the distillate to 100 mL with *water R* (test solution). Prepare a standard in the same manner by distillation, using 5 mL of *fluoride standard solution (10 ppm F) R* instead of the substance to be examined. Into two glass-stoppered cylinders introduce 20 mL of the test solution and 20 mL of the standard and 5 mL of *aminomethylalizarindiacetic acid reagent R*.

After 20 min, any blue colour in the test solution (originally red) is not more intense than that in the standard.

Limit Test for Heavy Metals

(Ph. Eur. method 2.4.8)

The methods described below require the use of *thioacetamide reagent R*. As an alternative, *sodium sulfide solution R1* (0.1 mL) is usually suitable. Since tests prescribed in monographs have been developed using *thioacetamide reagent R*, if *sodium sulfide solution R1* is used instead, it is necessary to include also for methods A, B and H a monitor solution, prepared from the quantity of the substance to be examined prescribed for the test, to which has been added the volume of lead standard solution prescribed for preparation of the reference solution. The test is invalid if the monitor solution is not at least as intense as the reference solution.

METHOD A

Test solution 12 mL of the prescribed aqueous solution of the substance to be examined.

Reference solution (standard) A mixture of 10 mL of *lead standard solution (1 ppm Pb) R* or *lead standard solution (2 ppm Pb) R*, as prescribed, and 2 mL of the prescribed aqueous solution of the substance to be examined.

Blank solution A mixture of 10 mL of *water R* and 2 mL of the prescribed aqueous solution of the substance to be examined.

To each solution, add 2 mL of *buffer solution pH 3.5 R*. Mix and add to 1.2 mL of *thioacetamide reagent R*. Mix immediately. Examine the solutions after 2 min.

System suitability The reference solution shows a slight brown colour compared to the blank solution.

Result Any brown colour in the test solution is not more intense than that in the reference solution.

If the result is difficult to judge, filter the solutions through a suitable membrane filter (nominal pore size 0.45 µm). Carry out the filtration slowly and uniformly, applying moderate and constant pressure to the piston. Compare the spots on the filters obtained with the different solutions.

METHOD B

Test solution 12 mL of the prescribed solution of the substance to be examined prepared using an organic solvent containing a minimum percentage of water (for example, dioxan containing 15 per cent of water or acetone containing 15 per cent of water).

Reference solution (standard) A mixture of 10 mL of lead standard solution (1 or 2 ppm Pb), as prescribed, and 2 mL of the prescribed solution of the substance to be examined in an organic solvent. Prepare the lead standard solution (1 or 2 ppm Pb) by dilution of *lead standard solution (100 ppm Pb) R* with the solvent used for the substance to be examined.

Blank solution A mixture of 10 mL of the solvent used for the substance to be examined and 2 mL of the prescribed solution of the substance to be examined in an organic solvent.

To each solution, add 2 mL of *buffer solution pH 3.5 R*. Mix and add to 1.2 mL of *thioacetamide reagent R*. Mix immediately. Examine the solutions after 2 min.

System suitability The reference solution shows a slight brown colour compared to the blank solution.

Result Any brown colour in the test solution is not more intense than that in the reference solution.

If the result is difficult to judge, filter the solutions through a suitable membrane filter (nominal pore size 0.45 µm). Carry out the filtration slowly and uniformly, applying moderate and constant pressure to the piston. Compare the spots on the filters obtained with the different solutions.

METHOD C

Test solution Place the prescribed quantity (not more than 2 g) of the substance to be examined in a silica crucible with 4 mL of a 250 g/L solution of *magnesium sulfate R* in *dilute sulfuric acid R*. Mix using a fine glass rod. Heat cautiously. If the mixture is liquid, evaporate gently to dryness on a water-bath. Progressively heat to ignition and continue heating until an almost white or at most greyish residue is obtained. Carry out the ignition at a temperature not exceeding 800 °C. Allow to cool. Moisten the residue with a few drops of *dilute sulfuric acid R*. Evaporate, ignite again and allow to cool. The total period of ignition must not exceed 2 h. Take up the residue in 2 quantities, each of 5 mL, of *dilute hydrochloric acid R*. Add 0.1 mL of *phenolphthalein solution R*, then *concentrated ammonia R* until a pink colour is obtained. Cool, add *glacial acetic acid R* until the solution is decolorised and add 0.5 mL in excess. Filter if necessary and wash the filter. Dilute to 20 mL with *water R*.

Reference solution (standard) Prepare as described for the test solution, using the prescribed volume of *lead standard solution (10 ppm Pb) R* instead of the substance to be examined. To 10 mL of the solution obtained add 2 mL of the test solution.

Monitor solution Prepare as described for the test solution, adding to the substance to be examined the volume of *lead standard solution (10 ppm Pb) R* prescribed for preparation of the reference solution. To 10 mL of the solution obtained add 2 mL of the test solution.

Blank solution A mixture of 10 mL of *water R* and 2 mL of the test solution.

To 12 mL of each solution, add 2 mL of *buffer solution pH 3.5 R*. Mix and add to 1.2 mL of *thioacetamide reagent R*. Mix immediately. Examine the solutions after 2 min.

System suitability:

- the reference solution shows a slight brown colour compared to the blank solution,
- the monitor solution is at least as intense as the reference solution.

Result Any brown colour in the test solution is not more intense than that in the reference solution.

If the result is difficult to judge, filter the solutions through a suitable membrane filter (nominal pore size 0.45 µm). Carry out the filtration slowly and uniformly, applying moderate and constant pressure to the piston. Compare the spots on the filters obtained with the different solutions.

METHOD D

Test solution In a silica crucible, mix thoroughly the prescribed quantity of the substance to be examined with 0.5 g of *magnesium oxide R1*. Ignite to dull redness until a

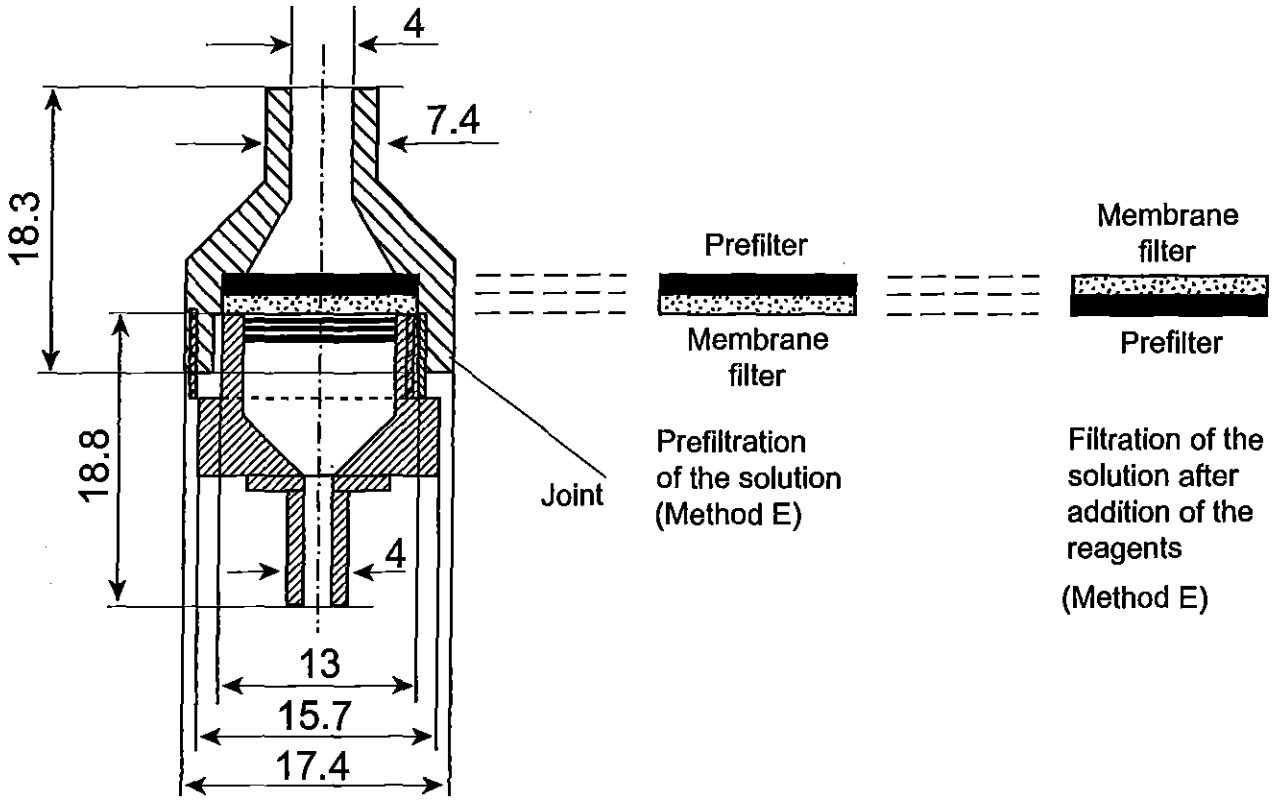


Figure 2.4.8.-1. – *Apparatus for the test for heavy metals*
Dimensions in millimetres

homogeneous white or greyish-white mass is obtained. If after 30 min of ignition the mixture remains coloured, allow to cool, mix using a fine glass rod and repeat the ignition. If necessary repeat the operation. Heat at 800 °C for about 1 h. Take up the residue in 2 quantities, each of 5 mL, of a mixture of equal volumes of *hydrochloric acid RI* and *water R*. Add 0.1 mL of *phenolphthalein solution R* and then *concentrated ammonia R* until a pink colour is obtained. Cool, add *glacial acetic acid R* until the solution is decolorised and add 0.5 mL in excess. Filter if necessary and wash the filter. Dilute to 20 mL with *water R*.

Reference solution (standard) Prepare as described for the test solution using the prescribed volume of *lead standard solution (10 ppm Pb) R* instead of the substance to be examined and drying in an oven at 100-105 °C. To 10 mL of the solution obtained add 2 mL of the test solution.

Monitor solution Prepare as described for the test solution, adding to the substance to be examined the volume of *lead standard solution (10 ppm Pb) R* prescribed for preparation of the reference solution and drying in an oven at 100-105 °C. To 10 mL of the solution obtained add 2 mL of the test solution.

Blank solution A mixture of 10 mL of *water R* and 2 mL of the test solution.

To 12 mL of each solution, add 2 mL of *buffer solution pH 3.5 R*. Mix and add to 1.2 mL of *thioacetamide reagent R*. Mix immediately. Examine the solutions after 2 min.

System suitability:
- the reference solution shows a slight brown colour compared to the blank solution,
- the monitor solution is at least as intense as the reference solution.

Result Any brown colour in the test solution is not more intense than that in the reference solution.

If the result is difficult to judge, filter the solutions through a suitable membrane filter (nominal pore size 0.45 µm). Carry out the filtration slowly and uniformly, applying moderate and constant pressure to the piston. Compare the spots on the filters obtained with the different solutions.

METHOD E

Test solution Dissolve the prescribed quantity of the substance to be examined in 30 mL of *water R* or the prescribed volume.

Reference solution (standard) Unless otherwise prescribed, dilute the prescribed volume of *lead standard solution (1 ppm Pb) R* to the same volume as the test solution.

Prepare the filtration apparatus by adapting the barrel of a 50 mL syringe without its piston to a support containing, on the plate, a membrane filter (nominal pore size 3 µm) and above it a prefilter (Figure 2.4.8.-1).

Transfer the test solution into the syringe barrel, put the piston in place and then apply an even pressure on it until the whole of the liquid has been filtered. In opening the support and removing the prefilter, check that the membrane filter remains uncontaminated with impurities. If this is not the case replace it with another membrane filter and repeat the operation under the same conditions.

To the prefiltrate or to the prescribed volume of the prefiltrate add 2 mL of *buffer solution pH 3.5 R*. Mix and add to 1.2 mL of *thioacetamide reagent R*. Mix immediately and allow to stand for 10 min and again filter as described above, but inverting the order of the filters, the liquid passing first through the membrane filter before passing through the prefilter (Figure 2.4.8.-1). The filtration must be carried out slowly and uniformly by applying moderate and constant

pressure to the piston of the syringe. After complete filtration, open the support, remove the membrane filter, and dry using filter paper.

In parallel, treat the reference solution in the same manner as the test solution.

Result The colour of the spot obtained with the test solution is not more intense than that obtained with the reference solution.

METHOD F

Test solution Place the prescribed quantity or volume of the substance to be examined in a clean, dry, 100 mL long-necked combustion flask (a 300 mL flask may be used if the reaction foams excessively). Clamp the flask at an angle of 45°. If the substance to be examined is a solid, add a sufficient volume of a mixture of 8 mL of *sulfuric acid R* and 10 mL of *nitric acid R* to moisten the substance thoroughly; if the substance to be examined is a liquid, add a few millilitres of a mixture of 8 mL of *sulfuric acid R* and 10 mL of *nitric acid R*. Warm gently until the reaction commences, allow the reaction to subside and add additional portions of the same acid mixture, heating after each addition, until a total of 18 mL of the acid mixture has been added. Increase the amount of heat and boil gently until the solution darkens. Cool, add 2 mL of *nitric acid R* and heat again until the solution darkens. Continue the heating, followed by the addition of *nitric acid R* until no further darkening occurs, then heat strongly until dense, white fumes are produced. Cool, cautiously add 5 mL of *water R*, boil gently until dense, white fumes are produced and continue heating to reduce to 2-3 mL. Cool, cautiously add 5 mL of *water R* and examine the colour of the solution. If the colour is yellow, cautiously add 1 mL of *strong hydrogen peroxide solution R* and again evaporate until dense, white fumes are produced and reduce to a volume of 2-3 mL. If the solution is still yellow in colour, repeat the addition of 5 mL of *water R* and 1 mL of *strong hydrogen peroxide solution R* until the solution is colourless. Cool, dilute cautiously with *water R* and rinse into a 50 mL colour comparison tube, ensuring that the total volume does not exceed 25 mL. Adjust the solution to pH 3.0-4.0, using short range pH indicator paper as external indicator, with *concentrated ammonia R1* (*dilute ammonia R1* may be used, if desired, as the specified range is approached), dilute with *water R* to 40 mL and mix. Add 2 mL of *buffer solution pH 3.5 R*. Mix and add to 1.2 mL of *thioacetamide reagent R*. Mix immediately. Dilute to 50 mL with *water R* and mix.

Reference solution (standard) Prepare at the same time and in the same manner as the test solution, using the prescribed volume of *lead standard solution (10 ppm Pb) R*.

Monitor solution Prepare as described for the test solution, adding to the substance to be examined the volume of *lead standard solution (10 ppm Pb) R* prescribed for the preparation of the reference solution.

Blank solution Prepare as described for the test solution, omitting the substance to be examined.

Examine the solutions vertically against a white background after 2 min.

System suitability:

— the reference solution shows a brown colour compared to the blank solution,

— the monitor solution is at least as intense as the reference solution.

Result Any brown colour in the test solution is not more intense than that in the reference solution.

If the result is difficult to judge, filter the solutions through a suitable membrane filter (nominal pore size 0.45 µm). Carry out the filtration slowly and uniformly, applying moderate and constant pressure to the piston. Compare the spots on the filters obtained with the different solutions.

METHOD G

CAUTION: when using high-pressure digestion vessels the safety precautions and operating instructions given by the manufacturer must be followed. The digestion cycles have to be elaborated depending on the type of microwave oven to be used (for example, energy-controlled microwave ovens, temperature-controlled microwave ovens or high-pressure ovens). The cycle must conform to the manufacturer's instructions. The digestion cycle is suitable if a clear solution is obtained.

Test solution Place the prescribed amount of the substance to be examined (not more than 0.5 g) in a suitable, clean beaker. Add successively 2.7 mL of *sulfuric acid R*, 3.3 mL of *nitric acid R* and 2.0 mL of *strong hydrogen peroxide solution R* using a magnetic stirrer. Allow the substance to react with a reagent before adding the next one. Transfer the mixture to a dry high-pressure-resistant digestion vessel (fluoropolymer or quartz glass).

Reference solution (standard) Prepare as described for the test solution, using the prescribed volume of *lead standard solution (10 ppm Pb) R* instead of the substance to be examined.

Monitor solution Prepare as prescribed for the test solution, adding to the substance to be examined the volume of *lead standard solution (10 ppm Pb) R* prescribed for the preparation of the reference solution.

Blank solution Prepare as described for the test solution, omitting the substance to be examined.

Close the vessels and place in a laboratory microwave oven. Digest using a sequence of 2 separate suitable programmes. Design the programmes in several steps in order to control the reaction, monitoring pressure, temperature or energy depending on the type of microwave oven available. After the first programme allow the digestion vessels to cool before opening. Add to each vessel 2.0 mL of *strong hydrogen peroxide solution R* and digest using the second programme. After the second programme allow the digestion vessels to cool before opening. If necessary to obtain a clear solution, repeat the addition of *strong hydrogen peroxide solution R* and the second digestion programme.

Cool, dilute cautiously with *water R* and rinse into a flask, ensuring that the total volume does not exceed 25 mL.

Using short-range pH indicator paper as external indicator, adjust the solutions to pH 3.0-4.0 with *concentrated ammonia R1* (*dilute ammonia R1* may be used as the specified range is approached). To avoid heating of the solutions use an ice-bath and a magnetic stirrer. Dilute to 40 mL with *water R* and mix. Add 2 mL of *buffer solution pH 3.5 R*. Mix and add to 1.2 mL of *thioacetamide reagent R*. Mix immediately. Dilute to 50 mL with *water R*, mix and allow to stand for 2 min.

Filter the solutions through a suitable membrane filter (nominal pore size 0.45 µm). Carry out the filtration slowly and uniformly, applying moderate and constant pressure to the piston. Compare the spots on the filters obtained with the different solutions.

System suitability:

— the spot obtained with the reference solution shows a brown colour compared to the spot obtained with the blank solution,

— the spot obtained with the monitor solution is at least as intense as the spot obtained with the reference solution.

Result The brown colour of the spot obtained with the test solution is not more intense than that of the spot obtained with the reference solution.

METHOD H

Test solution Dissolve the prescribed quantity of the substance to be examined in 20 mL of the solvent or solvent mixture prescribed.

Reference solution Dilute the prescribed volume of *lead standard solution (10 ppm Pb) R* to 20 mL with the solvent or solvent mixture prescribed.

Blank solution 20 mL of the solvent or solvent mixture prescribed.

To each solution, add 2 mL of *buffer solution pH 3.5 R*. Mix. *(In some cases precipitation occurs, in which case the specific monograph would describe re-dissolution in a defined volume of a given solvent.)* Add to 1.2 mL of *thioacetamide reagent R*. Mix immediately and allow to stand for 2 min. Filter the solutions through a suitable membrane filter (nominal pore size 0.45 µm). Compare the spots on the filters obtained with the different solutions.

System suitability The spot obtained with the reference solution shows a brownish-black colour compared to the spot obtained with the blank solution.

Result The brownish-black colour of the spot obtained with the test solution is not more intense than that of the spot obtained with the reference solution.

Limit Test for Iron

(Ph. Eur. method 2.4.9)

Dissolve the prescribed quantity of the substance to be examined in *water R* and dilute to 10 mL with the same solvent or use 10 mL of the prescribed solution. Add 2 mL of a 200 g/L solution of *citric acid monohydrate R* and 0.1 mL of *thioglycollic acid R*. Mix, make alkaline with *ammonia R* and dilute to 20 mL with *water R*. Prepare a standard in the same manner, using 10 mL of *iron standard solution (1 ppm Fe) R*.

After 5 min, any pink colour in the test solution is not more intense than that in the standard.

Limit Test for Lead in Sugars

(Ph. Eur. method 2.4.10)

Determine the lead by atomic absorption spectrometry (*2.2.23, Method II*).

Test solution Dissolve 20.0 g of the substance to be examined in a mixture of equal volumes of *dilute acetic acid R* and *water R* and dilute to 100.0 mL with the same mixture of solvents. Add 2.0 mL of a clear 10 g/L solution of *ammonium pyrrolidinedithiocarbamate R* and 10.0 mL of *methyl isobutyl ketone R* and then shake for 30 s protected from bright light. Allow the layers to separate and use the methyl isobutyl ketone layer.

Reference solutions Prepare 3 reference solutions in the same manner as the test solution but adding 0.5 mL, 1.0 mL and 1.5 mL respectively of *lead standard solution (10 ppm Pb) R* in addition to the 20.0 g of the substance to be examined.

Set the zero of the instrument using *methyl isobutyl ketone R* treated as described for the test solution without the substance to be examined. Measure the absorbance at 283.3 nm using a lead hollow-cathode lamp as source of radiation and an air-acetylene flame.

The substance to be examined contains not more than 0.5 ppm of lead, unless otherwise prescribed.

Limit Test for Magnesium

(Ph. Eur. method 2.4.6)

To 10 mL of the prescribed solution add 0.1 g of *disodium tetraborate R*. Adjust the solution, if necessary, to pH 8.8 to pH 9.2 using *dilute hydrochloric acid R* or *dilute sodium hydroxide solution R*. Shake with 2 quantities, each of 5 mL, of a 1 g/L solution of hydroxyquinoline R in *chloroform R*, for 1 min each time. Allow to stand. Separate and discard the organic layer. To the aqueous solution add 0.4 mL of *butylamine R* and 0.1 mL of *triethanolamine R*. Adjust the solution, if necessary, to pH 10.5 to pH 11.5. Add 4 mL of the solution of hydroxyquinoline in chloroform, shake for 1 min, allow to stand and separate. Use the lower layer for comparison. Prepare a standard in the same manner using a mixture of 1 mL of *magnesium standard solution (10 ppm Mg) R* and 9 mL of *water R*.

Any colour in the solution obtained from the substance to be examined is not more intense than that in the standard.

Limit Test for Magnesium and Alkaline-earth Metals

(Ph. Eur. method 2.4.7)

To 200 mL of *water R* add 0.1 g of *hydroxylamine hydrochloride R*, 10 mL of *ammonium chloride buffer solution pH 10.0 R*, 1 mL of *0.1 M zinc sulfate* and about 15 mg of *mordant black 11 triturate R*. Heat to about 40 °C. Titrate with *0.01 M sodium edetate* until the violet colour changes to full blue. To the solution add the prescribed quantity of the substance to be examined dissolved in 100 mL of *water R* or use the prescribed solution. If the colour of the solution changes to violet, titrate with *0.01 M sodium edetate* until the full blue colour is again obtained.

The volume of *0.01 M sodium edetate* used in the second titration does not exceed the prescribed quantity.

Limit Test for Heavy Metals in Herbal Drugs and Herbal Drug Preparations

(Ph. Eur. method 2.4.27)

CAUTION: when using closed high-pressure digestion vessels and microwave laboratory equipment, be familiar with the safety and operating instructions given by the manufacturer.

APPARATUS

The apparatus typically consists of the following:

— as digestion flasks, polytetrafluoroethylene, perfluoroalkoxy polymer, quartz or glass flasks with a volume of 20-150 mL, fitted with an airtight closure, a valve to adjust the pressure inside the container and a polytetrafluoroethylene tube to allow release of gas;

— a system to make flasks airtight, using the same torsional force for each of them;

— a programmable microwave oven (e.g. with a magnetron frequency of 2450 MHz, with a selectable output from 0 to 1500 ± 70 W in 1 per cent increments), a polytetrafluoroethylene-coated microwave cavity with a variable speed exhaust fan, a rotating turntable drive system and exhaust tubing to vent fumes;

— an atomic absorption spectrometer (*2.2.23*), an inductively coupled plasma-atomic emission spectrometer (*2.2.57*), or an inductively coupled plasma-mass spectrometer (*2.2.58*).

METHOD

Examine by atomic absorption spectrometry (AAS) (*2.2.23*), inductively coupled plasma-atomic emission spectrometry

(ICP-AES) (*2.2.57*), or inductively coupled plasma-mass spectrometry (ICP-MS) (*2.2.58*).

Deviations from the experimental parameters of the sample preparation procedure and the method description below are acceptable provided that the validation requirements are met and the system suitability test is fulfilled on the day of the analysis.

Sample preparation

Clean all the glassware and laboratory equipment with a 10 g/L solution of *nitric acid R* before use.

Test solution In a digestion flask, place the prescribed quantity of the substance to be examined (about 0.50 g of powdered herbal drug (1400) (*2.9.12*)). Add 4 mL of *heavy metal-free hydrochloric acid R* and 6 mL of *heavy metal-free nitric acid R*. Make the flask airtight.

Place the digestion flasks in the microwave oven. Carry out the digestion in 3 steps according to the following programme, used for 7 flasks each containing the test solution: 80 per cent power for 15 min, 100 per cent power for 5 min, 80 per cent power for 20 min.

At the end of the cycle, allow the flasks to cool in air or water. After cooling, open each digestion flask and introduce the clear, colourless solution obtained into a 50 mL volumetric flask. Rinse each digestion flask with 2 quantities, each of 15 mL, of *heavy metal-free dilute nitric acid R*, collect the rinsings in the volumetric flask and dilute to 50.0 mL with *water R*. Modifiers (e.g. in the case of AAS with electrothermal atomisation, 1.0 mL of a 10 g/L solution of *magnesium nitrate R* and 1.0 mL of a 100 g/L solution of *ammonium dihydrogen phosphate R*) and stabilising agents may be used, if necessary.

Blank solution Mix 4 mL of *heavy metal-free hydrochloric acid R* and 6 mL of *heavy metal-free nitric acid R* in a digestion flask. Carry out the digestion in the same manner as for the test solution.

DETERMINATION OF ARSENIC, CADMIUM, COPPER, NICKEL AND LEAD USING AAS (2.2.23) WITH ELECTROTHERMAL ATOMISATION

Measure the content of arsenic, cadmium, copper, nickel and lead by direct calibration (*2.2.23, Method I*) or by the standard additions method (*2.2.23, Method II*), using reference solutions of each heavy metal and the instrumental parameters recommended in Table 2.4.27.-1.

The absorbance value of the blank solution is subtracted from the value obtained with the test solution.

Table 2.4.27.-1. – *Instrumental parameters for AAS with electrothermal atomisation*

		As	Cd	Cu	Ni	Pb
Wavelength	nm	193.7	228.8	324.8	232	283.5
Slit width	nm	0.5	0.5	0.5	0.2	0.5
Lamp current	mA	10	6	7	10	5
Ignition temperature	°C	1400	800	800	800	800
Atomisation temperature	°C	2600	1800	2300	2500	2200
Gas flow rate	L/min	3	3	3	3	3

DETERMINATION OF ARSENIC AND MERCURY USING AAS (2.2.23) WITH COLD-VAPOUR OR HYDRIDE ATOMISATION

Measure the content of arsenic and mercury by direct calibration (*2.2.23, Method I*) or by the standard additions method (*2.2.23, Method II*), using reference solutions of arsenic or mercury and an automated continuous-flow hydride vapour generation system.

The absorbance value of the blank solution is subtracted from the value obtained with the test solution.

Arsenic

Sample solution To 19.0 mL of the test solution or of the blank solution as prescribed above, add 1 mL of a 200 g/L solution of *potassium iodide R*. Allow the test solution to stand at room temperature for about 50 min or at 70 °C for about 4 min.

Acid reagent *Heavy metal-free hydrochloric acid R.*

Reducing reagent 6 g/L solution of *sodium tetrahydroborate R* in a 5 g/L solution of *sodium hydroxide R.*

The recommended instrumental parameters in Table 2.4.27.-2 may be used.

Mercury

Sample solution Test solution or blank solution, as prescribed above.

Acid reagent 515 g/L solution of *heavy metal-free hydrochloric acid R.*

Reducing reagent 10 g/L solution of *stannous chloride R* in heavy metal-free dilute hydrochloric acid R.

The recommended instrumental parameters in Table 2.4.27.-2 may be used.

Table 2.4.27.-2. – *Instrumental parameters for AAS with cold-vapour or hydride atomisation*

		As	Hg
Wavelength	nm	193.7	253.7
Slit width	nm	0.2	0.5
Lamp current	mA	10	4
Acid reagent flow rate	mL/min	1.0	1.0
Reducing reagent flow rate	mL/min	1.0	1.0
Sample solution flow rate	mL/min	7.0	7.0
Absorption cell		Quartz (heated)	Quartz (unheated)
Nitrogen flow rate	L/min	0.1	0.1

DETERMINATION OF ARSENIC, CADMIUM, COPPER, MERCURY, NICKEL AND LEAD USING ICP-AES (2.2.57)

Measure the content of arsenic, cadmium, copper, mercury, nickel and lead by direct calibration (*2.2.23, Method I*), using reference solutions of each heavy metal or a mixture of all measured metals, and the instrumental parameters recommended in Table 2.4.27.-3.

The emission value of the blank solution is subtracted from the value obtained with the test solution.

DETERMINATION OF ARSENIC, CADMIUM, COPPER, MERCURY, NICKEL AND LEAD USING ICP-MS (2.2.58)

Measure the content of arsenic, cadmium, copper, mercury, nickel and lead by direct calibration (*2.2.23, Method I*) using reference solutions of each heavy metal and the analytical isotopes and additional masses recommended in Table 2.4.27.-4.

The signal intensity of the blank solution is subtracted from the value obtained with the test solution.

SYSTEM SUITABILITY

A system suitability test must be carried out on the day of the analysis to ensure that the sample preparation and measurement system are appropriate.

Table 2.4.27.-3. – *Instrumental parameters for ICP-AES*

		As	Cd	Cu	Hg	Ni	Pb
Wavelength	nm	193.696/	214.438/	324.754/	184.950/	231.604/	220.351/
		197.197/	226.502/	327.396/	253.652/	231.997/	283.306/
		189.042	228.802	224.700	435.835	352.454	168.215
Ar, Monitorline	nm	430.010	430.010	430.010	430.010	430.010	430.010
Plasma energy	W	1200	1200	1200	1200	1200	1200
Peak algorithm with background correction		yes	yes	yes	yes	yes	yes

Acceptance criterion for preparation of sample solution A clear solution is obtained.

Acceptance criterion for measurement system The measured concentration of a standard solution of the metal at a concentration within the range of the used calibration curve does not differ from the actual concentration by more than 20 per cent.

Table 2.4.27.-4. – *Recommended analytical isotopes and additional masses for ICP-MS*

Isotope	Element of Interest
75	Arsenic
106, 108, 111, 114	Cadmium
63, 65	Copper
202	Mercury
60, 62	Nickel
206, 207, 208	Lead

VALIDATION REQUIREMENTS

The analytical procedures used must be validated in accordance with the relevant general methods AAS (*2.2.23*), ICP-AES (*2.2.57*) and ICP-MS (*2.2.58*). Additionally, the following criteria must be fulfilled.

SPECIFICITY

Specificity is the ability to ensure that the analytical procedures for sample preparation and measurement allow a reliable determination of the metal(s) in the presence of components (e.g. carrier gas, impurities, matrix) that may be expected to be present.

Acceptance criteria The procedure must be able to assess unequivocally each heavy metal to be determined with this procedure in the presence of components that may be expected to be present, including other heavy metals, matrix components and other sources of interference; specificity is demonstrated by complying with the accuracy requirement for the metal(s) to be determined.

RANGE

The calibration range of each metal is within the linear range of the method; test solutions containing residues at a level outside the calibration range may be diluted to concentrations within the calibration range.

Acceptance criterion Range is demonstrated by complying with the recovery requirement.

ACCURACY

Verify the accuracy using a certified reference material (CRM) or by performing a test for recovery.

Recovery. Recovery may be determined on a sample of the substance to be examined, spiked with a known quantity of a reference standard of the metal (3 concentration levels in the range of 50-150 per cent of the intended specification limit, even if the original concentration of the reference standard is at the specified value), in triplicate.

Acceptance criterion Spike recovery is within 70 per cent and 150 per cent for the mean of 3 replicates at each concentration.

REPEATABILITY

Test samples Either 6 independent samples of the substance to be examined spiked with a suitable reference standard at the specified concentration level, or 3 concentration levels prepared in triplicate.

Acceptance criterion The relative standard deviation is in both cases not greater than the value indicated in Table 2.4.27.-5.

INTERMEDIATE PRECISION

The effect of random events (intra-laboratory variations) on the analytical precision of the method must be established. Acceptable experiments for establishing intermediate precision include performing the repeatability analysis on different days, or with different instrumentation, or with different analysts. Only 1 of the 3 experiments is required to demonstrate intermediate precision.

Acceptance criterion The relative standard deviation is not greater than the value indicated in Table 2.4.27.-5.

LIMIT OF QUANTIFICATION

Determine the lowest concentration meeting the acceptance criterion. Use the results from the accuracy study.

Acceptance criterion The limit of quantification is below the specification limit.

Table 2.4.27.-5

Concentration range of the metal (mg/kg)	Repeatability (RSD) (per cent)	Intermediate precision (RSD) (per cent)
0.01 - 1	20	32
> 1	10	16

LIMIT OF DETECTION (ONLY APPLICABLE TO LIMIT TESTS)

Determine the lowest concentration giving a signal clearly distinct from that obtained with a blank solution.

Acceptance criterion The limit of detection is not more than 0.1 times the concentration of the specification limit.

Limit Test for Nickel in Polyols

(Ph. Eur. method 2.4.15)

Determine the nickel by atomic absorption spectrometry (*2.2.23, Method II*).

Test solution Dissolve 20.0 g of the substance to be examined in a mixture of equal volumes of *dilute acetic acid R* and *water R* and dilute to 100.0 mL with the same mixture of solvents. Add 2.0 mL of a saturated solution of *ammonium pyrrolidinedithiocarbamate R* (about 10 g/L) and 10.0 mL of *methyl isobutyl ketone R* and then shake for 30 s protected from bright light. Allow the layers to separate and use the methyl isobutyl ketone layer.

Reference solutions Prepare 3 reference solutions in the same manner as the test solution but adding 0.5 mL, 1.0 mL and 1.5 mL respectively of *nickel standard solution (10 ppm Ni) R* in addition to the 20.0 g of the substance to be examined.

Set the zero of the instrument using *methyl isobutyl ketone R* treated as described for preparation of the test solution omitting the substance to be examined. Measure the absorbance at 232.0 nm using a nickel hollow-cathode lamp as source of radiation and an air-acetylene flame.

The substance to be examined contains not more than 1 ppm of nickel, unless otherwise prescribed.

Limit Test for Phosphates

(Ph. Eur. method 2.4.11)

To 100 mL of the solution prepared and, if necessary, neutralised as prescribed add 4 mL of *sulfomolybdic reagent R3*. Shake and add 0.1 mL of *stannous chloride solution R1*. Prepare a standard in the same manner using 2 mL of *phosphate standard solution (5 ppm PO_4) R* and 98 mL of *water R*. After 10 min, compare the colours using 20 mL of each solution.

Any colour in the test solution is not more intense than that in the standard.

Limit Test for Potassium

(Ph. Eur. method 2.4.12)

To 10 mL of the prescribed solution add 2 mL of a freshly prepared 10 g/L solution of *sodium tetraphenylborate R*. Prepare a standard in the same manner using a mixture of 5 mL of *potassium standard solution (20 ppm K) R* and 5 mL of *water R*.

After 5 min, any opalescence in the test solution is not more intense than that in the standard.

Limit Test for Sulfates

(Ph. Eur. method 2.4.13)

All solutions used for this test must be prepared with distilled water R.

Add 3 mL of a 250 g/L solution of *barium chloride R* to 4.5 mL of *sulfate standard solution (10 ppm SO_4) R1*. Shake and allow to stand for 1 min. To 2.5 mL of this suspension add 15 mL of the prescribed solution and 0.5 mL of *acetic acid R*. Prepare a standard in the same manner using 15 mL of *sulfate standard solution (10 ppm SO_4) R* instead of the prescribed solution.

After 5 min, any opalescence in the test solution is not more intense than that in the standard.

Appendix VIII

A. Non-aqueous Titration

(No Ph. Eur. method)

Method I

Dissolve the prescribed quantity of the substance being examined in a suitable volume of *anhydrous acetic acid* previously neutralised using the indicator specified in the monograph, warming and cooling if necessary, or prepare a solution as directed. When the substance is a salt of hydrochloric or hydrobromic acid, add 15 mL of *mercury(II) acetate solution* before neutralising the solvent, unless otherwise directed in the monograph. Titrate with *0.1M perchloric acid VS* to the colour change of the indicator that corresponds to the maximum absolute value of dE/dV (where E is the electromotive force and V is the volume of titrant) in a *potentiometric titration*, Appendix VIII B, of the substance being examined. The indicator specified in the monograph is also used for the neutralisation of the *mercury(II) acetate solution* and the standardisation of the titrant.

When the temperature (t_2) of the titrant at the time of the assay differs from the temperature (t_1) of the titrant when it was standardised, multiply the volume of the titrant required by $[1+0.0011(t_1 - t_2)]$ and calculate the result of the assay from the corrected volume.

Carry out a blank titration when necessary.

Method II

The titrant, solvent and, where necessary, the indicator to be used are stated in the monograph.

Protect the solution and titrant from atmospheric carbon dioxide and moisture throughout the determination.

Dissolve the substance being examined in a suitable volume of the solvent previously neutralised to the indicator, warming and cooling if necessary, or prepare a solution as directed. Titrate to the colour change of the indicator that corresponds to the maximum absolute value of dE/dV (where E is the electromotive force and V is the volume of titrant) in a *potentiometric titration*, Appendix VIII B, of the substance under examination. The titrant is standardised using the same solvent and indicator as specified for the substance.

Carry out a blank titration when necessary.

B. Amperometric, Potentiometric and Voltametric Titrations

Amperometric Titration

(Ph. Eur. method 2.2.19)

In an amperometric titration, the end-point is determined by following the variation of the current measured between 2 electrodes (either one indicator electrode and one reference electrode or 2 indicator electrodes) immersed in the solution to be examined and maintained at a constant potential difference as a function of the quantity of titrant added.

The potential of the measuring electrode is sufficient to ensure a diffusion current for the electroactive substance.

Apparatus The apparatus comprises an adjustable voltage source and a sensitive microammeter; the detection system generally consists of an indicator electrode (for example, a platinum electrode, a rotating-disc electrode or a carbon

electrode) and a reference electrode (for example, a silver-silver chloride electrode).

A three-electrode apparatus is sometimes used, consisting of an indicator electrode, a reference electrode and a polarised auxiliary electrode.

Method Set the potential of the indicator electrode as prescribed and plot a graph of the initial current and the values obtained during the titration as functions of the quantity of titrant added. Add the titrant in not fewer than 3 successive quantities equal to a total of about 80 per cent of the theoretical volume corresponding to the presumed equivalence point. The 3 values must fall on a straight line. Continue adding the titrant beyond the presumed equivalence point in not fewer than 3 successive quantities. The values obtained must fall on a straight line. The point of intersection of the 2 lines represents the end-point of the titration.

For amperometric titrations with 2 indicator electrodes, the whole titration curve is recorded and used to determine the end-point.

Potentiometric Titration

(Ph. Eur. method 2.2.20)

In a potentiometric titration (volumetric titration with potentiometric end-point determination) the end-point is determined by recording the variation of the potential difference between 2 electrodes (either 1 indicator electrode and 1 reference electrode, or a combined electrode) immersed in the solution to be examined as a function of the volume of titrant added.

Apparatus The apparatus used comprises a millivoltmeter. Commercial autotitrator instruments may be used and are operated in accordance with the manufacturer's instructions, using electrodes recommended for the type of titration described.

The indicator electrode to be used depends on the substance to be determined and may be a glass or metal electrode (e.g. platinum, gold or silver).

For acid-base titrations, a glass-silver-silver chloride electrode combination is generally used.

Method Prepare the sample solution as described. Add the titrant in suitable aliquots paying particular attention to the rate of addition and the volume increments near the end-point. Continue the titration beyond this point to allow a clear detection of the end-point.

The end-point of the titration is reached when the maximum change in potential occurs in a plot of potential versus volume of titrant, and is expressed as the corresponding volume of titrant. Recording the first or second derivative curve can facilitate the determination of the end-point.
In potentiometric titrations of weak acids or bases using non-aqueous solvents, if necessary, either carry out a blank determination or pre-neutralise the solvent mixture. Where it is impracticable to use potentiometric detection for this purpose, the solvent mixture can be pre-neutralised by titration using a suitable indicator. Some examples are given below:

Titrant	Indicator
Perchloric acid	*Crystal violet solution R*
Tetrabutylammonium hydroxide	3 g/L solution of *thymol blue R* in *methanol R*
Ethanolic sodium hydroxide	*Thymolphthalein solution R*

Voltametric Titrations

(Ph. Eur. method 2.2.65)

In voltametric titration the end-point of the titration is determined by following the variation of the voltage measured between 2 electrodes (either 1 indicator electrode and 1 reference electrode or 2 indicator electrodes) immersed in the solution to be examined and maintained at a constant current as a function of the quantity of titrant added.

Apparatus The apparatus comprises an adjustable current source and a voltmeter; the detection system generally consists of an indicator electrode (for example, a platinum electrode, a rotating-disc electrode or a carbon electrode) and a 2nd electrode (for example, a platinum electrode, a rotating-disc electrode or a carbon electrode).

Method Set the current to the indicator electrode as prescribed in the monograph and plot a graph of the initial voltage and the values obtained during the titration as functions of the quantity of titrant added. Add the titrant in not fewer than 3 successive quantities equal to a total of about 80 per cent of the theoretical volume corresponding to the presumed equivalence point. The 3 values must fall on a straight line. Continue adding the titrant beyond the presumed equivalence point in not fewer than 3 successive quantities. The values obtained must fall on another straight line. The point of intersection of the 2 lines represents the end-point of the titration.

Using titration systems for voltametric titration with 2 indicator electrodes, the whole titration curve is recorded and used to determine the end-point.

Determination of Primary Aromatic Amino-nitrogen

(Ph. Eur. method 2.5.8)

Dissolve the prescribed quantity of the substance to be examined in 50 mL of *dilute hydrochloric acid R* or in another prescribed solvent and add 3 g of *potassium bromide R*. Cool in ice-water and titrate by slowly adding *0.1 M sodium nitrite* with constant stirring.

Determine the end-point electrometrically or by the use of the prescribed indicator.

C. Oxygen-flask Combustion

(Ph. Eur. method 2.5.10)

Unless otherwise prescribed the combustion flask is a conical flask of at least 500 mL capacity of borosilicate glass with a ground-glass stopper fitted with a suitable carrier for the sample, for example in platinum or platinum-iridium.

Finely grind the substance to be examined, place the prescribed quantity in the centre of a piece of filter paper measuring about 30 mm by 40 mm provided with a small strip about 10 mm wide and 30 mm long. If paper impregnated with lithium carbonate is prescribed, moisten the centre of the paper with a saturated solution of *lithium carbonate R* and dry in an oven before use. Envelop the substance to be examined in the paper and place it in the sample carrier. Introduce into the flask *water R* or the prescribed solution designed to absorb the combustion products, displace the air with oxygen by means of a tube having its end just above the liquid, moisten the neck of the flask with *water R* and close with its stopper. Ignite the paper strip by suitable means with the usual precautions. Keep the flask firmly closed during the combustion. Shake the flask

vigorously to completely dissolve the combustion products. Cool and after about 5 min, unless otherwise prescribed, carefully unstopper the flask. Wash the ground parts and the walls of the flask, as well as the sample carrier, with *water R*. Combine the combustion products and the washings and proceed as prescribed in the monograph.

For Iodine

(No Ph. Eur. method)

Burn the specified quantity of the substance being examined in the prescribed manner using a mixture of 10 mL of *water* and 2 mL of 1M *sodium hydroxide* as the absorbing liquid. When the process is complete, add to the flask an excess of *acetic bromine solution* (between 5 and 10 mL) and allow to stand for 2 minutes. Remove the excess of bromine by the addition of *formic acid* (about 0.5 to 1 mL), rinse the sides of the flask with *water* and displace any bromine vapour above the liquid with a current of air. Add 1 g of *potassium iodide* and titrate with 0.02M *sodium thiosulfate VS* using *starch mucilage*, added towards the end of the titration, as indicator. Each mL of 0.02M *sodium thiosulfate VS* is equivalent to 0.4230 mg of I.

D. Complexometric Titrations

(Ph. Eur. method 2.5.11)

ALUMINIUM

Introduce 20.0 mL of the prescribed solution into a 500 mL conical flask, add 25.0 mL of *0.1 M sodium edetate* and 10 mL of a mixture of equal volumes of a 155 g/L solution of *ammonium acetate R* and *dilute acetic acid R*. Boil for 2 min, then cool. Add 50 mL of *ethanol R* and 3 mL of a freshly prepared 0.25 g/L solution of *dithizone R* in *ethanol R*. Titrate the excess of sodium edetate with *0.1 M zinc sulfate* until the colour changes from greenish-blue to reddish-violet.

1 mL of *0.1 M sodium edetate* is equivalent to 2.698 mg of Al.

BISMUTH

Introduce the prescribed solution into a 500 mL conical flask. Dilute to 250 mL with *water R* and then, unless otherwise prescribed, add dropwise, with shaking, *concentrated ammonia R* until the mixture becomes cloudy. Add 0.5 mL of *nitric acid R*. Heat to about 70 °C until the cloudiness disappears completely. Add about 50 mg of *xylenol orange triturate R* and titrate with *0.1 M sodium edetate* until the colour changes from pinkish-violet to yellow.

1 mL of *0.1 M sodium edetate* is equivalent to 20.90 mg of Bi.

CALCIUM

Introduce the prescribed solution into a 500 mL conical flask, and dilute to 300 mL with *water R*. Add 6.0 mL of *strong sodium hydroxide solution R* and about 200 mg of *calconecarboxylic acid triturate R*. Titrate with *0.1 M sodium edetate* until the colour changes from violet to full blue.

1 mL of *0.1 M sodium edetate* is equivalent to 4.008 mg of Ca.

MAGNESIUM

Introduce the prescribed solution into a 500 mL conical flask and dilute to 300 mL with *water R*. Add 10 mL of *ammonium chloride buffer solution pH 10.0 R* and about 50 mg of *mordant black 11 triturate R*. Heat to about 40 °C then titrate at this temperature with *0.1 M sodium edetate* until the colour changes from violet to full blue.

1 mL of *0.1 M sodium edetate* is equivalent to 2.431 mg of Mg.

LEAD

Introduce the prescribed solution into a 500 mL conical flask and dilute to 200 mL with *water R*. Add about 50 mg of *xylenol orange triturate R* and *hexamethylenetetramine R* until the solution becomes violet-pink. Titrate with *0.1 M sodium edetate* until the violet-pink colour changes to yellow.

1 mL of *0.1 M sodium edetate* is equivalent to 20.72 mg of Pb.

ZINC

Introduce the prescribed solution into a 500 mL conical flask and dilute to 200 mL with *water R*. Add about 50 mg of *xylenol orange triturate R* and *hexamethylenetetramine R* until the solution becomes violet-pink. Add 2 g of *hexamethylenetetramine R* in excess. Titrate with *0.1 M sodium edetate* until the violet-pink colour changes to yellow.

1 mL of *0.1 M sodium edetate* is equivalent to 6.54 mg of Zn.

E. Potentiometric Determination of Ionic Concentration Using Ion-selective Electrodes

(Ph. Eur. method 2.2.36)

Ideally, the potential E of an ion-selective electrode varies linearly with the logarithm of the activity a_i of a given ion, as expressed by the Nernst equation:

$$E = E_0 + 2.303\frac{RT}{z_i F}\log_{10} a_i$$

E_0 = part of the constant potential due to the apparatus used,
R = gas constant,
T = absolute temperature,
F = Faraday's number,
z_i = charge number of the ion including its sign.

At a constant ionic strength, the following holds:

$$E = E_0 + \frac{k}{z_i}\log_{10} f C_i$$

C_i = molar concentration of the ion,
f = the activity coefficient ($a_i = f C_i$),
k = $\frac{RT}{F}$

If: $E_0 + \frac{k}{z_i}\log_{10} f = E'_0$ and $S = \frac{k}{z_i}$

S = slope of the calibration curve of the electrode,

the following holds: $E = E'_0 + S\log_{10} C_i$

and for $-\log_{10} C_i = pC_i$: $E = E'_0 - SpC_i$.

The potentiometric determination of the ion concentration is carried out by measuring the potential difference between two suitable electrodes immersed in the solution to be examined; the indicator electrode is selective for the ion to be determined and the other is a reference electrode.

Apparatus Use a voltmeter allowing measurements at least to the nearest 0.1 millivolt and whose input impedance is at least one hundred times greater than that of the electrodes used.

Ion-selective electrodes may be primary electrodes with a crystal or non-crystal membrane or with a rigid matrix (for

example, glass electrodes), or electrodes with charged (positive or negative) or uncharged mobile carriers, or sensitised electrodes (enzymatic-substrate electrodes, gas-indicator electrodes). The reference electrode is generally a silver–silver chloride electrode with suitable junction liquids producing no interference.

Procedure Carry out each measurement at a temperature constant to ± 0.5 °C, taking into account the variation of the slope of the electrode with temperature (see Table 2.2.36.-1). Adjust the ionic strength and possibly the pH of the solution to be analysed using the buffer reagent described in the monograph and equilibrate the electrode by immersing it in the solution to be analysed, under slow and uniform stirring, until a constant reading is obtained.

Table 2.2.36.-1. – *Values of k at different temperatures*

Temperature (°C)	*k*
20	0.0582
25	0.0592
30	0.0602

If the electrode system is used frequently, check regularly the repeatability and the stability of responses, and the linearity of the calibration curve or the calculation algorithm in the range of concentrations of the test solution; if not, carry out the test before each set of measurements. The response of the electrode may be regarded as linear if the slope S of the calibration curve is approximately equal to k/z_i per unit of pC_i.

METHOD I (DIRECT CALIBRATION)

Measure at least three times in succession the potential of at least three reference solutions spanning the expected concentration of the test solution. Calculate the calibration curve, or plot on a chart the mean potential E obtained against the concentration of the ion to be determined expressed as $-\log_{10} C_i$ or pC_i.

Prepare the test solution as prescribed in the monograph; measure the potential three times and, from the mean potential, calculate the concentration of the ion to be determined using the calibration curve.

METHOD II (MULTIPLE STANDARD ADDITIONS)

Prepare the test solution as prescribed in the monograph. Measure the potential at equilibrium E_T of a volume V_T of this solution of unknown concentration C_T of the ion to be determined. Make at least three consecutive additions of a volume V_S negligible compared to V_T ($V_S \leq 0.01 V_T$) of a reference solution of a concentration C_S known to be within the linear part of the calibration curve. After each addition, measure the potential and calculate the difference of potential ΔE between the measured potential and E_T. ΔE is related to the concentration of the ion to be determined by the equation:

$$\Delta E = S \log_{10}\left(1 + \frac{C_S V_S}{C_T V_T}\right)$$

or

$$10^{\frac{\Delta E}{S}} = 1 + \frac{C_S V_S}{C_T V_T}$$

V_T = volume of the test solution,
C_T = concentration of the ion to be determined in the test solution,
V_S = added volume of the reference solution,
C_S = concentration of the ion to be determined in the reference solution,
S = slope of the electrode determined experimentally, at constant temperature, by measuring the difference between the potentials obtained with two reference solutions whose concentrations differ by a factor of ten and are situated within the range where the calibration curve is linear.

Plot on a graph $10^{\frac{\Delta E}{S}}$ (*y*-axis) against V_S (*x*-axis) and extrapolate the line obtained until it intersects the *x*-axis. At the intersection, the concentration C_T of the ion to be determined in the test solution is given by the equation:

$$C_T = -\frac{C_S V_S}{V_T}$$

METHOD III (SINGLE STANDARD ADDITION)

To a volume V_T of the test solution prepared as prescribed in the monograph, add a volume V_S of a reference solution containing an amount of the ion to be determined known to give a response situated in the linear part of the calibration curve. Prepare a blank solution in the same conditions. Measure at least three times the potentials of the test solution and the blank solution, before and after adding the reference solution. Calculate the concentration C_T of the ion to be analysed using the following equation and making the necessary corrections for the blank:

$$C_T = \frac{C_S V_S}{10^{\frac{\Delta E}{S}}(V_T + V_S) - V_T}$$

V_T = volume of the test solution or the blank,
C_T = concentration of the ion to be determined in the test solution,
V_S = added volume of the reference solution,
C_S = concentration of the ion to be determined in the reference solution,
ΔE = difference between the average potentials measured before and after adding V_S,
S = slope of the electrode determined experimentally, at constant temperature, by measuring the difference between the potentials obtained from two reference solutions whose concentrations differ by a factor of ten and are situated within the range where the calibration curve is linear.

F. Determination of Ethanol

Use Method I or, where appropriate, Method II unless otherwise prescribed in the monograph.

Method I

(No Ph. Eur. method)

Carry out the method for *gas chromatography*, Appendix III B, using the following solutions. Solution (1) contains 5.0% v/v of *absolute ethanol* and 5.0% v/v of *propan-1-ol* (internal standard). For solution (2) dilute a volume of the preparation being examined with *water* to contain between 4.0 and 6.0% v/v of ethanol. Prepare solution (3) in the same manner as solution (2) but adding sufficient of the internal standard to produce a final concentration of 5.0% v/v.

The chromatographic procedure may be carried out using a column (1.5 m × 4 mm) packed with porous polymer beads (100 to 120 mesh) (Porapak Q and Chromosorb 101 are suitable) and maintained at 150° with both the inlet port and the detector at 170°.

Calculate the percentage content of ethanol from the areas of the peaks due to ethanol in the chromatograms obtained with solutions (1) and (3).

Method II

(No Ph. Eur. method)

For preparations in which, in accordance with the authority given in the monographs, Industrial Methylated Spirit has been used, determine the content of ethanol as described in Method I but using as solution (2) a volume of the preparation being examined diluted with *water* to contain between 4.0 and 6.0% v/v of total ethanol and methanol.

Determine the concentration of methanol in the following manner. Carry out the chromatographic procedure described under Method I but using the following solutions. Solution (1) contains 0.25% v/v of *methanol* and 0.25% v/v of *propan-1-ol* (internal standard). For solution (2) dilute a volume of the preparation being examined with *water* to contain between 0.2% and 0.3% v/v of methanol. Prepare solution (3) in the same manner as solution (2) but adding sufficient of the internal standard to produce a final concentration of 0.25% v/v.

The sum of the contents of ethanol and methanol is within the range specified in the monograph and the ratio of the content of methanol to that of ethanol is commensurate with Industrial Methylated Spirit having been used.

Method III

(Ph. Eur. method 2.9.10)

These methods are intended for the examination of liquid pharmaceutical preparations and their ingredients that contain ethanol.

The ethanol content of a liquid is expressed as the number of volumes of ethanol contained in 100 volumes of the liquid, the volumes being measured at 20 ± 0.1 °C. This is known as the 'percentage of ethanol by volume' (per cent V/V). The content may also be expressed in grams of ethanol per 100 g of the liquid. This is known as the 'percentage of ethanol by mass' (per cent m/m).

METHOD A

Where preparations contain dissolved substances, the dissolved substances must be separated from the ethanol that is to be determined by distillation. Where distillation would distil volatile substances other than ethanol and water, the appropriate precautions are stated in the monograph.

The relation between the density at 20 ± 0.1 °C, the relative density (corrected to vacuum) and the ethanol content of a mixture of water and ethanol is given in the tables of the International Organisation for Legal Metrology (1972), International Recommendation No. 22.

Apparatus

The apparatus (see Figure 2.9.10.-1) consists of a round-bottomed flask (*A*) fitted with a distillation head (*B*) with a steam trap and attached to a vertical condenser (*C*). The latter is fitted at its lower part with a tube (*D*), which carries the distillate into the lower part of a 100 mL or 250 mL volumetric flask. The volumetric flask is immersed in a mixture of ice and water (*E*) during the distillation. A disc having a circular aperture 6 cm in diameter is placed under the flask (*A*) to reduce the risk of charring any dissolved substances.

Method

Pycnometer method/oscillating transducer density meter method Transfer 25.0 mL of the preparation to be examined, measured at 20 ± 0.1 °C, to the distillation flask. Dilute with 100-150 mL of *distilled water R* and add a few pieces of pumice. Attach the distillation head and condenser. Distil and collect not less than 90 mL of distillate in a 100 mL volumetric flask. Adjust the temperature to 20 ± 0.1 °C and dilute to 100.0 mL with *distilled water R* at 20 ± 0.1 °C. Determine the relative density at 20 ± 0.1 °C using a pycnometer or an oscillating transducer density meter.

The values indicated in Table 2.9.10.-1, column 3, are multiplied by 4 to obtain the percentage of ethanol by volume (*V/V*) contained in the preparation. After calculation of the ethanol content using the table, round off the result to 1 decimal place.

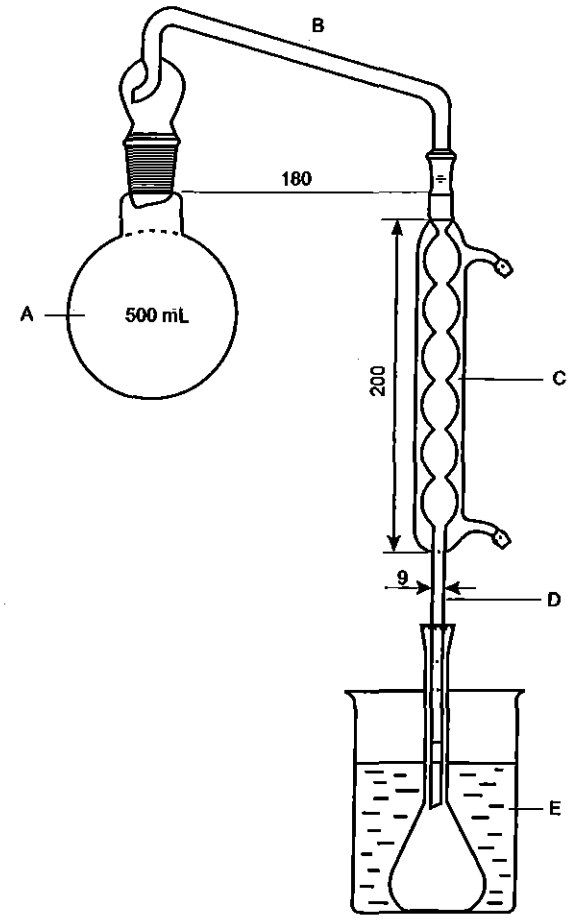


Figure 2.9.10.-1. – *Apparatus for the determination of ethanol content*

Dimensions in millimetres

Hydrometer method Transfer 50.0 mL of the preparation to be examined, measured at 20 ± 0.1 °C, to the distillation flask, add 200-300 mL of *distilled water R* and distil, as described above, into a volumetric flask until at least 180 mL has been collected. Adjust the temperature to 20 ± 0.1 °C and dilute to 250.0 mL with *distilled water R* at 20 ± 0.1 °C.

Transfer the distillate to a cylinder whose diameter is at least 6 mm wider than the bulb of the hydrometer. If the volume is insufficient, double the quantity of the sample and dilute the distillate to 500.0 mL with *distilled water R* at 20 ± 0.1 °C.

Multiply the strength by 5 to allow for the dilution during the determination. After calculation of the ethanol content using Table 2.9.10.-1, round off the result to 1 decimal place.

Table 2.9.10.-1. – *Relationship between density, relative density and ethanol content*

ρ_{20} (kg·m^{-3})	Relative density of the distillate measured in air d_{20}^{20}	Ethanol content in per cent *V/V* at 20 °C
968.0	0.9697	25.09
968.5	0.9702	24.64
969.0	0.9707	24.19
969.5	0.9712	23.74
970.0	0.9717	23.29
970.5	0.9722	22.83
971.0	0.9727	22.37
971.5	0.9733	21.91
972.0	0.9738	21.45
972.5	0.9743	20.98
973.0	0.9748	20.52
973.5	0.9753	20.05
974.0	0.9758	19.59
974.5	0.9763	19.12
975.0	0.9768	18.66
975.5	0.9773	18.19
976.0	0.9778	17.73
976.5	0.9783	17.25
977.0	0.9788	16.80
977.5	0.9793	16.34
978.0	0.9798	15.88
978.5	0.9803	15.43
979.0	0.9808	14.97
979.5	0.9813	14.52
980.0	0.9818	14.07
980.5	0.9823	13.63
981.0	0.9828	13.18
981.5	0.9833	12.74
982.0	0.9838	12.31
982.5	0.9843	11.87
983.0	0.9848	11.44
983.5	0.9853	11.02
984.0	0.9858	10.60
984.5	0.9863	10.18
985.0	0.9868	9.76
985.5	0.9873	9.35
986.0	0.9878	8.94
986.5	0.9883	8.53
987.0	0.9888	8.13
987.5	0.9893	7.73
988.0	0.9898	7.34
988.5	0.9903	6.95
989.0	0.9908	6.56
989.5	0.9913	6.17
990.0	0.9918	5.79
990.5	0.9923	5.42
991.0	0.9928	5.04
991.5	0.9933	4.67
992.0	0.9938	4.30
992.5	0.9943	3.94
993.0	0.9948	3.58
993.5	0.9953	3.22
994.0	0.9958	2.86
994.5	0.9963	2.51
995.0	0.9968	2.16
995.5	0.9973	1.82
996.0	0.9978	1.47
996.5	0.9983	1.13
997.0	0.9988	0.80
997.5	0.9993	0.46
998.0	0.9998	0.13

METHOD B

Head-space gas chromatography (*2.2.28*).

Internal standard solution Dilute 1.0 mL of *propanol R1* to 100.0 mL with *water R*. Dilute 1.0 mL of the solution to 20.0 mL with *water R*.

Test solution Dilute a volume of the preparation to be examined corresponding to 0.4 g of ethanol to 50.0 mL with *water R*. Dilute 1.0 mL of the solution to 20.0 mL with *water R*. To 2.0 mL of this solution add 1.0 mL of the internal standard solution and dilute to 20.0 mL with *water R*. Transfer 2.0 mL of this solution into an injection vial.

Reference solution (a) Dilute 5.0 mL of *anhydrous ethanol R* to 100.0 mL with *water R*. Dilute 25.0 mL of the solution to 100.0 mL with *water R*. Dilute 1.0 mL of this solution to 20.0 mL with *water R*.

Reference solution (b) Mix 0.5 mL of reference solution (a) and 1.0 mL of the internal standard solution and dilute to 20.0 mL with *water R*. Transfer 2.0 mL of this solution into an injection vial.

Reference solution (c) Mix 1.0 mL of reference solution (a) and 1.0 mL of the internal standard solution and dilute to 20.0 mL with *water R*. Transfer 2.0 mL of this solution into an injection vial.

Reference solution (d) Mix 1.5 mL of reference solution (a) and 1.0 mL of the internal standard solution and dilute to 20.0 mL with *water R*. Transfer 2.0 mL of this solution into an injection vial.

Reference solution (e) Dilute 1.0 mL of *methanol R2* to 100.0 mL with *water R*. Dilute 1.0 mL of the solution to 20.0 mL with *water R*.

Reference solution (f) Mix 1.0 mL of the internal standard solution, 2.0 mL of reference solution (a) and 2.0 mL of reference solution (e) and dilute to 20.0 mL with *water R*. Transfer 2.0 mL of this solution into an injection vial.

Close the vials immediately with a tight rubber membrane stopper coated with polytetrafluoroethylene and secure with an aluminium crimp cap.

Column:
- *material*: fused silica;
- *size*: *l* = 30 m, Ø = 0.53 mm;
- *stationary phase*: *cyanopropyl(3)phenyl(3)methyl(94) polysiloxane R* (film thickness 3 µm).

Carrier gas *helium for chromatography R*.

Flow rate 3 mL/min.

Split ratio 1:50.

Static head-space conditions that may be used:
- *equilibration temperature*: 85 °C;
- *equilibration time*: 20 min.

Temperature:

	Time (min)	Temperature (°C)
Column	0 - 1.6	40
	1.6 - 9.9	40 → 65
	9.9 - 13.6	65 → 175
	13.6 - 20	175
Injection port		200
Detector		200

Detection Flame ionisation.

Injection 1.0 mL of the gaseous phase of the test solution and reference solutions (b), (c), (d) and (f), at least 3 times.

Elution order Methanol, ethanol, propanol.

Relative retention With reference to ethanol (retention time = about 5.3 min): methanol = about 0.8; propanol = about 1.6.

System suitability Reference solution (f):
— *resolution*: minimum 5 between the peaks due to methanol and ethanol.

Establish a calibration curve with the concentration of ethanol in reference solutions (b), (c), (d) and (f) as the abscissa and the mean ratio of the peak area of ethanol to the peak area of the internal standard in the corresponding chromatograms as the ordinate.

Calculate the percentage content of ethanol in the preparation to be examined.

METHOD C

Gas chromatography (*2.2.28*).

Internal standard solution Dilute 1.0 mL of *propanol R1* to 100.0 mL with *water R*.

Test solution Dilute a volume of the preparation to be examined corresponding to 1 g of ethanol to 50.0 mL with *water R*. To 1.0 mL of this solution add 1.0 mL of the internal standard solution and dilute to 20.0 mL with *water R*.

Reference solution (a) Dilute 1.0 mL of *anhydrous ethanol R* to 50.0 mL with *water R*.

Reference solution (b). Dilute 1.0 mL of *methanol R2* to 100.0 mL with *water R*. Dilute 1.0 mL of the solution to 20.0 mL with *water R*.

Reference solution (c) Mix 1.0 mL of the internal standard solution, 1.0 mL of reference solution (a) and 2.0 mL of reference solution (b) and dilute to 20.0 mL with *water R*.

Column:
— *material*: fused silica;
— *size*: *l* = 30 m, Ø = 0.53 mm;
— *stationary phase*: *cyanopropyl(3)phenyl(3)methyl(94) polysiloxane R* (film thickness 3 µm).

Carrier gas *helium for chromatography R*.

Flow rate 3 mL/min.

Split ratio 1:50.

Temperature:

	Time (min)	Temperature (°C)
Column	0 - 1.6	40
	1.6 - 9.9	40 → 65
	9.9 - 13.6	65 → 175
	13.6 - 20	175
Injection port		200
Detector		200

Detection Flame ionisation.

Injection 1.0 µL of the test solution and reference solution (c), at least 3 times.

Elution order Methanol, ethanol, propanol.

Relative retention With reference to ethanol (retention time = about 5.3 min): methanol = about 0.8; propanol = about 1.6.

System suitability Reference solution (c):
— *resolution*: minimum 5 between the peaks due to methanol and ethanol.

Calculate the ethanol content in per cent *V/V* using the following expression:

$$\frac{A_1 \times I_2 \times 100}{A_2 \times I_1 \times V_1}$$

A_1 = area of the peak due to ethanol in the chromatogram obtained with the test solution;
A_2 = area of the peak due to ethanol in the chromatogram obtained with reference solution (c);
I_1 = area of the peak due to the internal standard in the chromatogram obtained with the test solution;
I_2 = area of the peak due to the internal standard in the chromatogram obtained with reference solution (c);
V_1 = volume of the preparation to be examined in the test solution, in millilitres.

G. Determination of Methanol and Propan-2-ol

(Ph. Eur. method 2.9.11)

METHOD A

Head-space gas chromatography (*2.2.28*).

Internal standard solution Dilute 1.0 mL of *propanol R1* to 100.0 mL with *water R*. Dilute 1.0 mL of the solution to 20.0 mL with *water R*.

Test solution Mix 1.0 mL of the internal standard solution and 4.0 mL of the preparation to be examined and dilute to 20.0 mL with *water R*. Transfer 2.0 mL of this solution into an injection vial.

Reference solution (a) Mix 1.0 mL of *methanol R2* and 1.0 mL of *2-propanol R2* and dilute to 100.0 mL with *water R*. Dilute 1.0 mL of the solution to 20.0 mL with *water R*.

Reference solution (b) Dilute 5.0 mL of *anhydrous ethanol R* to 100.0 mL with *water R*. Dilute 25.0 mL of the solution to 100.0 mL with *water R*. Dilute 1.0 mL of this solution to 20.0 mL with *water R*.

Reference solution (c) Mix 1.0 mL of the internal standard solution, 2.0 mL of reference solution (a) and 2.0 mL of reference solution (b) and dilute to 20.0 mL with *water R*. Transfer 2.0 mL of this solution into an injection vial.

Close the vials immediately with a tight rubber membrane stopper coated with polytetrafluoroethylene and secure with an aluminium crimp cap.

Column:
— *material*: fused silica;
— *size*: *l* = 30 m, Ø = 0.53 mm;
— *stationary phase*: *cyanopropyl(3)phenyl(3)methyl(94) polysiloxane R* (film thickness 3 µm).

Carrier gas *helium for chromatography R*.

Flow rate 3 mL/min.

Split ratio 1:50.

Static head-space conditions that may be used:
— *equilibration temperature*: 85 °C;
— *equilibration time*: 20 min.

Temperature:

	Time (min)	Temperature (°C)
Column	0 - 1.6	40
	1.6 - 9.9	40 → 65
	9.9 - 13.6	65 → 175
	13.6 - 20	175
Injection port		200
Detector		200

Detection Flame ionisation.

Injection 1.0 mL of the gaseous phase of the test solution and reference solution (c), at least 3 times.

Elution order Methanol, ethanol, 2-propanol, 1-propanol.

Relative retention With reference to ethanol (retention time = about 5.3 min): methanol = about 0.8; 2-propanol = about 1.2; 1-propanol = about 1.6.

System suitability Reference solution (c):
— *resolution*: minimum 5 between the peaks due to methanol and ethanol.

Calculate the methanol content in per cent *V/V* using the following expression:

$$\frac{A_1 \times I_2}{A_2 \times I_1 \times 40}$$

A_1 = area of the peak due to methanol in the chromatogram obtained with the test solution;
A_2 = area of the peak due to methanol in the chromatogram obtained with reference solution (c);
I_1 = area of the peak due to the internal standard in the chromatogram obtained with the test solution;
I_2 = area of the peak due to the internal standard in the chromatogram obtained with reference solution (c).

Calculate the 2-propanol content in per cent *V/V* using the following expression:

$$\frac{A_3 \times I_2}{A_4 \times I_1 \times 40}$$

A_3 = area of the peak due to 2-propanol in the chromatogram obtained with the test solution;
A_4 = area of the peak due to 2-propanol in the chromatogram obtained with reference solution (c);
I_1 = area of the peak due to the internal standard in the chromatogram obtained with the test solution;
I_2 = area of the peak due to the internal standard in the chromatogram obtained with reference solution (c).

METHOD B

Gas chromatography (*2.2.28*).

Internal standard solution Dilute 1.0 mL of *propanol R1* to 100.0 mL with *water R*.

Test solution Mix 1.0 mL of the internal standard solution and 4.0 mL of the preparation to be examined and dilute to 20.0 mL with *water R*.

Reference solution (a) Mix 1.0 mL of *methanol R2* and 1.0 mL of *2-propanol R2* and dilute to 100.0 mL with *water R*. Dilute 1.0 mL of the solution to 20.0 mL with *water R*.

Reference solution (b) Dilute 1.0 mL of *anhydrous ethanol R* to 50.0 mL with *water R*.

Reference solution (c) Mix 1.0 mL of the internal standard solution, 1.0 mL of reference solution (b) and 2.0 mL of reference solution (a) and dilute to 20.0 mL with *water R*.

Column:
— *material*: fused silica;
— *size*: *l* = 30 m, Ø = 0.53 mm;
— *stationary phase*: *cyanopropyl(3)phenyl(3)methyl(94) polysiloxane R* (film thickness 3 µm).

Carrier gas *helium for chromatography R*.

Flow rate 3 mL/min.

Split ratio 1:50.

Temperature:

	Time (min)	Temperature (°C)
Column	0 - 1.6	40
	1.6 - 9.9	40 → 65
	9.9 - 13.6	65 → 175
	13.6 - 20	175
Injection port		200
Detector		200

Detection Flame ionisation.

Injection 1.0 µL of the test solution and reference solution (c), at least 3 times.

Elution order Methanol, ethanol, 2-propanol, 1-propanol.

Relative retention With reference to ethanol (retention time = about 5.3 min): methanol = about 0.8; 2-propanol = about 1.2; 1-propanol = about 1.6.

System suitability Reference solution (c):
— *resolution*: minimum 5 between the peaks due to methanol and ethanol.

Calculate the methanol content in per cent *V/V* using the following expression:

$$\frac{A_1 \times I_2}{A_2 \times I_1 \times 40}$$

A_1 = area of the peak due to methanol in the chromatogram obtained with the test solution;
A_2 = area of the peak due to methanol in the chromatogram obtained with reference solution (c);
I_1 = area of the peak due to the internal standard in the chromatogram obtained with the test solution;
I_2 = area of the peak due to the internal standard in the chromatogram obtained with reference solution (c).

Calculate the 2-propanol content in per cent *V/V* using the following expression:

$$\frac{A_3 \times I_2}{A_4 \times I_1 \times 40}$$

A_3 = area of the peak due to 2-propanol in the chromatogram obtained with the test solution;
A_4 = area of the peak due to 2-propanol in the chromatogram obtained with reference solution (c);
I_1 = area of the peak due to the internal standard in the chromatogram obtained with the test solution;
I_2 = area of the peak due to the internal standard in the chromatogram obtained with reference solution (c).

H. Determination of Nitrogen

Method I

(Ph. Eur. method 2.5.9)

SEMI-MICRO METHOD

Place a quantity of the substance to be examined (*m* g) containing about 2 mg of nitrogen in a combustion flask, add 4 g of a powdered mixture of 100 g of *dipotassium sulfate R*, 5 g of *copper sulfate pentahydrate R* and 2.5 g of *selenium R*, and three glass beads. Wash any adhering particles from the neck into the flask with 5 mL of *sulfuric acid R*, allowing it to run down the sides of the flask, and mix the contents by rotation. Close the mouth of the flask loosely, for example by means of a glass bulb with a short stem, to avoid excessive loss of sulfuric acid. Heat gradually at first, then increase the temperature until there is vigorous boiling with condensation of sulfuric acid in the neck of the flask; precautions should be taken to prevent the upper part of the flask from becoming overheated. Continue the heating for 30 min, unless otherwise prescribed. Cool, dissolve the solid material by cautiously adding to the mixture 25 mL of *water R*, cool again and place in a steam-distillation apparatus. Add 30 mL of *strong sodium hydroxide solution R* and distil immediately by passing steam through the mixture. Collect about 40 mL of distillate in 20.0 mL of *0.01 M hydrochloric acid* and enough *water R* to cover the tip of the condenser. Towards the end of the distillation, lower the receiver so that the tip of the condenser is above the surface of the acid. Take precautions to prevent any water on the outer surface of the condenser from reaching the contents of the receiver. Titrate the distillate with *0.01 M sodium hydroxide*, using *methyl red mixed solution R* as indicator (n_1 mL of *0.01 M sodium hydroxide*).

Repeat the test using about 50 mg of *glucose R* in place of the substance to be examined (n_2 mL of *0.01 M sodium hydroxide*).

$$\text{Content of nitrogen} = \frac{0.01401(n_2 - n_1)}{m} \text{ per cent}$$

Method II (Determination of Protein in Blood Products)

(No Ph. Eur. method)

For dried blood products prepare a solution of the preparation as directed in the monograph.

To a volume expected to contain about 0.1 g of protein add sufficient *saline solution* to produce 20 mL. To 2 mL of the resulting solution, in a 75-mL boiling tube, add 2 mL of a solution containing 75% v/v of *nitrogen-free sulfuric acid*, 4.5% w/v of *potassium sulfate* and 0.5% w/v of *copper(II) sulfate*, mix and loosely stopper the tube. Heat gradually to boiling, boil vigorously for 1.5 hours and cool. If the solution is not clear add 0.25 mL of *hydrogen peroxide solution (20 vol)*, continue heating until a clear solution is produced and cool. During heating, take precautions to ensure that the upper part of the tube is not overheated.

Transfer the solution to a distillation apparatus using three 3-mL quantities of *water*, add 10 mL of 10M *sodium hydroxide* and distil rapidly for 4 minutes, collecting the distillate in a mixture of 5 mL of a saturated solution of *boric acid* and 5 mL of *water* and keeping the tip of the condenser below the level of the acid. Lower the collection flask so that the condenser can drain freely and continue the distillation for a further 1 minute. Titrate with 0.02M *hydrochloric acid VS* using *methyl red mixed solution* as indicator (V_1 mL).

To a further volume of the preparation being examined, or of the solution prepared from it, expected to contain about 0.1 g of protein, add 12 mL of *saline solution*, 2 mL of a 7.5% w/v solution of *sodium molybdate* and 2 mL of a mixture of 1 volume of *nitrogen-free sulfuric acid* and 30 volumes of *water*. Shake, allow to stand for 15 minutes, add sufficient *water* to produce 20 mL, shake again and centrifuge. Using 2 mL of the resulting clear supernatant liquid repeat the procedure described above beginning at the words 'in a 75-mL boiling tube...' (V_2 mL). Calculate the protein content in mg per mL of the preparation being examined, using the expression $6.25 \times 0.280(V_1 - V_2)$ and taking into account the initial dilution.

J. Tetrazolium Assay of Steroids

(No Ph. Eur. method)

The coloured reaction products tend to adsorb onto the surface of the glassware. To avoid low results, the glassware should be treated with the coloured reaction products before use. The treated glassware should be reserved for this assay and should be washed only with water between assays.

Carry out the following procedure protected from light. Dissolve a quantity of the substance being examined in sufficient *aldehyde-free absolute ethanol* to produce a solution containing 300 to 350 µg in 10 mL unless otherwise specified in the monograph. Transfer 10 mL to a 25-mL graduated flask, add 2 mL of *triphenyltetrazolium chloride solution*, displace the air in the flask with *oxygen-free nitrogen*, immediately add 2 mL of *dilute tetramethylammonium hydroxide solution* and again displace the air with *oxygen-free nitrogen*. Stopper the flask, mix the contents by gently swirling and allow to stand in a water bath at 30° for 1 hour unless otherwise specified in the monograph. Cool rapidly, add sufficient *aldehyde-free absolute ethanol* to produce 25 mL, mix and immediately determine the *absorbance* of the resulting solution in a stoppered cell at the maximum at 485 nm, Appendix II B, using in the reference cell a solution prepared at the same time and in the same manner using 10 mL of *aldehyde-free absolute ethanol*. Repeat the operation using the specified BPCRS or EPCRS in place of the substance being examined ensuring that the period of time that elapses between the addition of the tetramethylammonium hydroxide solution and the measurement of the absorbance is the same as for the test solution.

K. Ethylene Glycol and Diethylene Glycol in Ethoxylated Substances

(Ph. Eur. method 2.4.30)

Ethoxylated substances may contain varied amounts of ethylene glycol and diethylene glycol, as a result of the manufacturing process. The following method may be used for the quantitative determination of these substances, in particular in the case of the following surfactants: macrogolglycerol ricinoleate, macrogolglycerol hydroxystearate, macrogol 15 hydroxystearate, nonoxinol 9 and macrogol cetostearyl ether.

Gas chromatography (*2.2.28*).

Internal standard solution Dissolve 30.0 mg of *1,2-pentanediol R* in *acetone R* and dilute to 30.0 mL with the same solvent. Dilute 1.0 mL of this solution to 20.0 mL with *acetone R*.

Test solution Dissolve 0.500 g of the substance to be examined in the internal standard solution and dilute to 10.0 mL with the same solution.

Reference solution (a) Mix 30.0 mg of *ethylene glycol R* with *acetone R* and dilute to 100.0 mL with the same solvent. Dilute 1.0 mL to 10.0 mL with the internal standard solution.

Reference solution (b) Prepare a solution of *diethylene glycol R* with a concentration corresponding to the prescribed limit and using the same solvents as for the preparation of reference solution (a).

Column:
— *material*: fused silica,
— *size*: *l* = 30 m, Ø = 0.53 mm,
— *stationary phase*: *macrogol 20 000 R* (film thickness 1 µm).

Carrier gas *helium for chromatography R.*

Flow rate 10 mL/min.

Split ratio 1:3.

Temperature:

	Time (min)	Temperature (°C)
Column	0 - 40	80 → 200
	40 - 45	200 → 230
	45 - 65	230
Injection port		250
Detector		250

Detection Flame ionisation.

Injection 2 µL.

Relative retention With reference to 1,2-pentanediol (retention time = about 19 min): ethylene glycol = about 0.7; diethylene glycol = about 1.3.

L. Residual Solvents

(Ph. Eur. method 2.4.24)

The test procedures described in this general method may be used:

i. for the identification of the majority of Class 1 and Class 2 residual solvents in an active substance, excipient or medicinal product when the residual solvents are unknown;

ii. as a limit test for Class 1 and Class 2 solvents when present in an active substance, excipient or medicinal product;

iii. for the quantification of Class 2 solvents when the limits are greater than 1000 ppm (0.1 per cent) or for the quantification of Class 3 solvents when required.

Class 1, Class 2 and Class 3 residual solvents are listed in general chapter *5.4. Residual solvents*.

Three diluents are described for sample preparation and the conditions to be applied for head-space injection of the gaseous sample onto the chromatographic system.

Two chromatographic systems are prescribed but System A is preferred whilst System B is employed normally for confirmation of identity. The choice of sample preparation procedure depends on the solubility of the substance to be examined and in certain cases the residual solvents to be controlled.

The following residual solvents are not readily detected by the head-space injection conditions described: formamide, 2-ethoxyethanol, 2-methoxyethanol, ethylene glycol, *N*-methylpyrrolidone and sulfolane. Other appropriate procedures should be employed for the control of these residual solvents.

When the test procedure is applied quantitatively to control residual solvents in a substance, then it must be validated.

PROCEDURE

Examine by gas chromatography with static head-space injection (*2.2.28*).

Sample preparation 1

This is intended for the control of residual solvents in water-soluble substances.

Sample solution (1) Dissolve 0.200 g of the substance to be examined in *water R* and dilute to 20.0 mL with the same solvent.

Sample preparation 2

This is intended for the control of residual solvents in water-insoluble substances.

Sample solution (2) Dissolve 0.200 g of the substance to be examined in *dimethylformamide R* (DMF) and dilute to 20.0 mL with the same solvent.

Sample preparation 3

This is intended for the control of *N,N*-dimethylacetamide and/or *N,N*-dimethylformamide, when it is known or suspected that one or both of these substances are present in the substance to be examined.

Sample solution (3) Dissolve 0.200 g of the substance to be examined in *1,3-dimethyl-2-imidazolidinone R* (DMI) and dilute to 20.0 mL with the same solvent.

In some cases none of the above sample preparation procedures are appropriate, in which case the diluent to be used for the preparation of the sample solution and the static head-space conditions to be employed must be demonstrated to be suitable.

Solvent solution (a) To 1.0 mL of *Class 1 residual solvent solution CRS*, add 9 mL of *dimethyl sulfoxide R* and dilute to 100.0 mL with *water R*. Dilute 1.0 mL of this solution to 100 mL with *water R*. Dilute 1.0 mL of this solution to 10.0 mL with *water R*.

The reference solutions correspond to the following limits:
— benzene: 2 ppm;
— carbon tetrachloride: 4 ppm;
— 1,2-dichloroethane: 5 ppm;
— 1,1-dichloroethene: 8 ppm;
— 1,1,1-trichloroethane: 10 ppm.

Solvent solution (b) Dissolve appropriate quantities of the Class 2 residual solvents in *dimethyl sulfoxide R* and dilute to 100.0 mL with the same solvent. Dilute in *water R* to give a concentration of 1/20 of the limits stated in Table 2 (see *5.4. Residual solvents*).

Solvent solution (c) Dissolve 1.00 g of the solvent or solvents present in the substance to be examined in *dimethyl sulfoxide R* or *water R*, if appropriate, and dilute to 100.0 mL with *water R*. Dilute to give a concentration of 1/20 of the limit(s) stated in Table 1 or 2 (see *5.4. Residual solvents*).

Blank solution Prepare as described for solvent solution (c) but without the addition of solvent(s) (used to verify the absence of interfering peaks).

Test solution Introduce 5.0 mL of the sample solution and 1.0 mL of the blank solution into an injection vial.

Reference solution (a) (Class 1) Introduce 1.0 mL of solvent solution (a) and 5.0 mL of the appropriate diluent into an injection vial.

Reference solution (a_1) (Class 1) Introduce 5.0 mL of the sample solution and 1.0 mL of solvent solution (a) into an injection vial.

Reference solution (b) (Class 2) Introduce 1.0 mL of solvent solution (b) and 5.0 mL of the appropriate diluent into an injection vial.

Reference solution (c) Introduce 5.0 mL of the sample solution and 1.0 mL of solvent solution (c) into an injection vial.

Reference solution (d) Introduce 1.0 mL of the blank solution and 5.0 mL of the appropriate diluent into an injection vial.

Close the vials with a tight rubber membrane stopper coated with polytetrafluoroethylene and secure with an aluminium crimp cap. Shake to obtain a homogeneous solution.

The following static head-space injection conditions may be used:

	Sample preparation procedure		
Operating parameters	1	2	3
Equilibration temperature (°C)	80	105	80
Equilibration time (min)	60	45	45
Transfer-line temperature (°C)	85	110	105
Carrier gas: *nitrogen for chromatography R* or *helium for chromatography R* at an appropriate pressure			
Pressurisation time (s)	30	30	30
Injection volume (mL)	1	1	1

The chromatographic procedure may be carried out using:

SYSTEM A

- a fused-silica capillary or wide-bore column 30 m long and 0.32 mm or 0.53 mm in internal diameter coated with *cyanopropyl(3)phenyl(3)methyl(94)polysiloxane R* (film thickness 1.8 µm or 3 µm);
- *nitrogen for chromatography R* or *helium for chromatography R* as the carrier gas, split ratio 1:5 with a linear velocity of about 35 cm/s;
- a flame-ionisation detector (a mass spectrometer may also be used or an electron-capture detector for the chlorinated residual solvents of Class 1);

maintaining the temperature of the column at 40 °C for 20 min, then raising the temperature at a rate of 10 °C per min to 240 °C and maintaining it at 240 °C for 20 min and maintaining the temperature of the injection port at 140 °C and that of the detector at 250 °C; or, where there is interference from the matrix, use:

SYSTEM B

- a fused-silica capillary or wide-bore column 30 m long and 0.32 mm or 0.53 mm in internal diameter coated with *macrogol 20 000 R* (film thickness 0.25 µm);
- *nitrogen for chromatography R* or *helium for chromatography R* as the carrier gas, split ratio 1:5 with a linear velocity of about 35 cm/s;
- a flame-ionisation detector (a mass spectrophotometer may also be used or an electron-capture detector for the chlorinated residual solvents of Class 1);

maintaining the temperature of the column at 50 °C for 20 min, then raising the temperature at a rate of 6 °C per min to 165 °C and maintaining it at 165 °C for 20 min and maintaining the temperature of the injection port at 140 °C and that of the detector at 250 °C.

Inject 1 mL of the gaseous phase of reference solution (a) onto the column described in System A and record the chromatogram under such conditions that the signal-to-noise ratio for 1,1,1-trichloroethane can be measured. The signal-to-noise ratio must be at least 5. A typical chromatogram is shown in Figure 2.4.24.-1.

Inject 1 mL of the gaseous phase of reference solution (a_1) onto the column described in System A. The peaks due to the Class 1 residual solvents are still detectable.

Inject 1 mL of the gaseous phase of reference solution (b) onto the column described in System A and record the chromatogram under such conditions that the resolution between acetonitrile and dichloromethane can be determined. The system is suitable if the chromatogram obtained resembles the chromatogram shown in Figure 2.4.24.-2 and the resolution between acetonitrile and dichloromethane is at least 1.0.

Inject 1 mL of the gaseous phase of the test solution onto the column described in System A. If in the chromatogram obtained, there is no peak which corresponds to one of the residual solvent peaks in the chromatograms obtained with reference solution (a) or (b), then the substance to be examined meets the requirements of the test. If any peak in the chromatogram obtained with the test solution corresponds to any of the residual solvent peaks obtained with reference solution (a) or (b) then System B is to be employed.

Inject 1 mL of the gaseous phase of reference solution (a) onto the column described in System B and record the chromatogram under such conditions that the signal-to-noise ratio for benzene can be measured. The signal-to-noise ratio must be at least 5. A typical chromatogram is shown in Figure 2.4.24.-3.

Inject 1 mL of the gaseous phase of reference solution (a_1) onto the column described in System B. The peaks due to the Class 1 residual solvents are still detectable.

Inject 1 mL of the gaseous phase of reference solution (b) onto the column described in System B and record the chromatogram under such conditions that the resolution between acetonitrile and 1,1,2-trichloroethene can be determined. The system is suitable if the chromatogram obtained resembles the chromatogram shown in Figure 2.4.24.-4 and the resolution between acetonitrile and 1,1,2-trichloroethene is at least 1.0.

Inject 1 mL of the gaseous phase of the test solution onto the column described in System B. If in the chromatogram obtained, there is no peak which corresponds to any of the residual solvent peaks in the chromatogram obtained with the reference solution (a) or (b), then the substance to be examined meets the requirements of the test. If any peak in the chromatogram obtained with the test solution corresponds to any of the residual solvent peaks obtained with reference solution (a) or (b) and confirms the correspondence obtained when using System A, then proceed as follows.

Inject 1 mL of the gaseous phase of reference solution (c) onto the column described for System A or System B. If necessary, adjust the sensitivity of the system so that the height of the peak corresponding to the identified residual solvent(s) is at least 50 per cent of the full scale of the recorder.

Inject 1 mL of the gaseous phase of reference solution (d) onto the column. No interfering peaks should be observed.

Inject 1 mL of the gaseous phase of the test solution and 1 mL of the gaseous phase of reference solution (c) on to the column. Repeat these injections twice more.

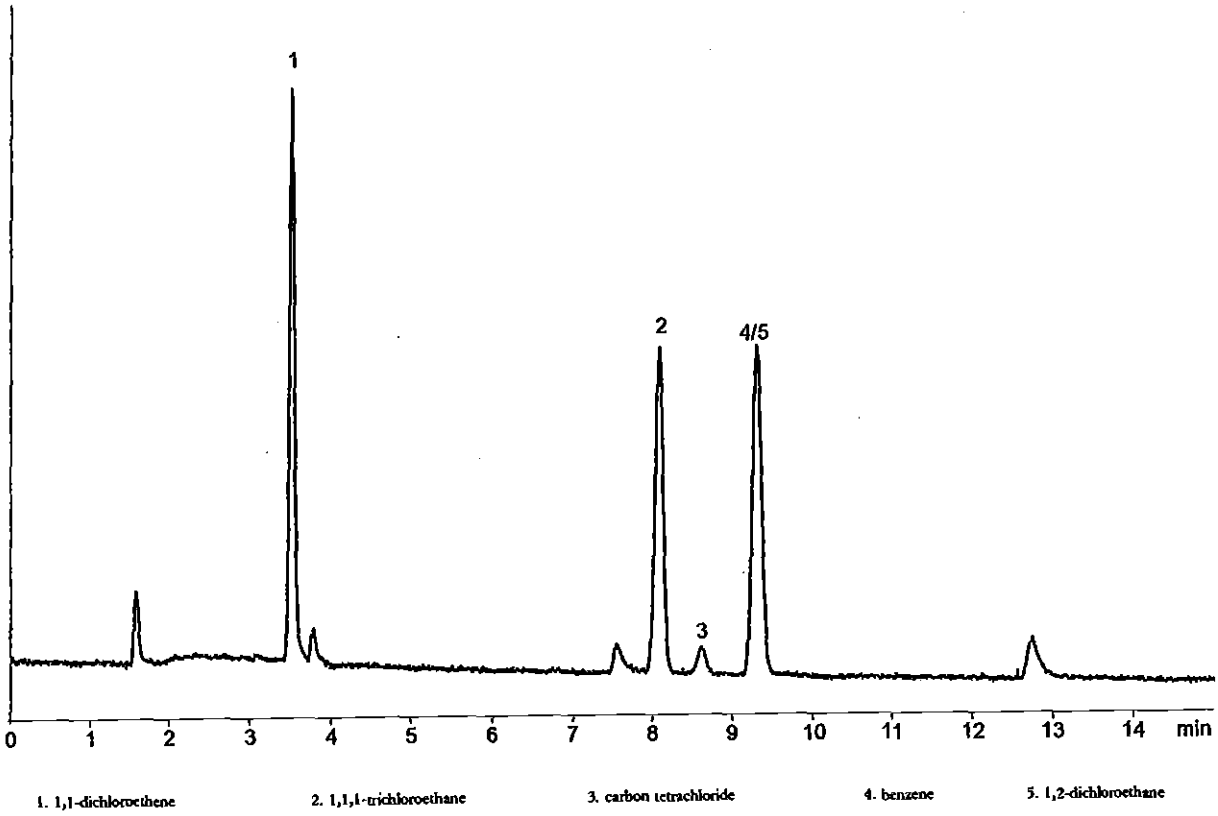


Figure 2.4.24.-1. – *Typical chromatogram of Class 1 solvents using the conditions described for System A and Procedure 1. Flame-ionisation detector*

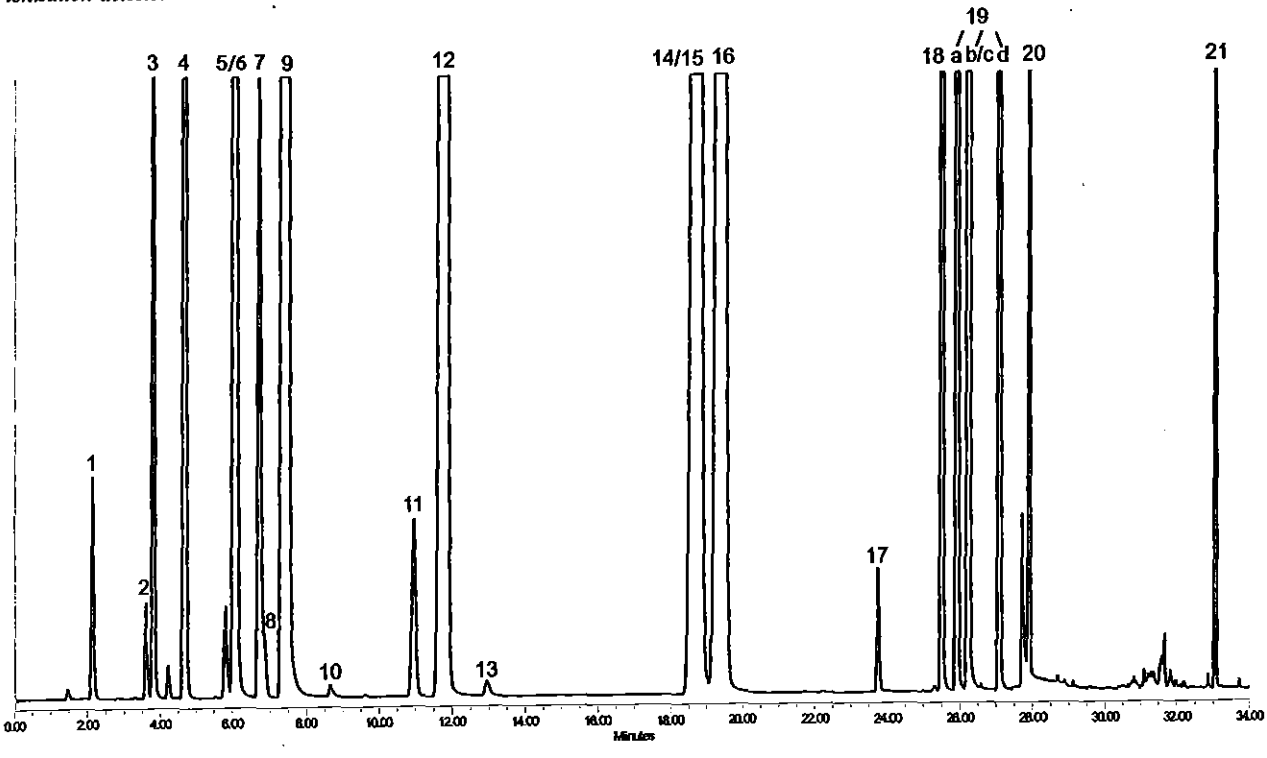


Figure 2.4.24.-2. – *Chromatogram of Class 2 solvents (solvent solution (b)) using the conditions described for System A and Procedure 1. Flame-ionisation detector*

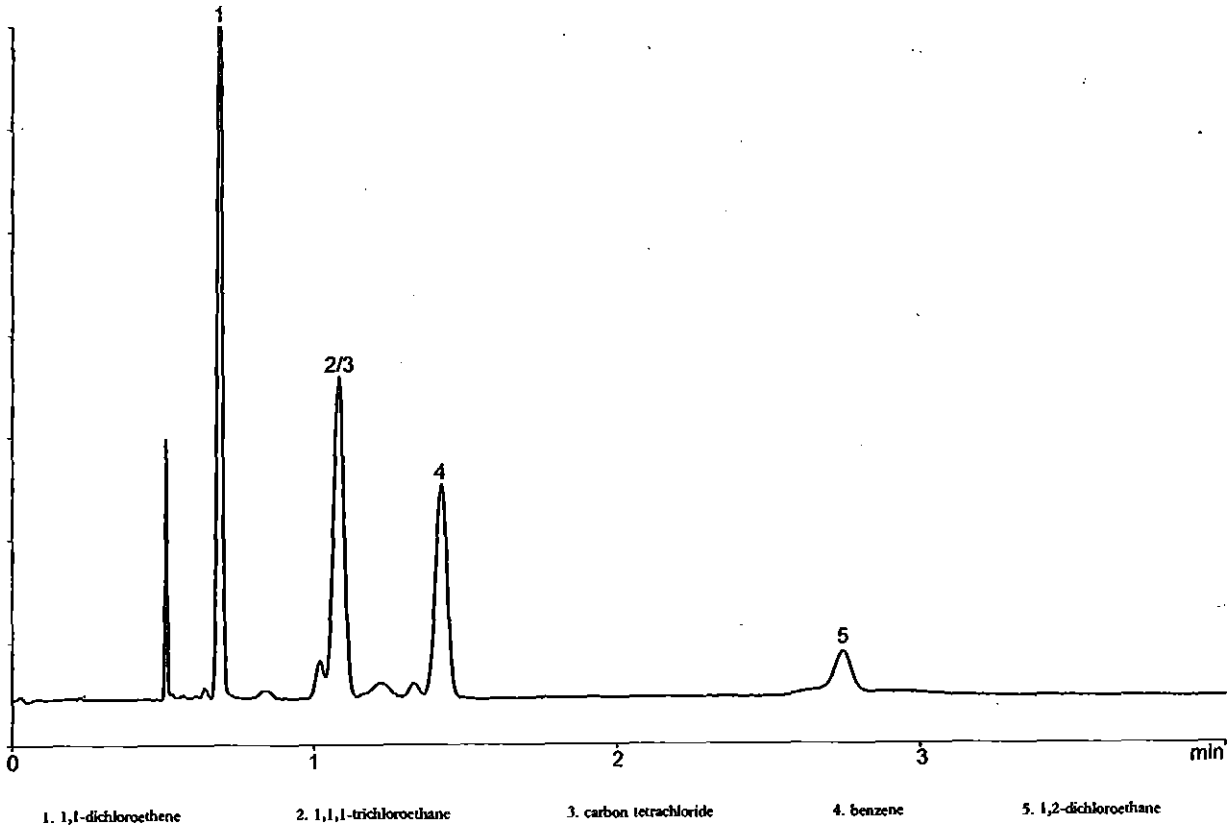


Figure 2.4.24.-3. – *Chromatogram of Class 1 residual solvents using the conditions described for System B and Procedure 1. Flame-ionisation detector*

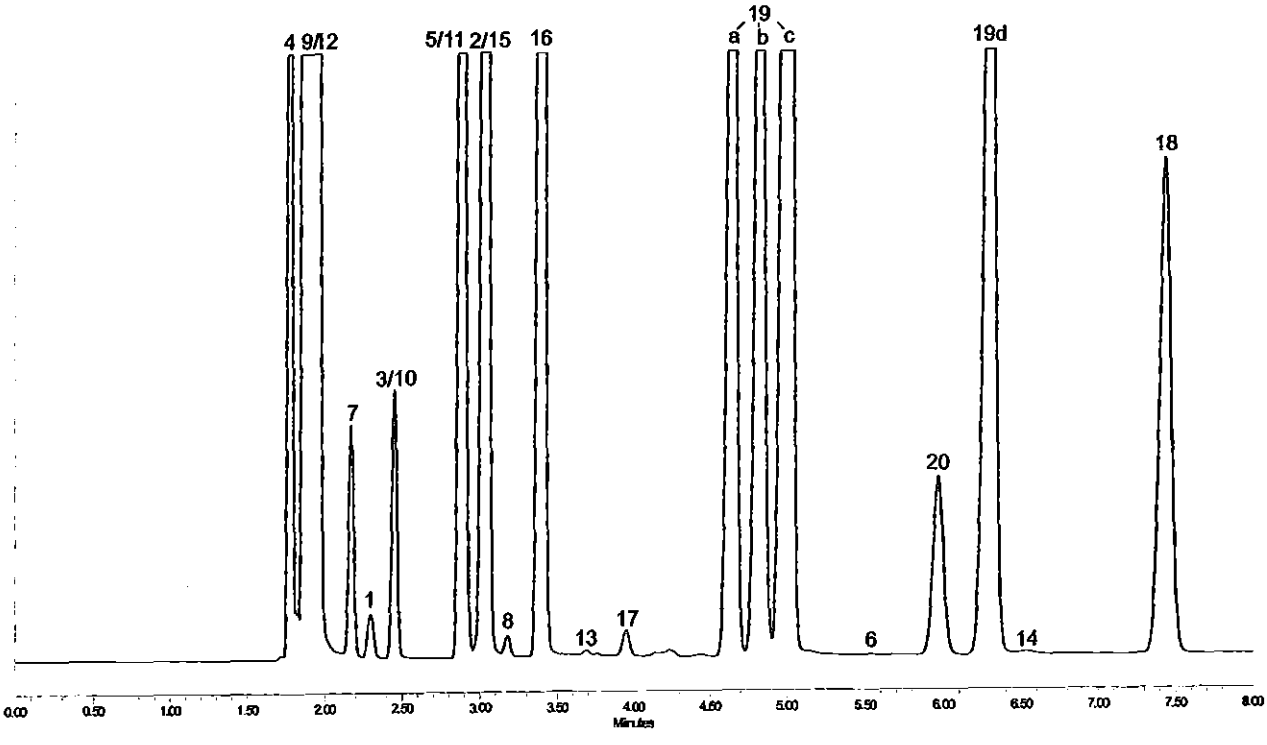


Figure 2.4.24.-4. – *Typical chromatogram of Class 2 residual solvents (solvent solution (b)) using the conditions described for System B and Procedure 1. Flame-ionisation detector*

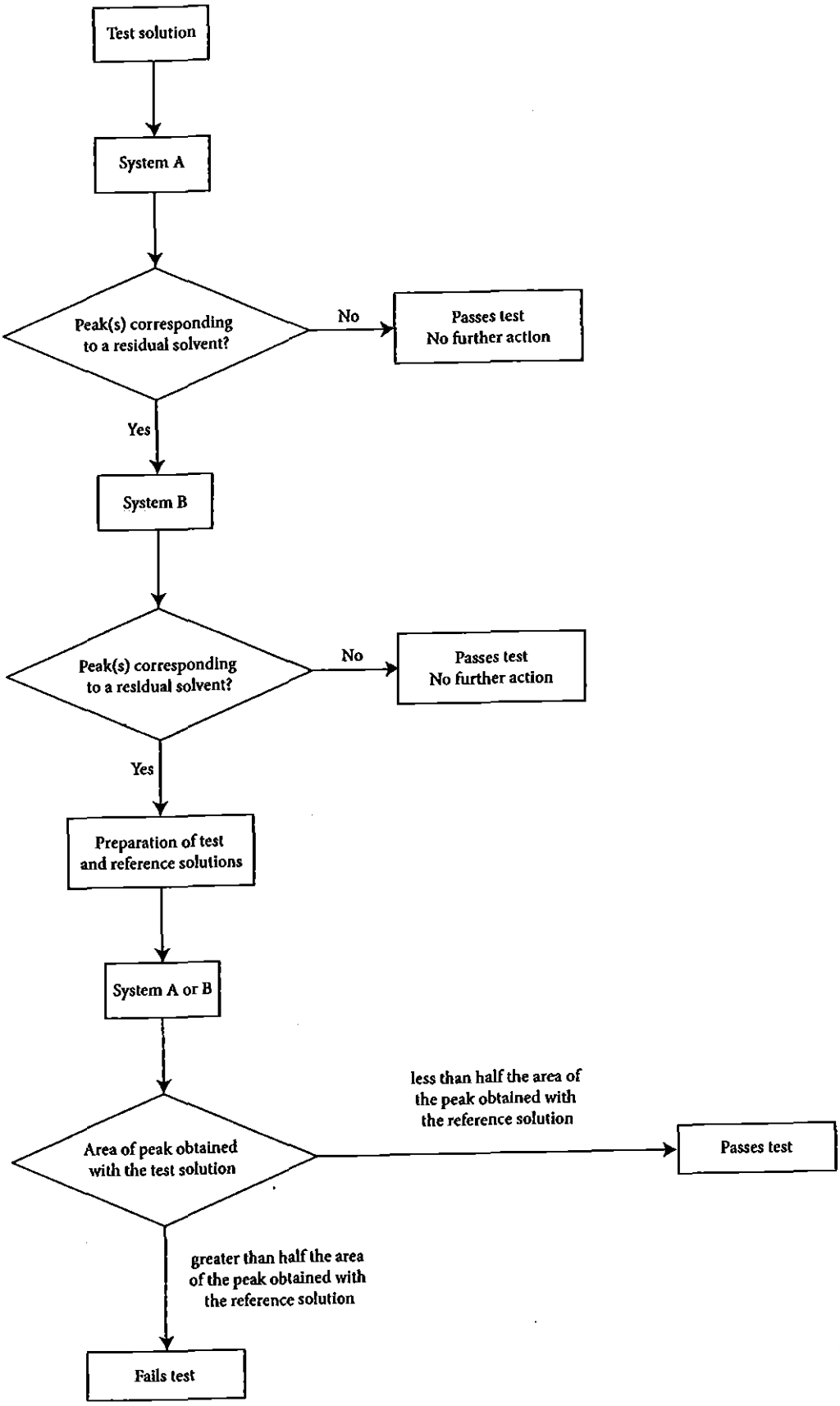


Figure 2.4.24.-5. – *Diagram relating to the identification of residual solvents and the application of limit tests*

The mean area of the peak of the residual solvent(s) in the chromatograms obtained with the test solution is not greater than half the mean area of the peak of the corresponding residual solvent(s) in the chromatograms obtained with reference solution (c). The test is not valid unless the relative standard deviation of the differences in areas between the analyte peaks obtained from 3 replicate paired injections of reference solution (c) and the test solution, is at most 15 per cent.

A flow diagram of the procedure is shown in Figure 2.4.24.-5.

When a residual solvent (Class 2 or Class 3) is present at a level of 0.1 per cent or greater then the content may be quantitatively determined by the method of standard additions.

M. Residual Ethylene Oxide and Dioxan

(Ph. Eur. method 2.4.25)

The test is intended for the determination of residual ethylene oxide and dioxan in samples soluble in water or dimethylacetamide. For substances that are insoluble or insufficiently soluble in these solvents, the preparation of the sample solution and the head-space conditions to be employed are given in the individual monograph.

Head-space gas chromatography (*2.2.28*).

A. For samples soluble in or miscible with water, the following procedure may be used.

Test solution Weigh 1.00 g (M_T) of the substance to be examined in a 10 mL vial (other sizes may be used depending on the operating conditions) and add 1.0 mL of *water R*. Close and mix to obtain a homogeneous solution. Allow to stand at 70 °C for 45 min.

Reference solution (a) Weigh 1.00 g (M_R) of the substance to be examined into an identical 10 mL vial, add 0.50 mL of *dioxan solution R2* and 0.50 mL of *ethylene oxide solution R3*. Close and mix to obtain a homogeneous solution. Allow to stand at 70 °C for 45 min.

Reference solution (b) To 0.50 mL of *ethylene oxide solution R3* in a 10 mL vial add 0.1 mL of a freshly prepared 10 mg/L solution of *acetaldehyde R* and 0.10 mL of *dioxan solution R1*. Close and mix to obtain a homogeneous solution. Allow to stand at 70 °C for 45 min.

B. For samples soluble in or miscible with dimethylacetamide, the following procedure may be used.

Test solution Weigh 1.00 g (M_T) of the substance to be examined in a 10 mL vial (other sizes may be used depending on the operating conditions) and add 0.20 mL of *water R* and 1.0 mL of *dimethylacetamide R*. Close and mix to obtain a homogeneous solution. Allow to stand at 90 °C for 45 min.

Reference solution (a) Weigh 1.00 g (M_R) of the substance to be examined into an identical 10 mL vial, add 0.10 mL of *dioxan solution R1*, 0.10 mL of *ethylene oxide solution R2* and 1.0 mL of *dimethylacetamide R*. Close and mix to obtain a homogeneous solution. Allow to stand at 90 °C for 45 min.

Reference solution (b) To 0.10 mL of *ethylene oxide solution R2* in a 10 mL vial, add 0.1 mL of a freshly prepared 10 mg/L solution of *acetaldehyde R* and 0.10 mL of *dioxan solution R1*. Close and mix to obtain a homogeneous solution. Allow to stand at 70 °C for 45 min.

Column:
- *material*: glass or fused silica;
- *size*: l = 30 m, Ø = 0.32 mm;
- *stationary phase*: *methylpolysiloxane R* (film thickness 1.0 µm).

Carrier gas *helium for chromatography R* or *nitrogen for chromatography R*.

Linear velocity 20 cm/s.

Split ratio 1:20.

Static head-space conditions that may be used:
- *equilibration temperature*: 70 °C (90 °C for solutions in dimethylacetamide);
- *equilibration time*: 45 min;
- *transfer-line temperature*: 75 °C (150 °C for solutions in dimethylacetamide);
- *carrier gas*: *helium for chromatography R*;
- *pressurisation time*: 1 min;
- *injection time*: 12 s.

Temperature:

	Time (min)	Temperature (°C)
Column	0 - 5	50
	5 - 31	50 → 180
	31 - 32.5	180 → 230
	32.5 - 37.5	230
Injection port		150
Detector		250

Detection Flame ionisation.

Injection A suitable volume, for example 1.0 mL, of the gaseous phase of the test solution and of reference solutions (a) and (b). Repeat the procedure twice more.

System suitability Reference solution (b):
- *resolution*: minimum 2.0 between the peaks due to acetaldehyde and ethylene oxide;
- *signal-to-noise ratio*: minimum 5 for the peaks due to ethylene oxide and dioxan.

Verification of precision

For each pair of injections, calculate for ethylene oxide and for dioxan the difference in area between the peaks obtained with the test solution and reference solution (a). The test is not valid unless the relative standard deviation of the 3 values obtained for ethylene oxide is not greater than 15 per cent and the relative standard deviation of the 3 values obtained for dioxan is not greater than 15 per cent. If the weighings used for the test solution and reference solution (a) differ from 1.00 g by more than 0.5 per cent, the appropriate corrections must be made.

Calculate the content of ethylene oxide or dioxan in parts per million from the following expressions:

$$\frac{A_T \times C}{(A_R \times M_T) - (A_T \times M_R)}$$

A_T = area of the peak due to ethylene oxide in the chromatogram obtained with the test solution;

A_R = area of the peak due to ethylene oxide in the chromatogram obtained with reference solution (a);

M_T = mass of the substance to be examined in the test solution, in grams;

M_R = mass of the substance to be examined in reference solution (a), in grams;

C = amount of ethylene oxide added to reference solution (a), in micrograms.

$$\frac{D_T \times C}{(D_R \times M_T) - (D_T \times M_R)}$$

D_T = area of the peak due to dioxan in the chromatogram obtained with the test solution;
D_R = area of the peak due to dioxan in the chromatogram obtained with reference solution (a);
C = amount of dioxan added to reference solution (a) in micrograms.

N. *N,N*-Dimethylaniline

(Ph. Eur. method 2.4.26)

METHOD A

Examine by gas chromatography (*2.2.28*), using *N,N-diethylaniline R* as the internal standard.

Internal standard solution Dissolve 50 mg of *N,N-diethylaniline R* in 4 mL of *0.1 M hydrochloric acid* and dilute to 50 mL with *water R*. Dilute 1 mL of this solution to 100 mL with *water R*.

Test solution Dissolve in a ground-glass-stoppered tube 0.50 g of the substance to be examined in 30.0 mL of *water R*. Add 1.0 mL of the internal standard solution. Adjust the solution to a temperature of 26-28 °C. Add 1.0 mL of *strong sodium hydroxide solution R* and mix until completely dissolved. Add 2.0 mL of *trimethylpentane R*. Shake for 2 min and allow the phases to separate. Use the upper layer.

Reference solution Dissolve 50.0 mg of *N,N-dimethylaniline R* in 4.0 mL of *0.1 M hydrochloric acid* and dilute to 50.0 mL with *water R*. Dilute 1.0 mL of this solution to 100.0 mL with *water R*. Dilute 1.0 mL of this solution to 30.0 mL with *water R*. Add 1.0 mL of the internal standard solution and 1.0 mL of *strong sodium hydroxide solution R*. Add 2.0 mL of *trimethylpentane R*. Shake for 2 min and allow the phases to separate. Use the upper layer.

The chromatographic procedure may be carried out using:
- a fused-silica capillary column 25 m long and 0.32 mm in internal diameter coated with *phenyl(50)methyl(50) polysiloxane R* (film thickness 0.52 µm),
- *helium for chromatography R* as the carrier gas with a split ratio 1:20, a column head pressure of 50 kPa and a split vent of 20 mL/min,
- a flame-ionisation detector,
- a split-liner consisting of a column about 1 cm long packed with *diatomaceous earth for gas chromatography R* impregnated with 10 per cent *m/m* of *methylpolysiloxane R*,

maintaining the temperature of the column at 150 °C for 5 min, then raising the temperature at a rate of 20 °C per min to 275 °C and maintaining it at 275 °C for 3 min and maintaining the temperature of the detector at 300 °C and that of the injection port at 220 °C.

The retention times are: *N,N*-dimethylaniline about 3.6 min, *N,N*-diethylaniline about 5.0 min.

Inject 1 µL of the test solution and 1 µL of the reference solution.

METHOD B

Examined by gas chromatography (*2.2.28*), using *naphthalene R* as the internal standard.

Internal standard solution Dissolve 50 mg of *naphthalene R* in *cyclohexane R* and dilute to 50 mL with the same solvent. Dilute 5 mL of this solution to 100 mL with *cyclohexane R*.

Test solution To 1.00 g of the substance to be examined in a ground-glass-stoppered tube add 5 mL of *1 M sodium hydroxide* and 1.0 mL of the internal standard solution. Stopper the tube and shake vigorously for 1 min. Centrifuge if necessary and use the upper layer.

Reference solution To 50.0 mg of *N,N-dimethylaniline R* add 2 mL of *hydrochloric acid R* and 20 mL of *water R*, shake to dissolve and dilute to 50.0 mL with *water R*. Dilute 5.0 mL of this solution to 250.0 mL with *water R*. To 1.0 mL of the latter solution in a ground-glass-stoppered tube add 5 mL of *1 M sodium hydroxide* and 1.0 mL of the internal standard solution. Stopper the tube and shake vigorously for 1 min. Centrifuge if necessary and use the upper layer.

The chromatographic procedure may be carried out using:
- a glass column 2 m long and 2 mm in internal diameter packed with *silanised diatomaceous earth for gas chromatography R* impregnated with 3 per cent *m/m* of *phenyl(50)methyl(50)polysiloxane R*,
- *nitrogen for chromatography R* as the carrier gas at a flow rate of 30 mL/min,
- a flame-ionisation detector,

maintaining the temperature of the column at 120 °C and that of the injection port and of the detector at 150 °C.

Inject 1 µL of the test solution and 1 µL of the reference solution.

O. 2-Ethylhexanoic Acid

(Ph. Eur. method 2.4.28)

Examine by gas chromatography (*2.2.28*), using *3-cyclohexylpropionic acid R* as the internal standard.

Internal standard solution Dissolve 100 mg of *3-cyclohexylpropionic acid R* in *cyclohexane R* and dilute to 100 mL with the same solvent.

Test solution To 0.300 g of the substance to be examined, add 4.0 mL of a 33 per cent *V/V* solution of *hydrochloric acid R*. Shake vigorously for 1 min with 1.0 mL of the internal standard solution. Allow the phases to separate (if necessary, centrifuge for a better separation). Use the upper layer.

Reference solution Dissolve 75.0 mg of *2-ethylhexanoic acid R* in the internal standard solution and dilute to 50.0 mL with the same solution. To 1.0 mL of the solution add 4.0 mL of a 33 per cent *V/V* solution of *hydrochloric acid R*. Shake vigorously for 1 min. Allow the phases to separate (if necessary, centrifuge for a better separation). Use the upper layer.

The chromatographic procedure may be carried out using:
- a wide-bore fused-silica column 10 m long and 0.53 mm in internal diameter coated with *macrogol 20 000 2-nitroterephthalate R* (film thickness 1.0 µm),
- *helium for chromatography R* as the carrier gas at a flow rate of 10 mL/min,
- a flame-ionisation detector,

with the following temperature programme:

	Time (min)	Temperature (°C)	Rate (°C/min)	Comment
Column	0 - 2	40	–	isothermal
	2 - 7.3	40 → 200	30	linear gradient
	7.3 - 10.3	200	–	isothermal
Injection port		200		
Detector		300		

Inject 1 µL of the test solution and 1 µL of the reference solution.

The test is not valid unless the resolution between the peaks due to 2-ethylhexanoic acid (first peak) and the internal standard is at least 2.0.

Calculate the percentage content of 2-ethylhexanoic acid from the expression:

$$\frac{A_T \times I_R \times m_R \times 2}{A_R \times I_T \times m_T}$$

A_T = area of the peak due to 2-ethylhexanoic acid in the chromatogram obtained with the test solution,

A_R = area of the peak due to 2-ethylhexanoic acid in the chromatogram obtained with the reference solution,

I_T = area of the peak due to the internal standard in the chromatogram obtained with the test solution,

I_R = area of the peak due to the internal standard in the chromatogram obtained with the reference solution,

m_T = mass of the substance to be examined in the test solution, in grams,

m_R = mass of 2-ethylhexanoic acid in the reference solution, in grams.

P. Total Protein

(Ph. Eur. method 2.5.33)

Many of the assay methods described in this chapter can be performed using kits from commercial sources.

METHOD 1

Protein in solution absorbs ultraviolet light at a wavelength of 280 nm, due to the presence of aromatic amino acids, mainly tyrosine and tryptophan, in the protein structure. This property can be used for assay purposes. If the buffer used to dissolve the protein has a high absorbance relative to that of water, an interfering substance is present. This interference may be obviated by using the buffer as compensation liquid but if the interfering substance produces a high absorbance, the results may nevertheless be compromised. At low concentrations, protein adsorbed onto the cell may significantly reduce the content in solution. This can be prevented by preparing samples at higher concentration or by using a non-ionic detergent in the preparation.

Test solution Dissolve a suitable quantity of the substance to be examined in the prescribed buffer to obtain a solution having a protein concentration between 0.2 mg/mL and 2 mg/mL.

Reference solution Prepare a solution of a suitable reference substance for the protein to be determined, in the same buffer and at the same protein concentration as the test solution.

Procedure

Keep the test solution, the reference solution and the compensation liquid at the same temperature during the performance of this test. Determine the absorbances (*2.2.25*) of the test solution and the reference solution in quartz cells at 280 nm, using the prescribed buffer as the compensation liquid. The response must be linear in the range of protein concentrations to be assayed to obtain accurate results.

Light scattering

The accuracy of the determination of protein can be diminished by the scattering of light by the test sample. If the proteins in solution exist as particles comparable in size to the wavelength of the measuring light (250 nm to 300 nm), scattering of the light beam results in an apparent increase in absorbance of the test sample. To calculate the absorbance at 280 nm due to light scattering, determine the absorbances of the test solution at wavelengths of 320 nm, 325 nm, 330 nm, 335 nm, 340 nm, 345 nm and 350 nm. Plot the logarithm of the observed absorbance against the logarithm of the wavelength and determine the standard curve best fitting the plotted points by linear regression. Extrapolate the curve to determine the logarithm of the absorbance at 280 nm. The antilogarithm of this value is the absorbance attributed to light scattering. Correct the observed values by subtracting the absorbance attributed to light scattering from the total absorbance at 280 nm to obtain the absorbance value of the protein in solution. Filtration with a 0.2 µm filter that does not adsorb protein or clarification by centrifugation may be performed to reduce the effect of light scattering, especially if the solution is noticeably turbid.

Calculations

Use corrected values for the calculations. Calculate the concentration of protein in the test solution (C_U) from the following equation:

$$C_U = C_S(A_U/A_S)$$

where C_S is the concentration of protein in the reference solution and A_U and A_S are the corrected absorbances of the test solution and the reference solution, respectively.

METHOD 2

This method (commonly referred to as the Lowry assay) is based on the reduction by protein of the phosphomolybdotungstic mixed acid chromogen in the phosphomolybdotungstic reagent, which results in an absorbance maximum at 750 nm.

The phosphomolybdotungstic reagent reacts primarily with tyrosine residues in the protein. Colour development reaches a maximum in 20 min to 30 min at room temperature, after which there is a gradual loss of colour. Because the method is sensitive to interfering substances, a procedure for precipitation of the protein from the test sample may be used. Most interfering substances cause a lower colour yield; however, some detergents cause a slight increase in colour. A high salt concentration may cause a precipitate to form. Because different protein species may give different colour response intensities, the reference substance and test protein must be the same. Where separation of interfering substances from the protein in the test sample is necessary, proceed as directed below for interfering substances prior to preparation of the test solution. The effect of interfering substances may be minimised by dilution, provided the concentration of the test protein remains sufficient for accurate measurement.

Use *distilled water R* to prepare all buffers and reagents used for this method.

Test solution Dissolve a suitable quantity of the substance to be examined in the prescribed buffer to obtain a solution having a concentration within the range of the standard curve. A suitable buffer will produce a solution of pH 10.0 to 10.5.

Reference solutions Dissolve the reference substance for the protein to be determined in the prescribed buffer. Dilute portions of this solution with the same buffer to obtain not fewer than five reference solutions having protein concentrations evenly spaced over a suitable range situated between 5 µg/mL and 100 µg/mL.

Blank Use the buffer used to prepare the test solution and the reference solutions.

Copper sulfate reagent Dissolve 100 mg of *copper sulfate pentahydrate R* and 0.2 g of *sodium tartrate R* in *distilled*

water R and dilute to 50 mL with the same solvent. Dissolve 10 g of *anhydrous sodium carbonate R* in *distilled water R* and dilute to 50 mL with the same solvent. Slowly pour the sodium carbonate solution into the copper sulfate solution with mixing. Use within 24 h.

Alkaline copper reagent Mix 1 volume of copper sulfate reagent, 2 volumes of a 50 g/L solution of *sodium dodecyl sulfate R* and 1 volume of a 32 g/L solution of *sodium hydroxide R*. Store at room temperature and use within 2 weeks.

Diluted phosphomolybdotungstic reagent Mix 5 mL of *phosphomolybdotungstic reagent R* with 55 mL of *distilled water R*. Store in an amber bottle, at room temperature.

Procedure

To 1.0 mL of each reference solution, of the test solution and of the blank, add 1.0 mL of alkaline copper reagent and mix. Allow to stand for 10 min. Add 0.5 mL of the diluted phosphomolybdotungstic reagent, mix and allow to stand at room temperature for 30 min. Determine the absorbances (*2.2.25*) of the solutions at 750 nm, using the solution from the blank as compensation liquid.

Calculations

The relationship of absorbance to protein concentration is non-linear; however, if the range of concentrations used to prepare the standard curve is sufficiently small, the latter will approach linearity. Plot the absorbances of the reference solutions against the protein concentrations and use linear regression to establish the standard curve. From the standard curve and the absorbance of the test solution, determine the concentration of protein in the test solution.

Interfering substances

In the following procedure, deoxycholate-trichloroacetic acid is added to a test sample to remove interfering substances by precipitation of proteins before determination; this technique can also be used to concentrate proteins from a dilute solution.

Add 0.1 mL of a 1.5 g/L solution of *sodium deoxycholate R* to 1 mL of a solution of the substance to be examined. Mix using a vortex mixer and allow to stand at room temperature for 10 min. Add 0.1 mL of a 720 g/L solution of *trichloroacetic acid R* and mix using a vortex mixer. Centrifuge at 3000 *g* for 30 min, decant the liquid and remove any residual liquid with a pipette. Redissolve the protein pellet in 1 mL of alkaline copper reagent.

METHOD 3

This method (commonly referred to as the Bradford assay) is based on the absorption shift from 470 nm to 595 nm observed when the acid blue 90 dye binds to protein. The acid blue 90 dye binds most readily to arginine and lysine residues in the protein which can lead to variation in the response of the assay to different proteins. The protein used as reference substance must therefore be the same as the protein to be determined. There are relatively few interfering substances, but it is preferable to avoid detergents and ampholytes in the test sample. Highly alkaline samples may interfere with the acidic reagent.

Use *distilled water R* to prepare all buffers and reagents used for this method.

Test solution Dissolve a suitable quantity of the substance to be examined in the prescribed buffer to obtain a solution having a concentration within the range of the standard curve.

Reference solutions Dissolve the reference substance for the protein to be determined in the prescribed buffer. Dilute portions of this solution with the same buffer to obtain not fewer than five reference solutions having protein concentrations evenly spaced over a suitable range situated between 0.1 mg/mL and 1 mg/mL.

Blank Use the buffer used to prepare the test solution and the reference solutions.

Acid blue 90 reagent Dissolve 0.10 g of *acid blue 90 R* in 50 mL of *alcohol R*. Add 100 mL of *phosphoric acid R*, dilute to 1000 mL with *distilled water R* and mix. Filter the solution and store in an amber bottle at room temperature. Slow precipitation of the dye occurs during storage. Filter the reagent before using.

Procedure

Add 5 mL of acid blue 90 reagent to 0.100 mL of each reference solution, of the test solution and of the blank. Mix by inversion. Avoid foaming, which will lead to poor reproducibility. Determine the absorbances (*2.2.25*) of the standard solutions and of the test solution at 595 nm, using the blank as compensation liquid. Do not use quartz (silica) spectrophotometer cells because the dye binds to this material.

Calculations

The relationship of absorbance to protein concentration is non-linear; however, if the range of concentrations used to prepare the standard curve is sufficiently small, the latter will approach linearity. Plot the absorbances of the reference solutions against protein concentrations and use linear regression to establish the standard curve. From the standard curve and the absorbance of the test solution, determine the concentration of protein in the test solution.

METHOD 4

This method (commonly referred to as the bicinchoninic acid or BCA assay) is based on reduction of the cupric (Cu^{2+}) ion to cuprous (Cu^{1+}) ion by protein. The bicinchoninic acid reagent is used to detect the cuprous ion. Few substances interfere with the reaction. When interfering substances are present their effect may be minimised by dilution, provided that the concentration of the protein to be determined remains sufficient for accurate measurement. Alternatively, the protein precipitation procedure given in Method 2 may be used to remove interfering substances. Because different protein species may give different colour response intensities, the reference protein and protein to be determined must be the same.

Use *distilled water R* to prepare all buffers and reagents used for this method.

Test solution Dissolve a suitable quantity of the substance to be examined in the prescribed buffer to obtain a solution having a concentration within the range of the concentrations of the reference solutions.

Reference solutions Dissolve the reference substance for the protein to be determined in the prescribed buffer. Dilute portions of this solution with the same buffer to obtain not fewer than five reference solutions having protein concentrations evenly spaced over a suitable range situated between 10 µg/mL and 1200 µg/mL.

Blank Use the buffer used to prepare the test solution and the reference solutions.

BCA reagent Dissolve 10 g of *disodium bicinchoninate R*, 20 g of *sodium carbonate monohydrate R*, 1.6 g of *sodium tartrate R*, 4 g of *sodium hydroxide R*, and 9.5 g of *sodium hydrogen carbonate R* in *distilled water R*. Adjust, if necessary, to pH 11.25 with a solution of *sodium hydroxide R* or a solution of *sodium hydrogen carbonate R*. Dilute to 1000 mL with *distilled water R* and mix.

Copper-BCA reagent Mix 1 mL of a 40 g/L solution of *copper sulfate pentahydrate R* and 50 mL of BCA reagent.

Procedure

Mix 0.1 mL of each reference solution, of the test solution and of the blank with 2 mL of the copper-BCA reagent. Incubate the solutions at 37 °C for 30 min, note the time and allow the mixtures to cool to room temperature. Within 60 min of the end of incubation, determine the absorbances (*2.2.25*) of the reference solutions and of the test solution in quartz cells at 562 nm, using the blank as compensation liquid. After the solutions have cooled to room temperature, the colour intensity continues to increase gradually.

Calculations

The relationship of absorbance to protein concentration is non-linear; however, if the range of concentrations used to prepare the standard curve is sufficiently small, the latter will approach linearity. Plot the absorbances of the reference solutions against protein concentrations and use linear regression to establish the standard curve. From the standard curve and the absorbance of the test solution, determine the concentration of protein in the test solution.

METHOD 5

This method (commonly referred to as the biuret assay) is based on the interaction of cupric (Cu^{2+}) ion with protein in alkaline solution and resultant development of absorbance at 545 nm. This test shows minimal difference between equivalent IgG and albumin samples. Addition of the sodium hydroxide and the biuret reagent as a combined reagent, insufficient mixing after the addition of the sodium hydroxide, or an extended time between the addition of the sodium hydroxide solution and the addition of the biuret reagent will give IgG samples a higher response than albumin samples. The trichloroacetic acid method used to minimise the effects of interfering substances also can be used to determine the protein content in test samples at concentrations below 500 µg/mL.

Use *distilled water R* to prepare all buffers and reagents used for this method.

Test solution Dissolve a suitable quantity of the substance to be examined in a 9 g/L solution of *sodium chloride R* to obtain a solution having a concentration within the range of the concentrations of the reference solutions.

Reference solutions Dissolve the reference substance for the protein to be determined in a 9 g/L solution of *sodium chloride R*. Dilute portions of this solution with a 9 g/L solution of *sodium chloride R* to obtain not fewer than three reference solutions having protein concentrations evenly spaced over a suitable range situated between 0.5 mg/mL and 10 mg/mL.

Blank Use a 9 g/L solution of *sodium chloride R*.

Biuret reagent Dissolve 3.46 g of *copper sulfate pentahydrate R* in 10 mL of hot *distilled water R*, and allow to cool (Solution A). Dissolve 34.6 g of *sodium citrate R* and 20.0 g of *anhydrous sodium carbonate R* in 80 mL of hot *distilled water R*, and allow to cool (Solution B).
Mix solutions A and B and dilute to 200 mL with *distilled water R*. Use within 6 months. Do not use the reagent if it develops turbidity or contains any precipitate.

Procedure

To one volume of the test solution add an equal volume of a 60 g/L solution of *sodium hydroxide R* and mix. Immediately add biuret reagent equivalent to 0.4 volumes of the test solution and mix rapidly. Allow to stand at a temperature between 15 °C and 25 °C for not less than 15 min. Within 90 min of addition of the biuret reagent, determine the absorbances (*2.2.25*) of the reference solutions and of the test solution at the maximum at 545 nm, using the blank as compensation liquid. Any solution that develops turbidity or a precipitate is not acceptable for calculation of protein concentration.

Calculations

The relationship of absorbance to protein concentration is approximately linear within the indicated range of protein concentrations for the reference solutions. Plot the absorbances of the reference solutions against protein concentrations and use linear regression to establish the standard curve. Calculate the correlation coefficient for the standard curve. A suitable system is one that yields a line having a correlation coefficient not less than 0.99. From the standard curve and the absorbance of the test solution, determine the concentration of protein in the test solution.

Interfering substances

To minimise the effect of interfering substances, the protein can be precipitated from the test sample as follows: add 0.1 volumes of a 500 g/L solution of *trichloroacetic acid R* to 1 volume of a solution of the test sample, withdraw the supernatant layer and dissolve the precipitate in a small volume of *0.5 M sodium hydroxide*. Use the solution obtained to prepare the test solution.

METHOD 6

This fluorimetric method is based on the derivatisation of the protein with *o*-phthalaldehyde, which reacts with the primary amines of the protein (*N*-terminal amino acid and the ε-amino group of lysine residues). The sensitivity of the assay can be increased by hydrolysing the protein before adding *o*-phthalaldehyde. Hydrolysis makes the α-amino group of the constituent amino acids available for reaction with the phthalaldehyde reagent. The method requires very small quantities of the protein. Primary amines, such as tris (hydroxymethyl)aminomethane and amino acid buffers, react with phthalaldehyde and must be avoided or removed. Ammonia at high concentrations reacts with phthalaldehyde. The fluorescence obtained when amine reacts with phthalaldehyde can be unstable. The use of automated procedures to standardise this procedure may improve the accuracy and precision of the test.

Use *distilled water R* to prepare all buffers and reagents used for this method.

Test solution Dissolve a suitable quantity of the substance to be examined in a 9 g/L solution of *sodium chloride R* to obtain a solution having a concentration within the range of the concentrations of the reference solutions. Adjust the solution to pH 8 to 10.5 before addition of the phthalaldehyde reagent.

Reference solutions Dissolve the reference substance for the protein to be determined in a 9 g/L solution of *sodium chloride R*. Dilute portions of this solution with a 9 g/L solution of *sodium chloride R* to obtain not fewer than five reference solutions having protein concentrations evenly spaced over a suitable range situated between 10 µg/mL and 200 µg/mL. Adjust the solutions to pH 8 to 10.5 before addition of the phthalaldehyde reagent.

Blank solution Use a 9 g/L solution of *sodium chloride R*.

Borate buffer solution Dissolve 61.83 g of *boric acid R* in *distilled water R* and adjust to pH 10.4 with a solution of *potassium hydroxide R*. Dilute to 1000 mL with *distilled water R* and mix.

Phthalaldehyde stock solution Dissolve 1.20 g of *phthalaldehyde R* in 1.5 mL of *methanol R*, add 100 mL of borate buffer solution and mix. Add 0.6 mL of a 300 g/L solution of *macrogol 23 lauryl ether R* and mix. Store at room temperature and use within 3 weeks.

Phthalaldehyde reagent To 5 mL of phthalaldehyde stock solution add 15 µL of *2-mercaptoethanol R*. Prepare at least 30 min before use. Use within 24 h.

Procedure

Mix 10 µL of the test solution and of each of the reference solutions with 0.1 mL of phthalaldehyde reagent and allow to stand at room temperature for 15 min. Add 3 mL of *0.5 M sodium hydroxide* and mix. Determine the fluorescent intensities (*2.2.21*) of solutions from the reference solutions and from the test solution at an excitation wavelength of 340 nm and an emission wavelength between 440 and 455 nm. Measure the fluorescent intensity of a given sample only once, since irradiation decreases the fluorescence intensity.

Calculations

The relationship of fluorescence to protein concentration is linear. Plot the fluorescent intensities of the reference solutions against protein concentrations and use linear regression to establish the standard curve. From the standard curve and the fluorescent intensity of the test solution, determine the concentration of protein in the test solution.

METHOD 7

This method is based on nitrogen analysis as a means of protein determination. Interference caused by the presence of other nitrogen-containing substances in the test sample can affect the determination of protein by this method. Nitrogen analysis techniques destroy the test sample during the analysis but are not limited to protein presentation in an aqueous environment.

Procedure A

Proceed as prescribed for the determination of nitrogen by sulfuric acid digestion (*2.5.9*) or use commercial instrumentation for Kjeldahl nitrogen assay.

Procedure B

Commercial instrumentation is available for nitrogen analysis. Most nitrogen analysis instruments use pyrolysis (i.e. combustion of the sample in oxygen at temperatures approaching 1000 °C), which produces nitric oxide (NO) and other oxides of nitrogen (NO_x) from the nitrogen present in the substance to be examined. Some instruments convert the nitric oxides to nitrogen gas, which is quantified using a thermal-conductivity detector. Other instruments mix nitric oxide (NO) with ozone (O_3) to produce excited nitrogen dioxide (NO_2*), which emits light when it decays and can be quantified with a chemiluminescence detector. A protein reference material that is relatively pure and is similar in composition to the test proteins is used to optimise the injection and pyrolysis parameters and to evaluate consistency in the analysis.

Calculations

The protein concentration is calculated by dividing the nitrogen content of the sample by the known nitrogen content of the protein. The known nitrogen content of the protein can be determined from the chemical composition of the protein or by comparison with a suitable reference substance.

Q. Acetic Acid in Synthetic Peptides

(Ph. Eur. method 2.5.34)

Examine by liquid chromatography (*2.2.29*).

Test solution Prepare as described in the monograph. The concentration of peptide in the solution may be adapted, depending on the expected amount of acetic acid in the sample.

Reference solution Prepare a 0.10 g/L solution of *glacial acetic acid R* in a mixture of 5 volumes of mobile phase B and 95 volumes of mobile phase A.

The chromatographic procedure may be carried out using:
- a stainless steel column 0.25 m long and 4.6 mm in internal diameter packed with *octadecylsilyl silica gel for chromatography R* (5 µm),
- as mobile phase at a flow rate of 1.2 mL/min:

Mobile phase A Dilute 0.7 mL of *phosphoric acid R* to 1000 mL with *water R*; adjust the pH to 3.0 with *strong sodium hydroxide solution R*,

Mobile phase B *Methanol R2*,

Time (min)	Mobile phase A (per cent *V/V*)	Mobile phase B (per cent *V/V*)
0 - 5	95	5
5 - 10	95 → 50	5 → 50
10 - 20	50	50
20 - 22	50 → 95	50 → 5
22 - 30	95	5

- as detector a spectrophotometer set at 210 nm.

Inject 10 µL of the reference solution and 10 µL of the test solution. In the chromatograms obtained, the peak corresponding to acetic acid has a retention time of 3-4 min. The baseline presents a steep rise after the start of the linear gradient, which corresponds to the elution of the peptide from the column. Determine the content of acetic acid in the peptide.

R. Nickel in Hydrogenated Vegetable Oils

(Ph. Eur. method 2.4.31)

Atomic absorption spectrometry (*2.2.23, Method I*).

The reagents magnesium nitrate R and ammonium dihydrogen phosphate R must be controlled for nickel before use. The actual nickel content is taken into account in the calculation of the nickel content of the sample.

Test solution Weigh 0.250 g (*m*) of the substance to be examined into a suitable high-pressure-resistant digestion vessel (fluoropolymer or quartz glass), add 6.0 mL of *nickel-free nitric acid R* and 2.0 mL of *strong hydrogen peroxide solution R*. Prepare a blank solution in the same manner. Place the closed vessels in a laboratory microwave oven and digest with an appropriate programme, e.g. 1000 W for 40 min. Allow the digestion vessels to cool before opening. Add 2.0 mL of *strong hydrogen peroxide solution R* and repeat the digestion step. Allow the digestion vessels to cool before opening. Quantitatively transfer to a 25 mL flask, add 0.5 mL of a 10 g/L solution of *magnesium nitrate R* and 0.5 mL of a 100 g/L solution of *ammonium dihydrogen phosphate R*, dilute to 25.0 mL with *water for chromatography R* and mix.

Reference solutions Into 4 volumetric flasks, introduce 25 µL, 50 µL, 75 µL and 100 µL of *nickel standard solution (5 ppm Ni) R*. To each flask, add 0.5 mL of a 10 g/L solution of *magnesium nitrate R*, 0.5 mL of a 100 g/L solution of *ammonium dihydrogen phosphate R* and 6.0 mL of *nickel-free nitric acid R* and dilute to 25.0 mL with *water for chromatography R*. Mix to obtain reference solutions containing respectively 5 ng/mL, 10 ng/mL, 15 ng/mL and 20 ng/mL (ppb) of nickel.

Zero solution In a volumetric flask, introduce 1.0 mL of a 10 g/L solution of *magnesium nitrate R*, 1.0 mL of a 100 g/L solution of *ammonium dihydrogen phosphate R* and 12.0 mL of *nickel-free nitric acid R*. Dilute to 50.0 mL with *water for chromatography R* and mix.

Method Determine the absorbance of each solution at 232.0 nm using a suitable graphite furnace atomic absorption (GFAA) spectrometer equipped with a background compensation system, a pyrolytically-coated tube and a nickel hollow-cathode lamp. The optimal temperature programme may be different for each instrument, so the programme recommended by the spectrometer manufacturer may be used. The following temperature parameters for GFAA analysis are given as an example: maintain the drying temperature of the furnace at 120 °C for 35 s after a 5 s ramp, the ashing temperature at 1100 °C for 10 s after a 30 s ramp, the cooling temperature at 800 °C for 5 s after a 5 s decrease, and the atomisation temperature at 2600 °C for 7 s. Use the zero solution to set the instrument to zero. Using the calibration curve, determine the concentrations of the test solution and the blank solution from the corresponding absorptions. If necessary, dilute with the zero solution to obtain a reading within the calibrated absorbance range.

Calculate the content of Ni in micrograms per gram (ppm) using the following expression:

$$\frac{c \times f}{m \times 40}$$

c = measured concentration of Ni, in nanograms per millilitre;
f = dilution factor of the test solution;
m = mass of the substance to be examined, in grams.

S. Methyl, Ethyl and Isopropyl Methanesulfonate in Methanesulfonic Acid

(Ph. Eur. method 2.5.37)

The following method has been validated for the methyl, ethyl and isopropyl esters of methanesulfonic acid at concentrations in the range of 0.5 ppm to 100 ppm.

If it is intended to be used to determine levels of methanesulfonic acid esters outside this validated range, for example in early steps of the synthesis prior to their removal, the concentration of the test solution has to be adjusted accordingly.

Gas chromatography (*2.2.28*) coupled with mass spectrometry (*2.2.43*).

Internal standard solution Dilute 7 µL of *butyl methanesulfonate CRS* (BMS) to 10.0 mL with *methylene chloride R*. Dilute 10 µL of the solution to 100.0 mL with *methylene chloride R*.

Test solution Add 0.74 g of the substance to be examined to 10.0 mL of *water R* and extract with 10.0 mL of the internal standard solution. Allow to separate and transfer the organic layer to a vial containing *anhydrous sodium sulfate R*. Shake and filter.

Reference solution (a) Dissolve 50 mg each of *methyl methanesulfonate R* (MMS), *ethyl methanesulfonate R* (EMS) and *isopropyl methanesulfonate R* (IMS) in the internal standard solution and dilute to 50.0 mL with the same solution. Dilute 74 µL of the solution to 10.0 mL with the internal standard solution. Dilute 100 µL of this solution to 10.0 mL with the internal standard solution.

Reference solution (b) Dilute 3.0 mL of reference solution (a) to 10.0 mL with the internal standard solution.

Column:
- *material*: fused silica;
- *size*: l = 15 m, Ø = 0.25 mm;
- *stationary phase*: *methylpolysiloxane R* (film thickness 1 µm).

Carrier gas *helium for chromatography R*.

Flow rate 1 mL/min.

Pulsed splitless 250 kPa, 0.25 min.

Temperature:

		Time (min)	Temperature (°C)
Column		0 - 1	55
		1 - 9	55 → 135
Injection port			240
Detector:	transfer line		280
	source		230
	analyser		150

Detection Mass spectrometer as described below; adjust the detector settings so as to comply with the system suitability criteria:
- quadrupole mass spectrometer equipped with an electron impact ionisation mode (70 eV);
- mass spectrometer parameters for the fragmentometric mode (single-ion monitoring (SIM)) set as follows:

Substance	*m/z*	Duration of monitoring
Butyl methanesulfonate (BMS)	56	t_R between 7.0 min and 9.0 min
Methyl methanesulfonate (MMS)	80	t_R between 2.0 min and 3.5 min
Ethyl methanesulfonate (EMS)	79	t_R between 4.0 min and 4.7 min
Isopropyl methanesulfonate (IMS)	123	t_R between 4.7 min and 5.5 min

Injection 2 µL.

Relative retention With reference to the internal standard (BMS) (retention time = about 7.6 min): MMS = about 0.3; EMS = about 0.5; IMS = about 0.6.

System suitability:
- *resolution*: minimum 3.0 between the peaks due to EMS and IMS in the chromatogram obtained with reference solution (a);
- *signal-to-noise ratio*: minimum 10 for the peaks due to MMS, EMS and IMS in the chromatogram obtained with reference solution (b).

Calculate the content of MMS, EMS or IMS in parts per million using the following expression:

$$\frac{A_2 \times I_1 \times W_1 \times C \times 0.148}{A_1 \times I_2 \times W_2}$$

A_1 = area of the peak due to MMS, EMS or IMS in the chromatogram obtained with reference solution (a);
A_2 = area of the peak due to MMS, EMS or IMS in the chromatogram obtained with the test solution;
C = percentage content of MMS, EMS or IMS;
I_1 = area of the peak due to the internal standard in the chromatogram obtained with reference solution (a);
I_2 = area of the peak due to the internal standard in the chromatogram obtained with the test solution;
W_1 = mass of MMS, EMS or IMS used to prepare reference solution (a), in milligrams;
W_2 = mass of the substance to be examined in the test solution, in milligrams;
0.148 = dilution factor.

T. Methyl, Ethyl and Isopropyl Methanesulfonate in Active Substances

(Ph. Eur. method 2.5.38)

The following general method has been validated for the determination of methyl, ethyl and isopropyl esters of methanesulfonic acid (in concentrations between 0.2 ppm and 5 ppm) in betahistine mesilate.

If it is intended to use the method for other active substances, particularly those that contain different concentrations of the methanesulfonic acid esters, the concentrations of the test solution and reference solutions must be adjusted accordingly and the method must be suitably validated.

METHOD

Head-space gas chromatography (*2.2.28*) coupled with mass spectrometry (*2.2.43*). *Prepare the test solution and reference solutions immediately before use.*

Solvent mixture *water R, acetonitrile R* (20:80 *V/V*). *The use of acetonitrile of appropriate purity is essential.*

Solution A Dissolve with the aid of ultrasound 30 mg of *anhydrous sodium thiosulfate R* and 60.0 g of *sodium iodide R* in *water R* and dilute to 50.0 mL with the same solvent.

Internal standard solution Dilute 10 µL of *butyl methanesulfonate CRS* (BMS) to 10.0 mL with the solvent mixture. Dilute 20 µL of the solution to 100.0 mL with the solvent mixture.

Blank solution Introduce 0.50 mL of solution A and 0.50 mL of the internal standard solution into a headspace vial and seal the vial immediately with a polytetrafluoroethylene-coated silicon membrane and an aluminium cap.

Test solution Weigh 25.0 mg of the substance to be examined into a 20 mL headspace vial. Add 0.50 mL of solution A and 0.50 mL of the internal standard solution and seal the vial immediately with a polytetrafluoroethylene-coated silicon membrane and an aluminium cap.

Following the derivatisation reaction, a precipitate may be observed, however this does not affect the validity of the quantification.

Reference solution (a) Dissolve 25.0 mg each of *methyl methanesulfonate R* (MMS), *ethyl methanesulfonate R* (EMS) and *isopropyl methanesulfonate R* (IMS) in *toluene R* and dilute to 5.0 mL with the same solvent. Dilute 50 µL of the solution to 25.0 mL with the internal standard solution.

Reference solution (b) Dilute 20 µL of reference solution (a) to 20.0 mL with the internal standard solution. Introduce 0.50 mL of this solution and 0.50 mL of solution A into a 20 mL headspace vial and seal the vial immediately with a polytetrafluoroethylene-coated silicon membrane and an aluminium cap.

Reference solution (c) Dilute 500 µL of reference solution (a) to 20.0 mL with the internal standard solution. Introduce 0.50 mL of this solution and 0.50 mL of solution A into a 20 mL headspace vial and seal the vial immediately with a polytetrafluoroethylene-coated silicon membrane and an aluminium cap.

Column:
— *material*: fused silica;
— *size*: *l* = 30 m, Ø = 0.25 mm;
— *stationary phase*: *polar-deactivated macrogol R* (film thickness 1 µm).

Carrier gas *helium for chromatography R.*

The use of an inert inlet liner without glass wool significantly reduces the effect of carry-over between the injections.

Flow rate 0.5 mL/min.

Split ratio 1:20.

Static head-space conditions that may be used:
— *equilibration temperature*: 60 °C;
— *equilibration time*: 30 min;
— *transfer-line temperature*: 120 °C.

Temperature:

		Time (min)	Temperature (°C)
Column		0 - 1	40
		1 - 10	40 → 130
Injection port			220
Detector	transfer line		280
	source		250
	analyser		200

At the end of analysis, the temperature of the column is raised to 240 °C and maintained at this temperature for 7 min.

Detection Mass spectrometer as described below; adjust the detector settings so as to comply with the system suitability criteria; alternatively a suitable electron-capture detector may be used:
— quadrupole mass spectrometer equipped with an electron impact ionisation mode (70 eV);
— mass spectrometer parameters for the fragmentometric mode (single-ion monitoring (SIM)) set as follows:

Substance	Quantitation ion (*m/z*)	Qualification ion (*m/z*)
Butyl iodide (BuI)*	184	127
Methyl iodide (MeI)*	142	127
Ethyl iodide (EtI)*	156	127
Isopropyl iodide (iPrI)*	170	127

* formed from BMS, MMS, EMS and IMS in the derivatisation reaction.

Injection 1 mL of the gas phase of the test solution, reference solutions (b) and (c) and the blank solution.

Relative retention With reference to the internal standard (BuI) (retention time = about 8.5 min): MeI = about 0.51; EtI = about 0.63; iPrI = about 0.68.

System suitability:
- *resolution*: minimum 1.5 between the peaks due to EtI and iPrI in the chromatogram obtained with reference solution (c);
- *signal-to-noise ratio*: minimum 10 for the peak due to each alkyl iodide in the chromatogram obtained with reference solution (b).

Calculate the content in parts per million of each alkyl methanesulfonate using the following expression:

$$\frac{A_2 \times I_1 \times W_1 \times C \times 0.05}{A_1 \times I_2 \times W_2}$$

A_1 = area of the peak due to each alkyl iodide in the chromatogram obtained with reference solution (c);
A_2 = area of the peak due to each alkyl iodide in the chromatogram obtained with the test solution;
C = percentage content of each ester;
I_1 = area of the peak due to the internal standard in the chromatogram obtained with reference solution (c);
I_2 = area of the peak due to the internal standard in the chromatogram obtained with the test solution;
W_1 = mass of each ester used to prepare reference solution (a), in milligrams;
W_2 = mass of the substance to be examined in the test solution, in milligrams;
0.05 = dilution factor.

V. Methanesulfonyl Chloride in Methanesulfonic Acid

(Ph. Eur. method 2.5.39)

The following method has been validated for the determination of methanesulfonyl chloride in methanesulfonic acid at concentrations in the range of 0.05 ppm to 50 ppm.

Gas chromatography (*2.2.28*) coupled with mass spectrometry (*2.2.43*).

Internal standard solution Dissolve 7 µL of *butyl methanesulfonate CRS* (BMS) in *methylene chloride R* and dilute to 10.0 mL with the same solvent. Dilute 5.0 mL of this solution to 50.0 mL with *methylene chloride R*.

Test solution To 5 mL of *water R*, add 7.4 g of the substance to be examined and mix slowly. After cooling, add 5.0 mL of *methylene chloride R* and 100 µL of the internal standard solution and shake. Allow to separate and transfer the organic layer to a vial containing 1 g of *anhydrous sodium sulfate R*. Repeat the extraction twice with 5.0 mL of *methylene chloride R* each time, combine the organic layers and filter.

Reference solution (a) Dissolve 50.0 mg of *methanesulfonyl chloride R* in *methylene chloride R* and dilute to 10.0 mL with the same solvent. Dilute 1.0 mL of the solution to 10.0 mL with *methylene chloride R*. Dilute 300 µL of this solution to 10.0 mL with *methylene chloride R*.

Reference solution (b) Dilute 500 µL of reference solution (a) and 100 µL of the internal standard solution to 15.0 mL with *methylene chloride R*.

Reference solution (c) Dilute 25 µL of reference solution (a) and 100 µL of the internal standard solution to 15.0 mL with *methylene chloride R*.

Column:
- *material*: fused silica;
- *size*: l = 15 m, Ø = 0.25 mm;
- *stationary phase*: *methylpolysiloxane R* (film thickness 1 µm).

Carrier gas *helium for chromatography R*.

Flow rate 1 mL/min.

Pulsed splitless 60 kPa, 0.1 min.

Temperature:

		Time (min)	Temperature (°C)
Column		0 - 4	40
		4 - 8	40 → 200
Injection port			240
Detector:	transfer line		280
	source		230
	analyser		150

At the end of analysis the temperature of the column is raised to 270 °C and maintained at this temperature for 8 min.

Detection Mass spectrometer as described below; adjust the detector settings so as to comply with the system suitability criteria:
- quadrupole mass spectrometer equipped with an electron impact ionisation mode (70 eV);
- mass spectrometer parameters for the fragmentometric mode (single-ion monitoring (SIM)) set as follows:

Substance	*m/z*	Duration of monitoring
Methanesulfonyl chloride	79	t_R between 3.3 min and 6.0 min
Butyl methanesulfonate (BMS)	56	t_R between 6.0 min and 8.0 min

Injection 5 µL of the test solution, reference solutions (b) and (c), the internal standard solution and *methylene chloride R*.

Relative retention With reference to the internal standard (BMS) (retention time = about 7.2 min): methanesulfonyl chloride = about 0.68.

System suitability:
- in the chromatogram obtained with the internal standard solution, there is no peak with the same retention time as methanesulfonyl chloride;
- *resolution*: minimum 5.0 between the peaks due to methanesulfonyl chloride and BMS in the chromatogram obtained with reference solution (b);
- *signal-to-noise ratio*: minimum 10 for the peak due to methanesulfonyl chloride in the chromatogram obtained with reference solution (c).

Calculate the content of methanesulfonyl chloride in parts per million using the following expression:

$$\frac{A_2 \times I_1 \times W_1 \times C \times 1.5}{A_1 \times I_2 \times W_2}$$

A_1 = area of the peak due to methanesulfonyl chloride in the chromatogram obtained with reference solution (b);
A_2 = area of the peak due to methanesulfonyl chloride in the chromatogram obtained with the test solution;
C = percentage content of methanesulfonyl chloride;
I_1 = area of the peak due to BMS in the chromatogram obtained with reference solution (b);

I_2 = area of the peak due to BMS in the chromatogram obtained with the test solution;
W_1 = mass of methanesulfonyl chloride used to prepare reference solution (a), in milligrams;
W_2 = mass of the sample in the test solution, in milligrams;
1.5 = dilution factor.

W. Determination of Elemental Impurities

(Ph. Eur. method 2.4.20)

INTRODUCTION

This chapter describes the general approach for the determination of elemental impurities in medicinal products or substances for pharmaceutical use. As the chemical composition of the considered samples and the specification limits for the element(s) of interest vary considerably, it is not possible to describe all suitable sample preparation and measurement methods. Therefore, any method that fulfils the requirements described in this chapter may be used.

The results of the analysis are acceptable only if the system suitability has been demonstrated by a suitable test. Before the initial use of a method, the analyst must ensure that the method is appropriate for the samples and instruments used. This is accomplished by applying a validation procedure to methods not described in the individual monograph or by a system suitability test for methods which are described in the monograph. Decision trees for the choice of the sample preparation and the measurement procedures are presented in Figures 2.4.20.-1 and 2.4.20.-2.

PROCEDURES

As a reference procedure is not provided for each element, matrix and concentration, the choice of procedure according to Figures 2.4.20.-1 and 2.4.20.-2, including sample preparation, detection technique and instrument parameters, is the responsibility of the user.

Use the flow chart in Figure 2.4.20.-1 to define the sample preparation method and the flow chart in Figure 2.4.20.-2 to define the measurement method. The sample preparation method should yield a sufficient quantity of sample to allow quantification of each element at the specified limit stated in the individual monograph or the general chapter.

All suitable sample preparation methods and measurement techniques (e.g. *2.2.22. Atomic emission spectrometry* (AES), *2.2.23. Atomic absorption spectrometry* (AAS), *2.2.37. X-ray fluorescence spectrometry* (XRFS), *2.2.57. Inductively coupled plasma-atomic emission spectrometry* (ICP-AES), *2.2.58. Inductively coupled plasma-mass spectrometry* (ICP-MS), *2.4.2. Arsenic, 2.4.8. Heavy metals, 2.4.9. Iron, 2.4.10. Lead in sugars, 2.4.15. Nickel in polyols, 2.4.31. Nickel in hydrogenated vegetable oils*) can be used for the determination of elemental impurities, if the method has been verified before the initial use by a system suitability test or a validation procedure according to this chapter.

If no sample preparation and/or measurement method is described in the individual monograph, a suitable sample preparation and/or measurement method must be developed and validated (see Figures 2.4.20.-1 and 2.4.20.-2).

SAMPLE PREPARATION

Sample preparation is critical to the success of elemental analysis. Many techniques not using direct measurement are heavily dependent on sample transport.

If an atomisation system is used, the most conventional means by which samples are introduced into the atomisation system is by solution nebulisation. In this case, solid samples must be dissolved in order to be introduced into the atomisation system. Samples may be dissolved in any appropriate solvent. The use of aqueous or dilute nitric acid solutions is strongly recommended, due to minimal interference with these solvents compared to other solvents. Hydrochloric acid, hydrofluoric acid, perchloric acid, sulfuric acid and hydrogen peroxide, at various concentrations, can be used to dissolve the samples. The viscosity of sulfuric acid is greater than that of the other acids and is to be taken into account as it can affect the overall fluidity of the solution.

The choice of solvents also includes, but is not limited to, the use of dilute bases, straight or diluted organic solvents, combinations of acids or bases, and combinations of organic solvents.

Acids, bases, and hydrogen peroxide of high purity must be used, especially when ICP-MS is employed. For aqueous solutions, use *deionised distilled water R*. Diluents must be checked for interference if they are used in an analysis. Because it is not always possible to obtain organic solvents that are free from elemental impurities, organic solvents of the highest purity possible with regard to these contaminants must be used. Specifically for ICP techniques, where samples are introduced into the plasma via solution nebulisation, it is important to consider the potential matrix effects and interferences that might arise from the solvent. The use of an appropriate internal standard and/or matching the standard matrix with samples should be applied for ICP-AES and ICP-MS analyses in cases where accuracy and precision are not sufficient. In any case, the selection of an appropriate internal standard should take into account the element(s) of interest, ionisation energy, wavelengths or masses, and the nature of the sample matrix.

Where a sample is found not to be soluble in any acceptable solvent, a variety of digestion or incineration techniques can be employed. These include hot-plate digestion, incineration and microwave-assisted digestions, using an open- or closed-vessel.

The decision regarding the type of digestion technique to be used depends on the nature of the sample being digested, as well as on the element(s) of interest and the concentration range of the elements to be quantified. Open-vessel digestion is not recommended for the analysis of volatile elements. The suitability of a digestion technique, whether open- or closed-vessel, should be supported by spike recovery experiments in order to verify that, within an acceptable tolerance, volatile elements have not been lost during sample preparation. The digestion cycle is suitable if a clear solution is obtained.

It is important to consider the selection of the type, the material of construction, the pretreatment, and the cleaning of analytical labware used in elemental analyses. The material must be inert and, depending on the specific application, resistant to caustics, acids, and/or organic solvents. For some analyses, care must be exercised to prevent the adsorption of elemental impurities onto the surface of a vessel, particularly in ultra-trace analyses. Contamination of sample solutions by elemental impurities and ions present in the container can also lead to inaccurate results.

The use of volumetric glassware that does not comply with Class A requirements of the appropriate International Standard of the International Organization for Standardization (ISO) is acceptable if the validation or the system suitability test of the method using such glassware

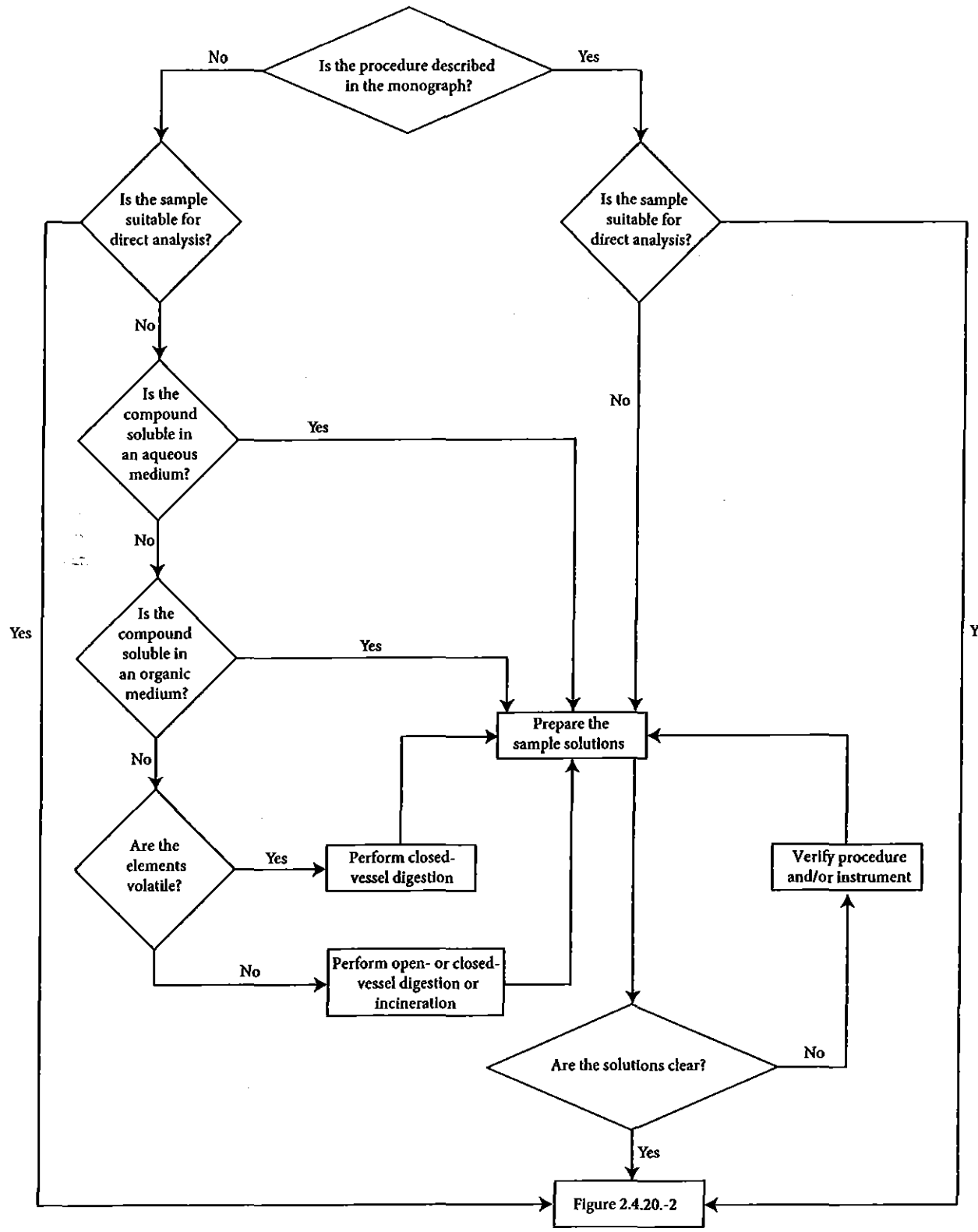


Figure 2.4.20.-1. – *Elemental impurities decision tree: sample preparation*

have experimentally demonstrated that the method is suitable for the intended purpose.

CAUTION: when using high-pressure digestion vessels and microwave laboratory equipment, the safety precautions and operating instructions given by the manufacturer must be followed.

MEASUREMENT

Method

The choice of the techniques depends mainly on the sample matrix and the characteristics and specification limits of the element(s) of interest. Analyse according to the instructions of the manufacturer of the equipment regarding program and wavelength.

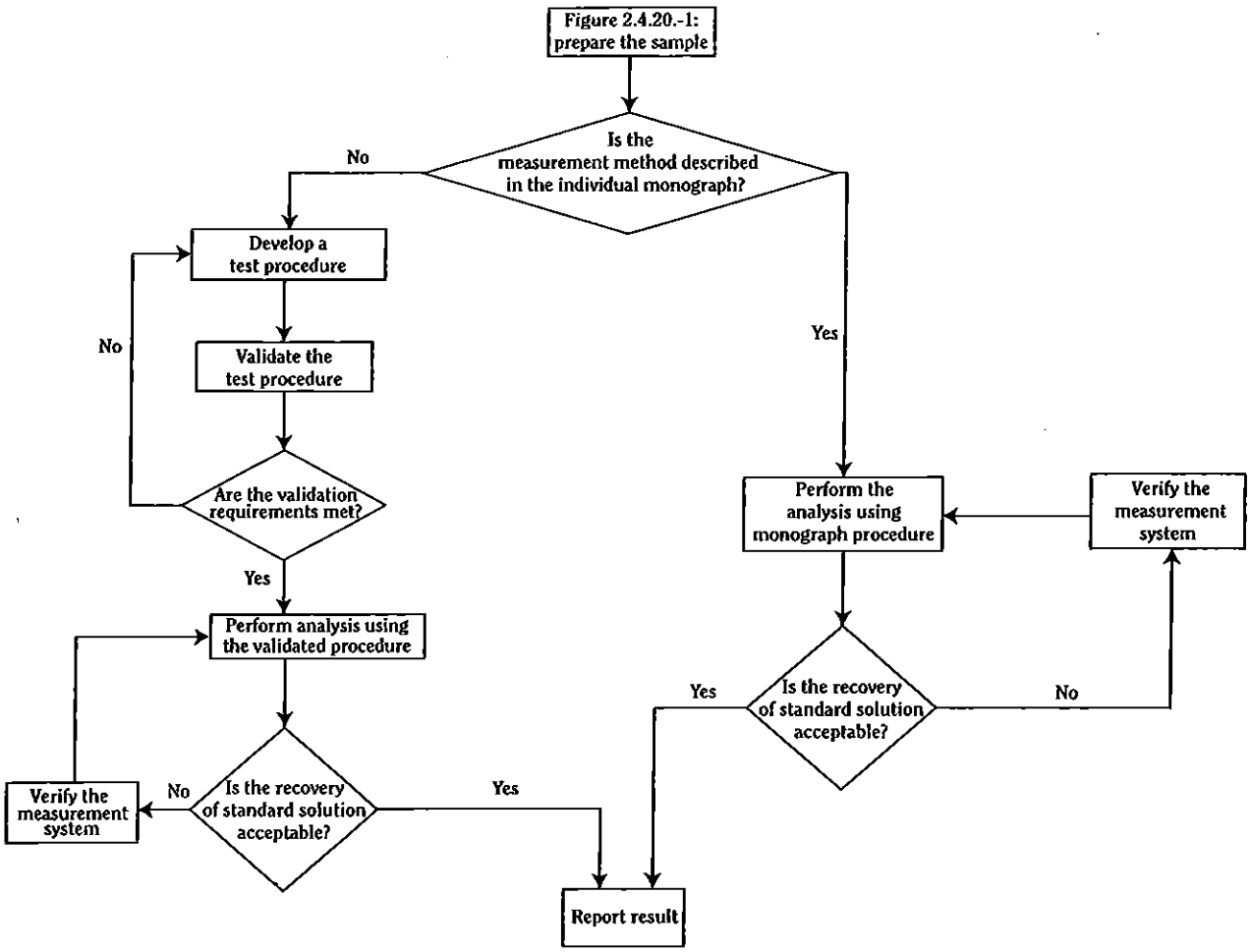


Figure 2.4.20.-2. – *Elemental impurities decision tree: measurement*

System suitability

A system suitability test must be carried out on the day of the analysis to ensure that the sample preparation and measurement system are appropriate.

Acceptance criterion for preparation of sample solution A clear solution is obtained.

Acceptance criterion for measurement system The measured concentration of a standard solution of the element at a concentration within the range of the used calibration curve does not differ from the actual concentration by more than 20 per cent.

Calculation

The blank value of reagents must be taken into account for the calculation of the content. Upon completion of the analysis, the concentration of a given element in the sample is calculated by the software of the instrument from the concentration of the element in the test solution. If no calculation software is available or no indication for calculation is given in the general chapter corresponding to the method used, the concentration of a given element in the sample can be calculated from the concentration of the element in the solution using the following expression:

$$C = A \times \frac{V_1}{m} \times \frac{V_2}{V_3}$$

C = concentration of element in the analysed sample, in micrograms per gram;

A = instrument reading of the concentration of the element in the sample solution, in micrograms per millilitre;

m = mass of the sample in the initial sample solution, in grams;

V_1 = volume of the initial sample preparation, in millilitres;

V_2 = total volume of any dilution performed, in millilitres;

V_3 = volume of initial sample preparation used in any dilution performed, in millilitres.

VALIDATION REQUIREMENTS

Some validation requirements provided below may differ from those provided in general chapters of the Ph. Eur. (e.g. *2.2.22* (AES), *2.2.23* (AAS), *2.2.57* (ICP-AES), *2.2.58* (ICP-MS)).

Before the initial use of the selected procedure, the analyst must ensure that the sample preparation and measurement method are appropriate for the element(s) of interest, sample matrix and instrument used. This is accomplished by following the validation procedure before the initial use and the system suitability test on the day of the analysis.

For elemental impurities, validation of a limit test must include specificity and limit of detection.

The following section defines the characteristics for the acceptability of a quantitative procedure. It must be demonstrated experimentally that such a procedure complies with the validation requirements, with an appropriate system suitability test using material spiked with a suitable reference material. The test materials must be spiked before any sample preparation steps. For example, if a test material is to be digested, the material must be spiked at the beginning of the digestion procedure.

SPECIFICITY

Specificity is the ability to ensure that the analytical procedure (sample preparation and measurement) allows a reliable determination of the element(s) of interest in the presence of components (e.g. carrier gas, impurities, matrix) that may be expected to be present.

Acceptance criteria The procedure must be able to assess unequivocally each elemental impurity to be determined with this procedure in the presence of components that may be expected to be present, including other elemental impurities, matrix components and other sources of interference; specificity is demonstrated by complying with the accuracy requirement for the element(s) to be determined.

RANGE

Acceptance criterion Range is demonstrated by complying with the recovery requirement.

ACCURACY

Verify the accuracy using a certified reference material or by performing a test for recovery. *Elemental impurity solutions CRS* may be used.

The recovery may be determined on a sample of the substance to be examined, spiked with a known quantity of a reference standard of the element of interest (3 concentration levels in the range of 50-150 per cent of the intended specification limit, even if the original concentration of the reference standard is at the specified value), in triplicate.

Acceptance criterion Spike recovery is within 70 per cent and 150 per cent for the mean of 3 replicates at each concentration.

REPEATABILITY

Test samples Either 6 independent samples of the substance to be examined spiked with a suitable reference standard at the specified concentration level, or 3 concentration levels prepared in triplicate.

Acceptance criterion The relative standard deviation is in both cases not more than 20 per cent.

INTERMEDIATE PRECISION

The effect of random events (intra-laboratory variations) on the analytical precision of the method must be established. Acceptable experiments for establishing intermediate precision include performing the repeatability analysis on different days, or with different instrumentation, or by different analysts. Only 1 of the 3 experiments is required to demonstrate intermediate precision.

Acceptance criterion The relative standard deviation is not more than 25 per cent.

LIMIT OF QUANTIFICATION

Use the results from the accuracy study. Determine the lowest concentration meeting the acceptance criterion.

Acceptance criterion The limit of quantification is below the specification limit.

LIMIT OF DETECTION (ONLY APPLICABLE TO LIMIT TESTS)

Determine the lowest concentration giving a signal clearly distinct from that obtained with a blank solution.

Acceptance criterion The limit of detection is not more than 0.5 times the concentration of the specification limit.

X. Methyl, Ethyl and Isopropyl Toluenesulfonate in Active Substances

(Ph. Eur. method 2.5.40)

The following general method has been validated for the determination of methyl, ethyl and isopropyl esters of toluenesulfonic acid (in concentrations between 0.2 ppm and 5 ppm) in sultamicillin tosilate dihydrate.

If it is intended to use the method for other active substances, particularly those that contain different concentrations of the toluenesulfonic acid esters, the concentrations of the test solution and reference solutions must be adjusted accordingly and the method must be suitably validated.

METHOD

Head-space gas chromatography (*2.2.28*) coupled with mass spectrometry (*2.2.43*). *Prepare the test solution and reference solutions immediately before use.*

Solvent mixture *water R, acetonitrile R* (20:80 *V/V*). *The use of acetonitrile of appropriate purity is essential.*

Solution A Dissolve 30 mg of *anhydrous sodium thiosulfate R* and 60.0 g of *sodium iodide R* in *water R* using sonication and dilute to 50.0 mL with the same solvent.

Internal standard solution Dilute 10 µL of *butyl methanesulfonate CRS* (BMS) to 10.0 mL with the solvent mixture. Dilute 20 µL of the solution to 100.0 mL with the solvent mixture.

Blank solution Introduce 0.5 mL of solution A and 0.5 mL of the internal standard solution into a headspace vial and seal the vial immediately with a polytetrafluoroethylene-coated silicon membrane and an aluminium cap.

Test solution Weigh 25.0 mg of the substance to be examined into a 20 mL headspace vial. Add 0.50 mL of solution A and 0.50 mL of the internal standard solution and seal the vial immediately with a polytetrafluoroethylene-coated silicon membrane and an aluminium cap.

Following the derivatisation reaction, a precipitate may be observed, however this does not affect the validity of the quantification.

Reference solution (a) Dissolve 25.0 mg each of *methyl toluenesulfonate R* (MTS), *ethyl toluenesulfonate R* (ETS) and *isopropyl toluenesulfonate R* (ITS) in *toluene R* and dilute to 5.0 mL with the same solvent. Dilute 50 µL of the solution to 25.0 mL with the internal standard solution.

Reference solution (b) Dilute 40 µL of reference solution (a) to 20.0 mL with the internal standard solution. Introduce 0.50 mL of this solution and 0.50 mL of solution A into a 20 mL headspace vial and seal the vial immediately with a polytetrafluoroethylene-coated silicon membrane and an aluminium cap.

Reference solution (c) Dilute 500 µL of reference solution (a) to 20.0 mL with the internal standard solution. Introduce 0.50 mL of this solution and 0.50 mL of solution A into a 20 mL headspace vial and seal the vial immediately with a polytetrafluoroethylene-coated silicon membrane and an aluminium cap.

Column:

— *material*: fused silica;
— *size*: *l* = 30 m, Ø = 0.25 mm;
— *stationary phase*: *polar-deactivated macrogol R* (film thickness 1 µm).

Carrier gas *helium for chromatography R.*

The use of an inert inlet liner without glass wool significantly reduces the effect of carry-over between the injections.

Flow rate 0.5 mL/min.

Split ratio 1:20.

Static head-space conditions that may be used:
— *equilibration temperature*: 60 °C;
— *equilibration time*: 30 min;
— *transfer-line temperature*: 120 °C.

Temperature:

		Time (min)	Temperature (°C)
Column		0 - 1	40
		1 - 10	40 → 130
Injection port			220
Detector	transfer line		280
	source		250
	analyser		200

At the end of analysis the temperature of the column is raised to 240 °C and maintained at this temperature for 7 min.

Detection Mass spectrometer as described below; adjust the detector settings so as to comply with the system suitability criteria:
— quadrupole mass spectrometer equipped with an electron impact ionisation mode (70 eV);
— mass spectrometer parameters for the fragmentometric mode (single-ion monitoring (SIM)) set as follows:

Substance	Quantitation Ion (m/z)	Qualification Ion (m/z)
Butyl iodide (BuI)*	184	127
Methyl iodide (MeI)*	142	127
Ethyl iodide (EtI)*	156	127
Isopropyl iodide (iPrI)*	170	127
* formed from BMS, MTS, ETS and ITS in the derivatisation reaction.		

Injection 1 mL of the gas phase of the test solution, reference solutions (b) and (c) and of the blank solution.

Relative retention With reference to the internal standard (BuI) (retention time = about 8.5 min): MeI = about 0.51; EtI = about 0.63; iPrI = about 0.68.

System suitability:
— *resolution*: minimum 1.5 between the peaks due to EtI and iPrI in the chromatogram obtained with reference solution (c);
— *signal-to-noise ratio*: minimum 10 for the peak due to each alkyl iodide in the chromatogram obtained with reference solution (b).

Calculate the content in parts per million of each alkyl toluenesulfonate using the following expression:

$$\frac{A_2 \times I_1 \times W_1 \times C \times 0.05}{A_1 \times I_2 \times W_2}$$

A_1 = area of the peak due to each alkyl iodide in the chromatogram obtained with reference solution (c);
A_2 = area of the peak due to each alkyl iodide in the chromatogram obtained with the test solution;
C = percentage content of each ester;
I_1 = area of the peak due to the internal standard in the chromatogram obtained with reference solution (c);
I_2 = area of the peak due to the internal standard in the chromatogram obtained with the test solution;
W_1 = mass of each ester used to prepare reference solution (a), in milligrams;
W_2 = mass of the substance to be examined in the test solution, in milligrams;
0.05 = dilution factor.

Y. Methyl, Ethyl and Isopropyl Benzenesulfonate in Active Substances

(Ph. Eur. method 2.5.41)

The following general method has been validated for the determination of methyl, ethyl and isopropyl esters of benzenesulfonic acid (in concentrations between 2.5 ppm and 40 ppm) in amlodipine besilate.

If it is intended to use the method for other active substances, particularly those that contain different concentrations of the benzenesulfonic acid esters, the concentrations of the test solution and reference solutions must be adjusted accordingly and the method must be suitably validated.

This method is not suitable for clopidogrel besilate as it was observed that methyl benzenesulfonate was obtained during the gas chromatographic analysis as an artefact originating from degradation.

METHOD

Head-space gas chromatography (*2.2.28*) coupled with mass spectrometry (*2.2.43*). *Prepare the test solution and reference solutions immediately before use.*

Solvent mixture *water R, acetonitrile R* (20:80 *V/V*). *The use of acetonitrile of appropriate purity is essential.*

Solution A Dissolve with the aid of ultrasound 30 mg of *anhydrous sodium thiosulfate R* and 60.0 g of *sodium iodide R* in *water R* and dilute to 50.0 mL with the same solvent.

Internal standard solution Dilute 10 µL of *butyl methanesulfonate CRS* (BMS) to 10.0 mL with the solvent mixture. Dilute 20 µL of the solution to 100.0 mL with the solvent mixture.

Blank solution Introduce 0.50 mL of solution A and 0.50 mL of the internal standard solution into a headspace vial and seal the vial immediately with a polytetrafluoroethylene-coated silicon membrane and an aluminium cap.

Test solution Weigh 25.0 mg of the substance to be examined into a 20 mL headspace vial. Add 0.50 mL of solution A and 0.50 mL of the internal standard solution and seal the vial immediately with a polytetrafluoroethylene-coated silicon membrane and an aluminium cap.

Following the derivatisation reaction, a precipitate may be observed, however this does not affect the validity of the quantification.

Reference solution (a) Dissolve 25.0 mg each of *methyl benzenesulfonate R* (MBS), *ethyl benzenesulfonate R* (EBS) and *isopropyl methanesulfonate R* (IMS) in *toluene R* and dilute to 5.0 mL with the same solvent. Dilute 50 µL of the solution to 25.0 mL with the internal standard solution.

Reference solution (b) Dilute 40 µL of reference solution (a) to 20.0 mL with the internal standard solution. Introduce 0.50 mL of this solution and 0.50 mL of solution A into a 20 mL headspace vial and seal the vial immediately with a polytetrafluoroethylene-coated silicon membrane and an aluminium cap.

Reference solution (c) Dilute 500 µL of reference solution (a) to 20.0 mL with the internal standard solution. Introduce 0.50 mL of this solution and 0.50 mL of solution A into a 20 mL headspace vial and seal the vial immediately with a polytetrafluoroethylene-coated silicon membrane and an aluminium cap.

Column:
— *material*: fused silica;
— *size*: *l* = 30 m, Ø = 0.25 mm;
— *stationary phase*: *polar-deactivated macrogol R* (film thickness 1 µm).

Carrier gas *helium for chromatography R.*

The use of an inert inlet liner without glass wool significantly reduces the effect of carry-over between the injections.

Flow rate 0.5 mL/min.

Split ratio 1:20.

Static head-space conditions that may be used:
— *equilibration temperature*: 60 °C;
— *equilibration time*: 30 min;
— *transfer-line temperature*: 120 °C.

Temperature:

		Time (min)	Temperature (°C)
Column		0 - 1	40
		1 - 10	40 → 130
Injection port			220
Detector	transfer line		280
	source		250
	analyser		200

At the end of analysis, the temperature of the column is raised to 240 °C and maintained at this temperature for 7 min.

Detection Mass spectrometer as described below; adjust the detector settings so as to comply with the system suitability criteria:
— quadrupole mass spectrometer equipped with an electron impact ionisation mode (70 eV);
— mass spectrometer parameters for the fragmentometric mode (single-ion monitoring (SIM)) set as follows:

Substance	Quantitation ion (*m/z*)	Qualification ion (*m/z*)
Butyl iodide (BuI)*	184	127
Methyl iodide (MeI)*	142	127
Ethyl iodide (EtI)*	156	127
Isopropyl iodide (iPrI)*	170	127
* formed from BMS, MBS, EBS and IMS in the derivatisation reaction.		

Injection 1 mL of the gas phase of the test solution, reference solutions (b) and (c) and the blank solution.

Relative retention With reference to the internal standard (BuI) (retention time = about 8.5 min): MeI = about 0.51; EtI = about 0.63; iPrI = about 0.68.

System suitability:
— *resolution*: minimum 1.5 between the peaks due to EtI and iPrI in the chromatogram obtained with reference solution (c);
— *signal-to-noise ratio*: minimum 10 for the peak due to each alkyl iodide in the chromatogram obtained with reference solution (b).

Calculate the content in parts per million of each alkyl benzenesulfonate using the following expression:

$$\frac{A_2 \times I_1 \times W_1 \times C \times 0.05}{A_1 \times I_2 \times W_2}$$

A_1 = area of the peak due to each alkyl iodide in the chromatogram obtained with reference solution (c);
A_2 = area of the peak due to each alkyl iodide in the chromatogram obtained with the test solution;
C = percentage content of each ester;
I_1 = area of the peak due to the internal standard in the chromatogram obtained with reference solution (c);
I_2 = area of the peak due to the internal standard in the chromatogram obtained with the test solution;
W_1 = mass of each ester used to prepare reference solution (a), in milligrams;
W_2 = mass of the substance to be examined in the test solution, in milligrams;
0.05 = dilution factor.

Z. Tetrabutylammonium in Radiopharmaceutical Preparations

(Ph. Eur. method 2.4.33 Tetrabutylammonium in Radiopharmaceutical Preparations)

Thin-layer chromatography (*2.2.27*).

Solvent mixture *anhydrous ethanol R, water R* (10:90 *V/V*).

Test solution The preparation to be examined.

Reference solution Dissolve 0.86 g of *tetrabutylammonium hydroxide R* in the solvent mixture and dilute to 100 mL with the solvent mixture. Dilute 1 mL of the solution to *V* with the solvent mixture, *V* being the maximum recommended dose in millilitres.

Plate *TLC silica gel plate R*; use a polyester plate.

Mobile phase:
— *mobile phase A*: *concentrated ammonia R, methanol R* (10:90 *V/V*);
— *mobile phase B*: *concentrated ammonia R, methanol R* (0.5:100 *V/V*).

Use either mobile phase A or mobile phase B. The choice of mobile phase depends on the matrix (for example, mobile phase B is suitable for preparations stabilised with ascorbate). Spiking experiments can be done to verify the suitability of the mobile phase and to show that the sample matrix has no influence on the results.

Application 2 µL.

Development Over 4/5 of the plate.

Drying At 50 °C for maximum 30 s.

Detection Place 0.5 g of *iodine R* at the bottom of a chromatographic tank; close the tank and heat it with a stream of hot air (about 60-80 °C) until it is filled with pink vapour; place the plate in the tank; leave the plate in contact with the iodine vapour for 1 min; withdraw the plate.

System suitability:
— the chromatogram obtained with the reference solution shows a clearly visible spot.

Limit:
— *tetrabutylammonium*: any spot due to tetrabutylammonium is not more intense than the corresponding spot in the chromatogram obtained with the reference solution (2.6mg/V).

Appendix IX

A. Determination of Sulfated Ash

Use Method I unless otherwise directed.

Method I

(No Ph. Eur. method)

Heat a platinum dish to redness for 10 minutes, allow to cool in a desiccator and weigh. Unless otherwise specified in the monograph, place 1 g of the substance being examined in the dish, moisten with *sulfuric acid*, ignite gently, again moisten with *sulfuric acid* and ignite at about 800°. Cool, weigh again, ignite for 15 minutes and repeat this procedure until two successive weighings do not differ by more than 0.5 mg.

Method II[1]

(Ph. Eur. method 2.4.14)

Ignite a suitable crucible (for example, silica, platinum, porcelain or quartz) at 600 ± 50 °C for 30 min, allow to cool in a desiccator over silica gel or other suitable desiccant and weigh. Place the prescribed amount of the substance to be examined in the crucible and weigh. Moisten the substance to be examined with a small amount of *sulfuric acid R* (usually 1 mL) and heat gently at as low a temperature as practicable until the sample is thoroughly charred. After cooling, moisten the residue with a small amount of *sulfuric acid R* (usually 1 mL), heat gently until white fumes are no longer evolved and ignite at 600 ± 50 °C until the residue is completely incinerated. Ensure that flames are not produced at any time during the procedure. Allow the crucible to cool in a desiccator over silica gel or other suitable desiccant, weigh it again and calculate the percentage of residue.

If the amount of the residue so obtained exceeds the prescribed limit, repeat the moistening with *sulfuric acid R* and ignition, as previously, for 30 min periods until 2 consecutive weighings do not differ by more than 0.5 mg or until the percentage of residue complies with the prescribed limit.

The amount of substance used for the test (usually 1-2 g) is chosen so that at the prescribed limit the mass of the residue (usually about 1 mg) can be measured with sufficient accuracy.

B. Determination of Sulfur Dioxide

Method I

(No Ph. Eur. method)

Apparatus A round-bottomed flask of 1000- to 1500-mL capacity is fitted with a water-cooled reflux condenser the upper end of which is connected to two absorption tubes in series. The flask is fitted with a gas inlet tube which reaches nearly to the bottom of the flask. Each absorption tube contains 10 mL of *hydrogen peroxide solution (20 vol)* previously neutralised with 0.1M *sodium hydroxide VS* using *bromophenol blue solution* as indicator.

Method Place in the flask 500 mL of *water* and 20 mL of *hydrochloric acid*. Pass through the flask a steady current of *nitrogen* or *carbon dioxide* that has been bubbled through *dilute sodium carbonate solution* and gradually heat the liquid until it boils. Maintain the current of *nitrogen* or *carbon dioxide*, allow the solution to boil for about 10 minutes and cool the flask by gradual immersion in water. Introduce, while momentarily removing the stopper of the flask, a weighed quantity of 50 to 100 g of the substance being examined, heat gently and boil for 45 minutes. Disconnect the absorption tubes before turning off the current of *nitrogen* or *carbon dioxide* and titrate the combined contents with 0.1M *sodium hydroxide VS*. Each mL of 0.1M *sodium hydroxide VS* is equivalent to 3.203 mg of sulfur dioxide.

Repeat the operation without the substance being examined. The solution in the absorption tubes remains neutral.

Method II

(Ph. Eur. method 2.5.29)

EQUIPMENT

The apparatus as shown in Figure 2.5.29.-1 comprises:
— a ground-glass 3-neck round-bottomed flask (*A*);
— a dropping funnel (*B*);
— a reflux condenser (*C*);
— a receiving tube (*D*);
— a transfer tube (*E*);
— a gas port.

PROCEDURE

Method

Introduce 150 mL of *water R* into the flask (*A*) and equilibrate the whole system by passing *carbon dioxide R* for 15 min at a rate of about 100 mL/min.

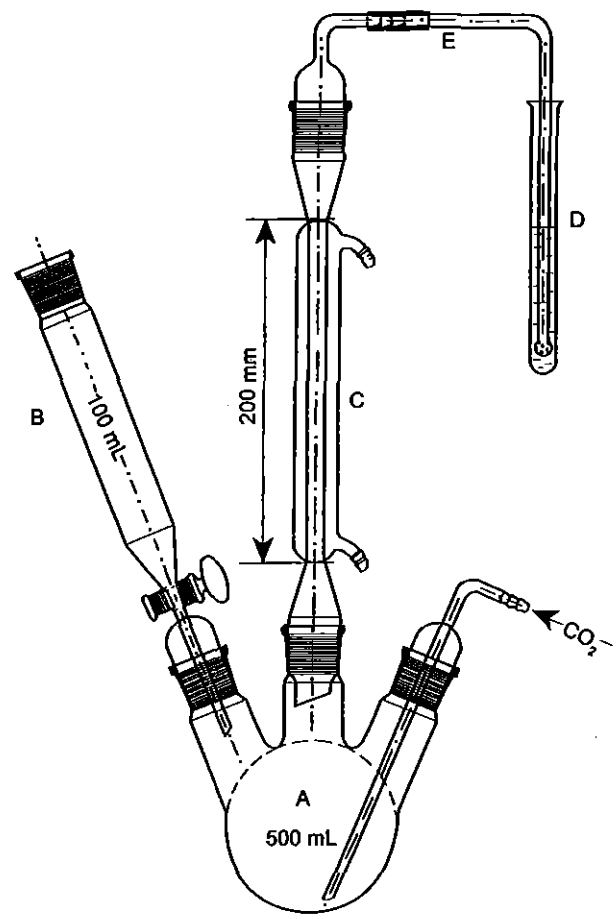


Figure 2.5.29.-1.– *Apparatus for the determination of sulfur dioxide content*

[1] *This chapter has undergone pharmacopoeial harmonisation. See chapter 5.8 Pharmacopoeial harmonisation.*

To 10 mL of *dilute hydrogen peroxide solution R*, add 0.15 mL of a 1 g/L solution of *bromophenol blue R* in *ethanol (20 per cent V/V) R*. Add *0.1 M sodium hydroxide* until a violet-blue colour is obtained, without exceeding the end-point. Pour the solution into the receiving tube (*D*) and mount the tube on the apparatus as shown in Figure 2.5.29.-1.

Without interrupting the stream of carbon dioxide, remove the dropping funnel (*B*) and introduce into the flask (*A*) 25.0 g (*m*) of the substance to be examined, rinsing with 100 mL of *water R*. Replace the dropping funnel, close the tap and pour 80 mL of *dilute hydrochloric acid R* into the funnel. Open the tap to allow the hydrochloric acid solution to flow into the flask. Make sure that no sulfur dioxide escapes by closing the tap before the last few millilitres of hydrochloric acid solution drain out. Boil for 1 h.

Open the tap of the dropping funnel then stop the flow of carbon dioxide . Transfer the contents of the receivingtube (*D*) to a 200 mL conical flask, rinsing the tube with a little *water R*. Heat on a water-bath for 15 min and allow to cool. Add 0.1 mL of a 1 g/L solution of *bromophenol blue R* in *ethanol (20 per cent V/V) R* and titrate with *0.1 M sodium hydroxide* until the colour changes from yellow to violet-blue (V_1). Carry out a blank titration (V_2).

Results

Calculate the content of sulfur dioxide, in parts per million, using the following expression:

$$32\,030 \times (V_1 - V_2) \times \frac{n}{m}$$

V_1 = volume of titrant used in the titration, in millilitres;
V_2 = volume of titrant used in the blank titration, in millilitres;
n = molarity of the sodium hydroxide solution used as titrant, in moles per litre;
m = mass of the sample, in grams.

C. Determination of Water

Use Method IA unless otherwise directed.

Method I Semi-micro Determination of Water

(Ph. Eur. method 2.5.12)

The semi-micro determination of water is based upon the quantitative reaction of water with sulfur dioxide and iodine in a suitable anhydrous medium in the presence of a base with sufficient buffering capacity.

APPARATUS

The apparatus consists of a titration vessel with:
— 2 identical platinum electrodes;
— tight inlets for introduction of solvent and titrant;
— an inlet for introduction of air via a desiccant;
— a sample inlet fitted with a stopper or, for liquids, a septum.

Inlet systems for introduction of dry nitrogen or for aspiration of solvents may also be fitted.

The titration is carried out according to the instrument supplier's instructions. Care is taken throughout the determination to avoid exposure of reagents and solvents to atmospheric moisture. The end-point is determined using 2 identical indicator electrodes connected to an electrical source that maintains between the electrodes either a constant current (*2.2.65. Voltametric titration*) or a constant voltage (*2.2.19. Amperometric titration*). Where direct titration is used (method A), addition of titrant causes either a decrease in voltage where constant current is maintained or an increase in current where constant voltage is maintained, until the end-point is reached. Instruments with automatic end-point detection are commonly used. Instrument qualification is carried out according to established quality system procedures, for example using a suitable certified reference material (*sodium aminosalicylate dihydrate for equipment qualification CRS* may be used).

STANDARDISATION

To the titration vessel, add *methanol R*, dried if necessary, or the solvent recommended by the supplier of the titrant. Where applicable for the apparatus used, eliminate residual water from the measurement cell or carry out a pre-titration. Introduce a suitable amount of water in an appropriate form (*water R* or a certified reference material) and carry out the titration, stirring for the necessary time. The water equivalent is not less than 80 per cent of that indicated by the supplier. Standardise the titrant before the first use and at suitable intervals thereafter.

Unless otherwise prescribed, use Method A.

METHOD A

Introduce into the titration vessel *methanol R*, or the solvent indicated in the monograph or recommended by the supplier of the titrant. Where applicable for the apparatus used, eliminate residual water from the measurement cell or carry out a pre-titration. Introduce the substance to be examined rapidly and carry out the titration, stirring for the necessary extraction time.

METHOD B

Introduce into the titration vessel *methanol R*, or the solvent indicated in the monograph or recommended by the supplier of the titrant. Where applicable for the apparatus used, eliminate residual water from the measurement cell or carry out a pre-titration. Introduce the substance to be examined rapidly and in a suitable state of division. Add an accurately measured volume of the titrant, sufficient to give an excess of about 1 mL or the prescribed volume. Allow to stand protected from light for 1 min or the prescribed time, with stirring. Titrate the excess of reagent using *methanol R* or the prescribed solvent, containing an accurately known quantity of water.

SUITABILITY

The accuracy of the determination with the chosen titrant must be verified for each combination of substance, titrant and solvent to be examined. The following procedure, given as an example, is suitable for samples containing 2.5-25 mg of water.

The water content of the substance to be examined is determined using the reagent/solvent system chosen. Thereafter, in the same titration vessel, sequential known amounts of water, corresponding to about 50-100 per cent of the amount found in the substance to be examined, are added in an appropriate form (at least 5 additions) and the water content is determined after each addition. Calculate the percentage recovery (r) after each addition using the following expression:

$$r = 100\frac{W_2}{W_1}$$

W_1 = amount of water added, in milligrams;
W_2 = amount of water found, in milligrams.

Calculate the mean percentage recovery ($\bar{r}$).
The reagent/solvent system is considered to be acceptable if $\bar{r}$ is between 97.5 per cent and 102.5 per cent.

Calculate the regression line. The x-axis represents the cumulative water added whereas the y-axis represents the sum of the initial water content determined for the substance (M) and the cumulative water determined after each addition. Calculate the slope (b), the intercept with the y-axis (a) and the intercept of the extrapolated calibration line with the x-axis (d).

Calculate the percentage errors (e_1 and e_2) using the following expressions:

$$e_1 = 100\frac{a - M}{M}$$

$$e_2 = 100\frac{|d| - M}{M}$$

a = the y-axis intercept, in milligrams of water;
d = the x-axis intercept, in milligrams of water;
M = water content of the substance, in milligrams of water.

The reagent/solvent system is considered to be acceptable if:
— $|e_1|$ and $|e_2|$ are not greater than 2.5 per cent;
— b is between 0.975 and 1.025.

Method II Determination of Water by Distillation
(Ph. Eur. method 2.2.13)

The apparatus (see Figure 2.2.13.-1) consists of a glass flask (*A*) connected by a tube (*D*) to a cylindrical tube (*B*) fitted with a graduated receiving tube (*E*) and reflux condenser (*C*). The receiving tube (*E*) is graduated in 0.1 mL. The source of heat is preferably an electric heater with rheostat control or an oil bath. The upper portion of the flask and the connecting tube may be insulated.

Method Clean the receiving tube and the condenser of the apparatus, thoroughly rinse with water, and dry.

Introduce 200 mL of *toluene R* and about 2 mL of *water R* into the dry flask. Distil for 2 h, then allow to cool for about 30 min and read the water volume to the nearest 0.05 mL. Place in the flask a quantity of the substance, weighed with an accuracy of 1 per cent, expected to give about 2 mL to 3 mL of water. If the substance has a pasty consistency, weigh it in a boat of metal foil. Add a few pieces of porous material and heat the flask gently for 15 min. When the toluene begins to boil, distil at the rate of about two drops per second until most of the water has distilled over, then increase the rate of distillation to about four drops per second. When the water has all distilled over, rinse the inside of the condenser tube with *toluene R*. Continue the distillation for 5 min, remove the heat, allow the receiving tube to cool to room temperature and dislodge any droplets of water which adhere to the walls of the receiving tube. When the water and toluene have completely separated, read the volume of water and calculate the content present in the substance as millilitres per kilogram, using the formula:

$$\frac{1000(n_2 - n_1)}{m}$$

m = the mass in grams of the substance to be examined,
n_1 = the number of millilitres of water obtained in the first distillation,
n_2 = the total number of millilitres of water obtained in the 2 distillations.

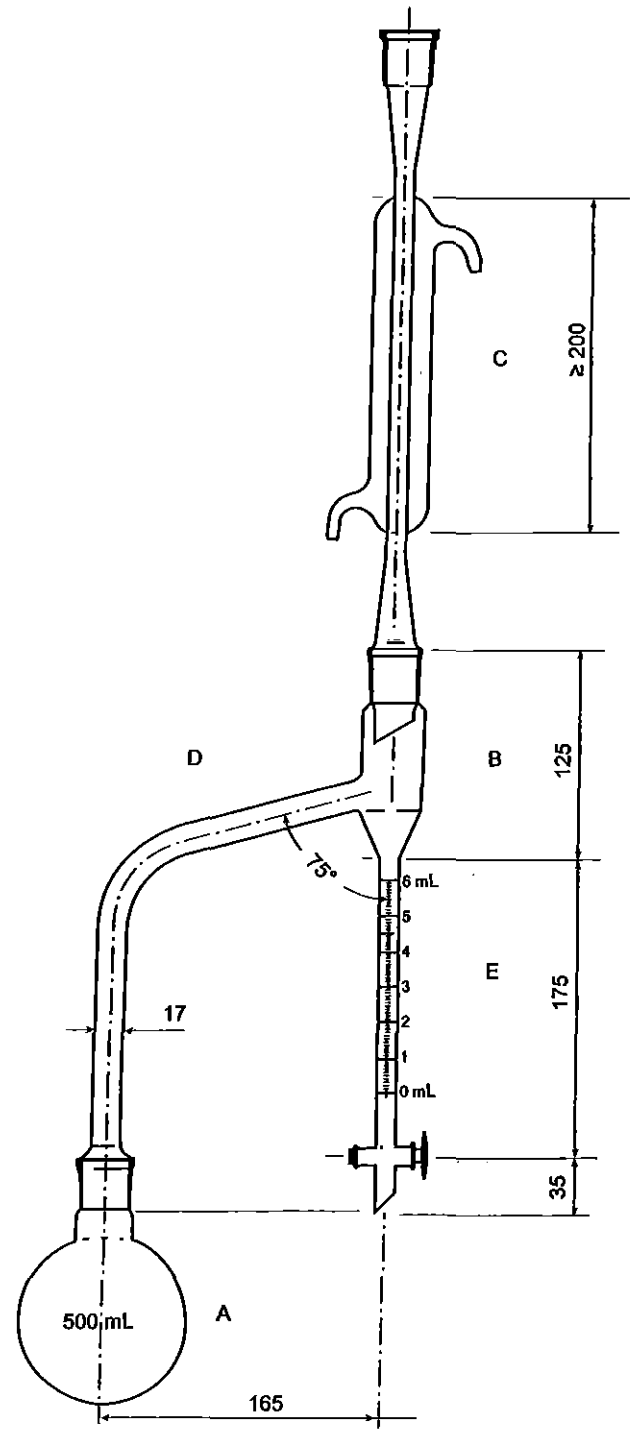


Figure 2.2.13.-1. – *Apparatus for the determination of water by distillation*
Dimensions in millimetres

Method III Coulometric Titration
(Ph. Eur. method 2.5.32)

PRINCIPLE

The coulometric titration of water is based upon the quantitative reaction of water with sulfur dioxide and iodine in an anhydrous medium in the presence of a base with sufficient buffering capacity. In contrast to the volumetric method described in general chapter *2.5.12. Water: semi-micro determination*, iodine is produced electrochemically in the reaction cell by oxidation of iodide. The iodine produced at

the anode reacts immediately with the water and the sulfur dioxide contained in the reaction cell. The quantity of water in the substance is directly proportional to the quantity of electricity (in coulombs), corresponding to electric current (in amperes) multiplied by time (in seconds), used for iodine generation up until the titration end-point. When all of the water in the reaction cell has been consumed, the end-point is reached and thus an excess of iodine appears. 1 mole of iodine corresponds to 1 mole of water, an amount of electricity of 10.71 C corresponds to 1 mg of water.

Moisture is eliminated from the reaction cell by pre-titration, i.e. the electrolyte reagent is titrated to dryness before starting the sample analysis. Individual determinations can be carried out successively in the same reagent solution, under the following conditions:

— each component of the test mixture is compatible with the other components;
— no other reactions take place;
— the volume and the water capacity of the electrolyte reagent are sufficient.

Coulometric titration is intended for the quantitative determination of small quantities of water (from 10 µg), however a working range of 100 µg to 10 mg of water is recommended for reproducibility reasons.

Accuracy and precision of the method are predominantly governed by the sample preparation and the extent to which atmospheric moisture is excluded from the system. Control of the system must be monitored by measuring the amount of baseline drift.

APPARATUS

The apparatus consists of a reaction cell, electrodes and a magnetic stirrer. The reaction cell consists of a large anode compartment and a smaller cathode compartment. Depending on the design of the electrode, both compartments can be separated by a diaphragm. Each compartment contains a platinum electrode. Liquid or solubilised samples are introduced through a septum, using a syringe. Alternatively, an evaporation technique may be used in which the sample is heated in an oven and the water is evaporated and carried into the cell by means of a stream of dry inert gas. The introduction of solid samples into the cell should in general be avoided. However, if it has to be done it is effected through a sealable port; appropriate precautions must be taken to avoid the introduction of moisture from air, such as working in a glove box in an atmosphere of dry inert gas. The analytical procedure is controlled by a suitable electronic device, which also displays the results.

Instrument qualification is carried out according to established quality system procedures, for example using a suitable certified reference material. *Sodium aminosalicylate dihydrate for equipment qualification CRS* may be used when proceeding by direct or liquid sample introduction, whereas *amoxicillin trihydrate for performance verification CRS* may be used with the evaporation technique.

METHOD

Fill the compartments of the reaction cell with *electrolyte reagent for the micro determination of water R* according to the manufacturer's instructions and perform the coulometric pre-titration to a stable end-point. Introduce the prescribed quantity of the substance to be examined into the reaction cell and titrate again to a stable end-point, stirring for at least 30 s, unless otherwise indicated in the monograph. If an oven is used, the prescribed quantity of sample is introduced into the oven and heated. After evaporation of the water from the sample into the reaction cell, the titration is started.

Alternatively, the evaporated moisture is immediately titrated while heating the sample in the oven to avoid loss of evaporated water already collected in the reagent solution during prolonged heating. Read the value from the instrument's output and calculate if necessary the percentage or quantity of water that is present in the substance. When appropriate to the type of sample and the sample preparation, perform a blank titration.

VERIFICATION OF ACCURACY

At appropriate intervals, such as at least at the beginning and the end of a series of sample titrations, introduce a defined quantity of water, in the same order of magnitude as the quantity of water in the sample, using a suitable certified reference material and perform the coulometric titration. The recovery is within the range of 97.5 per cent to 102.5 per cent for an addition of 1000 µg of H_2O and within the range of 90.0 per cent to 110.0 per cent for the addition of 100 µg of H_2O.

D. Determination of Loss on Drying

(Ph. Eur. method 2.2.32)

PRINCIPLE

Loss on drying is the loss of mass after drying under specified conditions, calculated as a percentage (*m/m*).

Drying to constant mass means that 2 consecutive weighings do not differ by more than 0.5 mg, the 2nd weighing following an additional period of at least 30 min of drying under the conditions prescribed for the substance to be examined.

EQUIPMENT

The equipment typically consists of:

— weighing bottles that are made of suitable inert material and can easily be dried to constant mass; their diameter is large enough so that the layer of the substance to be examined does not exceed about 5 mm;
— an analytical balance by which it is possible to determine a change in mass of 0.1 mg;
— depending on the procedure to be applied, a desiccator, a vacuum cabinet, a vacuum oven or an ordinary laboratory oven; in any case, the temperature of ovens is adjustable to the specified temperature ± 2 °C; vacuum ovens in which the pressure can at least be reduced to about 2 kPa are suitable; ovens are qualified according to established quality system procedures, for example by using a suitable certified reference material (*sodium aminosalicylate dihydrate for equipment qualification CRS* may be used).

Equipment using other means of drying such as microwaves, halogen lamps, infrared lamps or mixed technologies may be used provided they are demonstrated to be fit for purpose.

PROCEDURE

It is recommended to perform the test in an environment that has minimal impact on sample measurement (e.g. humidity).

Weigh an empty weighing bottle that has been previously dried under the conditions prescribed for the substance to be examined for at least 30 min, then weigh the weighing bottle filled with the prescribed quantity of substance to be examined. Dry to constant mass or for the prescribed time. Where the drying temperature is indicated by a single value rather than a range, drying is carried out at the prescribed

temperature ± 2 °C. Use one of the following procedures, unless otherwise prescribed in the monograph.

— In a desiccator: the drying is carried out over about 100 g of *molecular sieve R* at atmospheric pressure and at room temperature.
— *In vacuo*: the drying is carried out over about 100 g of *molecular sieve R* at a pressure not exceeding 2.5 kPa, at room temperature or at the temperature prescribed in the monograph.
— In an oven at a specified temperature: the drying is carried out at atmospheric pressure in an oven at the temperature prescribed in the monograph.

After drying in an oven, allow the weighing bottle and the sample to cool to room temperature in a desiccator and weigh the weighing bottle containing the dried sample.

The mass of the sample is the difference between the mass of the filled weighing bottle and the mass of the dried empty weighing bottle.

The loss on drying is the difference in the mass of the sample before and after drying, expressed as a percentage, *m/m* being implicit.

E. Limit Test for Carbon Monoxide in Medicinal Gases

(Ph. Eur. method 2.5.25)

METHOD I

Apparatus The apparatus (Figure 2.5.25.-1) consists of the following parts connected in series:

— a U-tube (U_1) containing *anhydrous silica gel R* impregnated with *chromium trioxide R*;
— a wash bottle (F_1) containing 100 mL of a 400 g/L solution of *potassium hydroxide R*;
— a U-tube (U_2) containing pellets of *potassium hydroxide R*;
— a U-tube (U_3) containing *diphosphorus pentoxide R* dispersed on previously granulated, fused pumice;
— a U-tube (U_4) containing 30 g of *recrystallised iodine pentoxide R* in granules, previously dried at 200 °C and kept at a temperature of 120 °C (T) during the test; the iodine pentoxide is packed in the tube in 1 cm columns separated by 1 cm columns of glass wool to give an effective length of 5 cm;
— a reaction tube (F_2) containing 2.0 mL of *potassium iodide solution R* and 0.15 mL of *starch solution R*.

Method Flush the apparatus with 5.0 L of *argon R* and, if necessary, discharge the blue colour in the iodide solution by adding the smallest necessary quantity of freshly prepared *0.002 M sodium thiosulfate*. Continue flushing until not more than 0.045 mL of *0.002 M sodium thiosulfate* is required after passage of 5.0 L of *argon R*. Pass the gas to be examined from the cylinder through the apparatus, using the prescribed volume and the flow rate. Flush the last traces of liberated iodine into the reaction tube by passing through the apparatus 1.0 L of *argon R*. Titrate the liberated iodine with *0.002 M sodium thiosulfate*. Carry out a blank test, using the prescribed volume of *argon R*. The difference between the volumes of *0.002 M sodium thiosulfate* used in the titrations is not greater than the prescribed limit.

METHOD II

Gases absorb light at one or more specific wavelengths. This property is widely used to allow highly selective measurement of their concentrations.

Description and principle of measurement

The concentration of carbon monoxide in other gases can be determined using an infrared analyser.

The infrared analyser generally consists of a light source emitting broadband infrared radiation, an optical device, a sample cell and a detector. The optical device may be positioned either before or after the sample cell; it consists of one or several optical filters, through which the broadband radiation is passed. The optical device in this case is selected for carbon monoxide. The measurement light beam passes through the sample cell and may also pass through a reference cell if the analyser integrates such a feature (some use an electronic system instead of a reference cell).

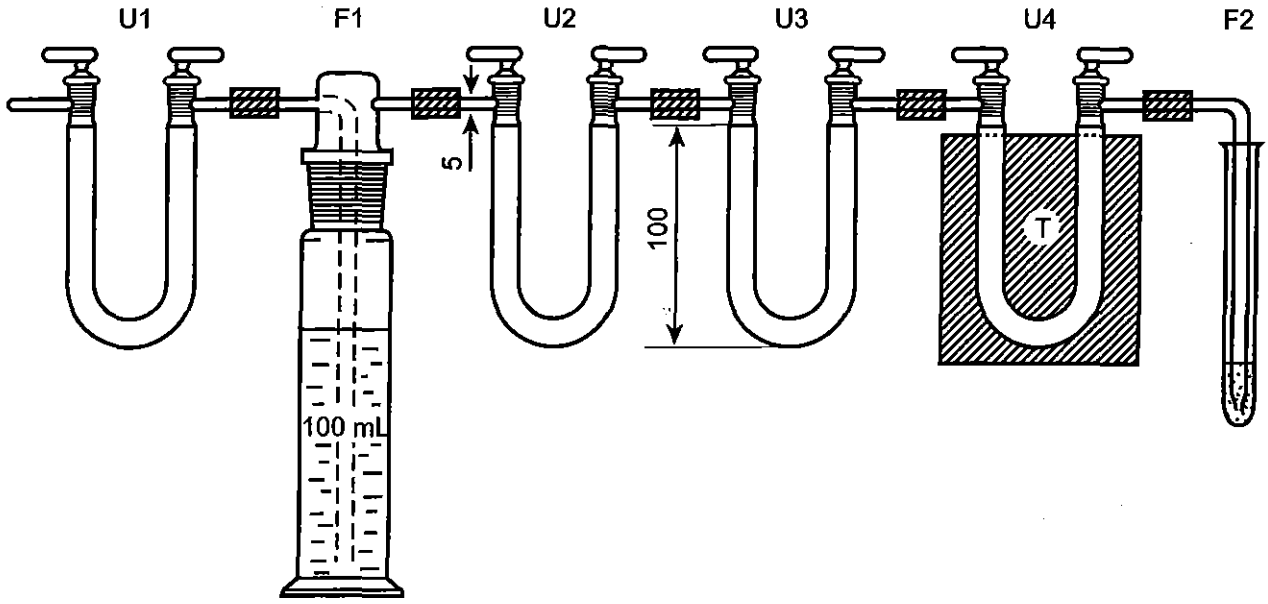


Figure 2.5.25.-1. – *Apparatus for the determination of carbon monoxide*
Dimensions in millimetres

When carbon monoxide is present in the sample cell, absorption of energy in the measurement light beam will occur according to the Beer-Lambert law and this produces a change in the detector signal. This measurement signal is compared to a reference signal to generate an output related to the concentration of carbon monoxide. The generated signal is linearised in order to obtain the carbon monoxide concentration. To prevent the entry of particles into the sensors, which could cause stray-light phenomena, the apparatus is fitted with a suitable filter.

Required technical specifications

When used for a limit test, the carbon monoxide infrared analyser meets the following technical specifications:

— *limit of detection*: (generally defined as a signal-to-noise ratio of 2) maximum 20 per cent of the maximum admissible concentration;

— *repeatability*: maximum relative standard deviation of 10 per cent of the maximum admissible concentration, determined on 6 measurements;

— *linearity*: maximum 10 per cent of the maximum admissible concentration.

The technical specifications must be met in the presence of the other gas impurities in the sample.

F. Determination of Carbon Dioxide in Medicinal Gases

(Ph. Eur. method 2.5.24)

Gases absorb light at one or more specific wavelengths. This property is widely used to allow highly selective measurement of their concentrations.

Description and principle of measurement

The concentration of carbon dioxide in other gases can be determined using an infrared analyser.

The infrared analyser generally consists of a light source emitting broadband infrared radiation, an optical device, a sample cell and a detector. The optical device may be positioned either before or after the sample cell and it consists of one or several optical filters, through which the broadband radiation is passed. The optical device in this case is selected for carbon dioxide. The measurement light beam passes through the sample cell and may also pass through a reference cell if the analyser integrates such a feature (some use an electronic system instead of a reference cell).

When carbon dioxide is present in the sample cell, absorption of energy in the measurement light beam will occur according to the Beer-Lambert law and this produces a change in the detector signal. This measurement signal is compared to a reference signal to generate an output related to the concentration of carbon dioxide. The generated signal is linearised in order to obtain the carbon dioxide concentration. To prevent the entry of particles into the sensors, which could cause stray-light phenomena, the apparatus is fitted with a suitable filter.

Required technical specifications

When used for a limit test, the infrared analyser meets the following technical specifications:

— *limit of detection*: (generally defined as a signal-to-noise ratio of 2) maximum 20 per cent of the maximum admissible concentration;

— *repeatability*: maximum relative standard deviation of 10 per cent of the maximum admissible concentration, determined on 6 measurements;

— *linearity*: maximum 10 per cent of the maximum admissible concentration.

The technical specifications must be met in the presence of the other gas impurities in the sample.

G. Determination of Nitrogen Monoxide and Nitrogen Dioxide in Medicinal Gases

(Ph. Eur. method 2.5.26)

Nitrogen monoxide and nitrogen dioxide in gases are determined using a chemiluminescence analyser (Figure 2.5.26.-1).

The apparatus consists of the following:

— a device for filtering, checking and controlling the flow of the gas to be examined,

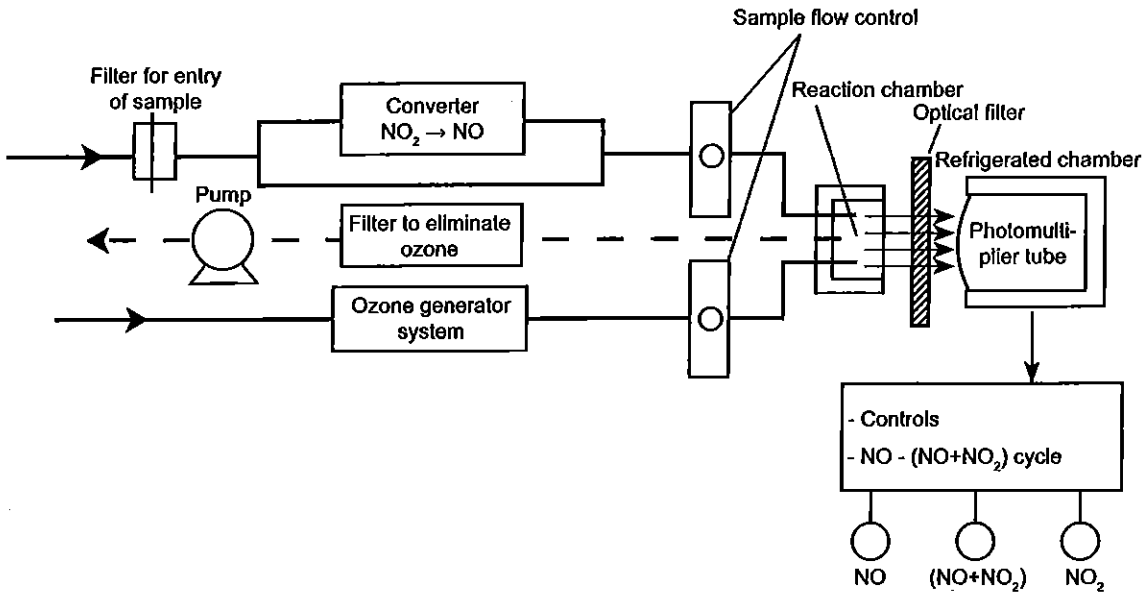


Figure 2.5.26.-1. – *Chemiluminescence analyser*

— a converter that reduces nitrogen dioxide to nitrogen monoxide, to determine the combined content of nitrogen monoxide and nitrogen dioxide. The efficiency of the converter has to be verified prior to use,
— a controlled-flow-rate ozone generator; the ozone is produced by high-voltage electric discharges across two electrodes; the ozone generator is supplied with pure oxygen or with dehydrated ambient air and the concentration of ozone obtained must greatly exceed the maximum content of any detectable nitrogen oxides,
— a chamber in which nitrogen monoxide and ozone can react,
— a system for detecting light radiation emitted at a wavelength of 1.2 µm, consisting of a selective optical filter and a photomultiplier tube.

H. Determination of Oxygen in Medicinal Gases

(Ph. Eur. method 2.5.27)

Oxygen in gases is determined using a paramagnetic analyser.

The principle of the method is based on the high paramagnetic sensitivity of the oxygen molecule. Oxygen exerts a strong interaction on magnetic fields, which is measured electronically, amplified and converted to a reading of oxygen concentration. The measurement of oxygen concentration is dependent upon the pressure and temperature and, if the analyser is not automatically compensated for variations in temperature and pressure, it must be calibrated immediately prior to use. As the paramagnetic effect of oxygen is linear, the instrument must have a suitable range with a readability of 0.1 per cent or better.

Calibration of the instrument Make the setting in the following manner:
— set the zero by passing *nitrogen R1* through the instrument until a constant reading is obtained;
— set the scale to 100 per cent by passing *oxygen R* through the instrument at the same flow rate as for *nitrogen R1* until a constant reading is obtained.

Assay Pass the gas to be examined through the instrument at a constant flow rate until a constant reading is obtained. Record the concentration of oxygen in the gas to be examined.

J. Determination of Water in Medicinal Gases

(Ph. Eur. method 2.5.28)

Water in gases is determined using an electrolytic hygrometer, described below.

The measuring cell consists of a thin film of diphosphorus pentoxide, between 2 coiled platinum wires which act as electrodes. The water vapour in the gas to be examined is absorbed by the diphosphorus pentoxide, which is transformed to phosphoric acid, an electrical conductor. A continuous voltage applied across the electrodes produces electrolysis of the water and the regeneration of the diphosphorus pentoxide. The resulting electric current, which is proportional to the water content in the gas to be examined, is measured. This system is self-calibrating since it obeys Faraday's law.

Take a sample of the gas to be examined. Allow the gas to stabilise at room temperature. Purge the cell continuously until a stable reading is obtained. Measure the water content in the gas to be examined, making sure that the temperature is constant throughout the device used to introduce the gas into the apparatus.

The electrolytic hygrometer achieves accurate sample flows by using a mass flow controller to deliver a constant volumetric flow rate to ensure that the water content is determined accurately. The calibration of the mass flow controller is normally performed using nitrogen. When using gases other than nitrogen for calibration, consult the manufacturer's instructions for the appropriate conversion factors and ensure that the correct cell is used for the type of gas to be examined.

K. Gas Detector Tubes

(Ph. Eur. method 2.1.6)

Gas detector tubes are cylindrical, sealed tubes consisting of an inert transparent material and are constructed to allow the passage of gas. They contain reagents adsorbed onto inert substrates that are suitable for the visualisation of the substance to be detected and, if necessary, they also contain preliminary layers and/or adsorbent filters to eliminate substances that interfere with the substance to be detected. The layer of indicator contains either a single reagent for the detection of a given impurity or several reagents for the detection of several substances (monolayer tube or multilayer tube).

The test is carried out by passing the required volume of the gas to be examined through the indicator tube. The length of the coloured layer or the intensity of a colour change on a graduated scale gives an indication of the impurities present.

The calibration of the detector tubes is verified according to the manufacturer's instructions.

Operating conditions Examine according to the manufacturer's instructions or proceed as follows.

The gas supply is connected to a suitable pressure regulator and needle valve. Connect the flexible tubing fitted with a Y-piece to the valve and adjust the flow of gas to be examined to purge the tubing in order to obtain an appropriate flow (Figure 2.1.6.-1). Prepare the indicator tube and fit to the metering pump, following the manufacturer's instructions. Connect the open end of the indicator tube to the short leg of the tubing and operate the pump by the appropriate number of strokes to pass a suitable volume of gas to be examined through the tube. Read the value corresponding to the length of the coloured layer or the intensity of the colour on the graduated scale. If a negative result is achieved, indicator tubes can be verified with a calibration gas containing the appropriate impurity.

In view of the wide variety of available compressor oils, it is necessary to verify the reactivity of the oil detector tubes for the oil used. Information on the reactivity for various oils is given in the leaflet supplied with the tube. If the oil used is not cited in the leaflet, the tube manufacturer must verify the reactivity and if necessary provide a tube specific for this oil.

Arsine detector tube

Sealed glass tube containing adsorbent filters and suitable supports for the gold salt or other appropriate indicator.

The minimum value indicated is 0.25 ppm or less, with a relative standard deviation of at most 20 per cent.

Carbon dioxide detector tube
Sealed glass tube containing adsorbent filters and suitable supports for hydrazine and crystal violet indicators. The minimum value indicated is 100 ppm with a relative standard deviation of at most 15 per cent.

Carbon monoxide detector tube
Sealed glass tube containing adsorbent filters and suitable supports for di-iodine pentoxide, selenium dioxide and fuming sulfuric acid indicators. The minimum value indicated is 5 ppm or less, with a relative standard deviation of at most 15 per cent.

Hydrogen sulfide detector tube
Sealed glass tube containing adsorbent filters and suitable supports for an appropriate lead salt indicator. The minimum value indicated is 0.2 ppm or less, with a relative standard deviation of at most 10 per cent.

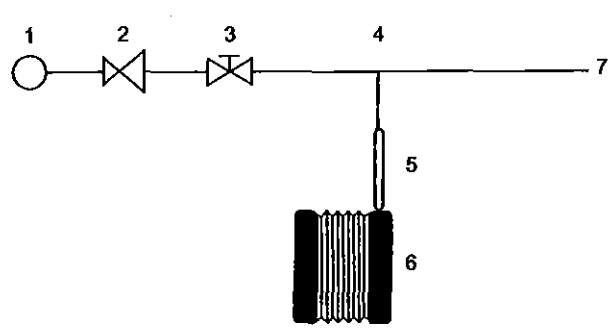
Nitrogen monoxide and nitrogen dioxide detector tube
Sealed glass tube containing adsorbent filters and suitable supports for an oxidising layer (Cr(VI) salt) and the diphenylbenzidine indicator. The minimum value indicated is 0.5 ppm with a relative standard deviation of at most 15 per cent.

Oil detector tube
Sealed glass tube containing adsorbent filters and suitable supports for the sulfuric acid indicator. The minimum value indicated is 0.1 mg/m^3 with a relative standard deviation of at most 30 per cent.

Phosphine detector tube
Sealed glass tube containing adsorbent filters and suitable supports for the gold salt or other appropriate indicator. The minimum value indicated is 0.2 ppm or less, with a relative standard deviation of at most 20 per cent.

Sulfur dioxide detector tube
Sealed glass tube containing adsorbent filters and suitable supports for the iodine and starch indicator. The minimum value indicated is 0.5 ppm with a relative standard deviation of at most 15 per cent.

Water vapour detector tube
Sealed glass tube containing adsorbent filters and suitable supports for the magnesium perchlorate indicator. The minimum value indicated is 67 ppm or less, with a relative standard deviation of at most 20 per cent.



1. Gas supply
2. Pressure regulator
3. Needle valve
4. Y-piece
5. Indicator tube
6. Indicator tube pump
7. End open to atmosphere

Figure 2.1.6.-1. – *Apparatus for gas detector tubes*

L. Determination of Nitrous Oxide in Gases

(Ph. Eur. method 2.5.35)

Gases absorb light at one or more specific wavelengths. This property is widely used to allow highly selective measurement of their concentrations.

Description and principle of measurement
The concentration of nitrous oxide in other gases can be determined using an infrared analyser.

The infrared analyser generally consists of a light source emitting broadband infrared radiation, an optical device, a sample cell and a detector. The optical device may be positioned either before or after the sample cell and it consists of one or several optical filters, through which the broadband radiation is passed. The optical device in this case is selected for nitrous oxide. The measurement light beam passes through the sample cell and may also pass through a reference cell if the analyser integrates such a feature (some use an electronic system instead of a reference cell).

When nitrous oxide is present in the sample cell, absorption of energy in the measurement light beam will occur according to the Beer-Lambert law and this produces a change in the detector signal. This measurement signal is compared to a reference signal to generate an output related to the concentration of nitrous oxide. The generated signal is linearised in order to obtain the nitrous oxide concentration. To prevent the entry of particles into the sensors, which could cause stray-light phenomena, the apparatus is fitted with a suitable filter.

M. Water-Solid Interactions: Determination of Sorption-Desorption Isotherms and of Water Activity[1]

(Ph. Eur. method 2.9.39)

INTRODUCTION
Pharmaceutical solids as raw materials or as constituents of dosage forms most often come in contact with water during processing and storage. This may occur (a) during crystallisation, lyophilisation, wet granulation, or spray drying; and (b) because of exposure upon handling and storage to an atmosphere containing water vapour or exposure to other materials in a dosage form that contain water capable of distributing it to other ingredients. Some properties known to be altered by the association of solids with water include rates of chemical degradation in the "solid-state", crystal growth and dissolution, dispersibility and wetting, powder flow, lubricity, powder compactibility, compact hardness and microbial contamination.

Although precautions can be taken when water is perceived to be a problem, i.e. eliminating all moisture, reducing contact with the atmosphere, or controlling the relative humidity of the atmosphere, such precautions generally add expense to the process with no guarantee that during the life of the product further problems associated with moisture will be avoided. It is also important to recognise that there are many situations where a certain level of water in a solid is required for proper performance, e.g. powder compaction.

[1] *This chapter has undergone pharmacopoeial harmonisation. See chapter 5.8. Pharmacopoeial harmonisation.*

It is essential for both reasons, therefore, that as much as possible is known about the effects of moisture on solids before strategies are developed for their handling, storage and use.

Some of the more critical pieces of required information concerning water-solid interactions are:

- total amount of water present;
- the extent to which adsorption and absorption occur;
- whether or not hydrates form;
- specific surface area of the solid, as well as such properties as degree of crystallinity, degree of porosity, and glass transition and melting temperature;
- site of water interaction, the extent of binding, and the degree of molecular mobility;
- effects of temperature and relative humidity;
- essentially irreversible hydration;
- kinetics of moisture uptake;
- various factors that might influence the rate at which water vapour can be taken up by a solid;
- for water-soluble solids capable of being dissolved by the sorbed water, under which conditions dissolution will take place.

PHYSICAL STATES OF SORBED WATER

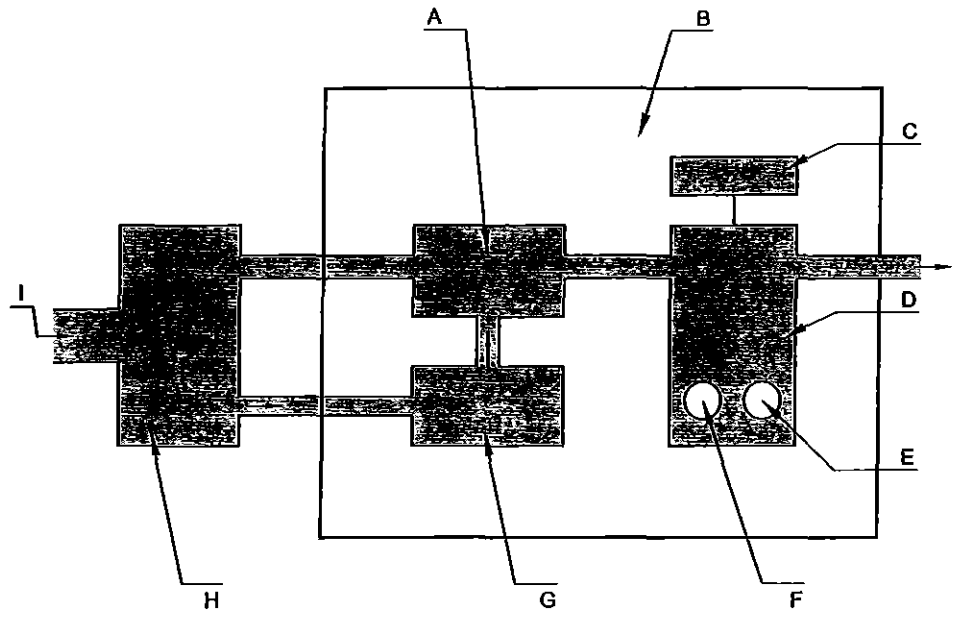
Water can physically interact with solids in different ways. It can interact at the surface (adsorption) or it can penetrate the bulk solid structure (absorption). When both adsorption and absorption occur, the term sorption is often used. Adsorption is particularly critical in affecting the properties of solids when the specific surface area is large. Large values of specific surface area are seen with solids having very small particles, as well as with solids having a high degree of intraparticle porosity. Absorption is characterised by an association of water per gram of solid that is much greater than that which can form a monomolecular layer on the available surface, and an amount that is generally independent of the specific surface area.

Most crystalline solids will not absorb water into their bulk structures because of the close packing and high degree of order of the crystal lattice. Indeed, it has been shown that the degree of absorption into solids exhibiting partial crystallinity and partial amorphous structure is often inversely proportional to the degree of crystallinity. With some crystalline solids, however, crystal hydrates may form. These hydrates may exhibit a stoichiometric relationship, in terms of water molecules bound per solid molecule, or they may be non-stoichiometric. Upon dehydration, crystal hydrates may either retain their original crystal structure, or lose their crystallinity and become amorphous, or transform into a new anhydrous or less-hydrated crystal form.

Amorphous or partially amorphous solids are capable of taking up significant amounts of water because there is sufficient molecular disorder in the solid to permit penetration, swelling or dissolution. Such behaviour is observed with most amorphous polymers and with small-molecular-mass solids rendered amorphous during preparation, e.g. by lyophilisation, or after milling. The introduction of defects into highly crystalline solids will also produce this behaviour. The greater the chemical affinity of water for the solid, the greater the total amount that can be absorbed. When water is absorbed by amorphous solids, the bulk properties of the solid can be significantly altered. It is well established, for example, that amorphous solids, depending on the temperature, can exist in at least one of 2 states, "glassy" or "fluid"; the temperature at which one state transforms into the other is the glass transition temperature, T_g.

Water absorbed into the bulk solid structure, by virtue of its effect on the free volume of the solid, can act as an efficient plasticiser and reduce the value of T_g. Since the rheological properties of "fluid" and "glassy" states are quite different, i.e. the "fluid" state exhibits much less viscosity as one goes increasingly above the glass transition temperature, it is not surprising that a number of important bulk properties dependent on the rheology of the solid are affected by moisture content. Since amorphous solids are metastable relative to the crystalline form of the material, with small-molecular-mass materials, it is possible for absorbed moisture to initiate reversion of the solid to the crystalline form, particularly if the solid is transformed by the sorbed water to a "fluid" state. This is the basis of "cake collapse" often observed during the lyophilisation process. An additional phenomenon noted specifically with water-soluble solids is their tendency to deliquesce, i.e. to dissolve in their own sorbed water, at relative humidities, RH_i, in excess of the relative humidity of a saturated solution of the solid, RH_0. Deliquescence arises because of the high water solubility of the solid and the significant effect it has on the colligative properties of water. It is a dynamic process that continues to occur as long as RH_i is greater than RH_0.

The key to understanding the effects water can have on the properties of solids, and vice versa, rests with an understanding of the location of the water molecule and its physical state. More specifically, water associated with solids can exist in a state that is directly bound to the solid, as well as in a state of mobility approaching that of bulk water. This difference in mobility has been observed through such measurements as heats of sorption, freezing point, nuclear magnetic resonance, dielectric properties and diffusion. Such changes in mobility have been interpreted as arising because of changes in the thermodynamic state of water as more and more water is sorbed. Thus, water bound directly to a solid is often thought as unavailable to affect the properties of the solid, whereas larger amounts of sorbed water may become more clustered and form water more like that exhibiting solvent properties. In the case of crystal hydrates, the combination of intermolecular forces (hydrogen bonding) and crystal packing can produce very strong water-solid interactions. Recognising that the presence of water in an amorphous solid can affect the glass transition temperature and hence the physical state of the solid, at low levels of water, most polar amorphous solids are in a highly viscous glassy state because of their high values of T_g. Hence, water is "frozen" into the solid structure and is rendered immobile by the high viscosity, e.g. 10^{13} Pa·s. As the amount of water sorbed increases and T_g decreases, approaching ambient temperatures, the glassy state approaches that of a "fluid" state and water mobility along with the mobility of the solid itself increases significantly. At high RH, the degree of water plasticisation of the solid can be sufficiently high so that water and the solid can now achieve significant amounts of mobility. In general, therefore, this picture of the nature of sorbed water helps to explain the rather significant effect moisture can have on a number of bulk properties of solids such as chemical reactivity and mechanical deformation. It suggests strongly that methods of evaluating chemical and physical stability of solids and solid dosage forms take into account the effects water can have on the solid when it is sorbed, particularly when it enters the solid structure and acts as a plasticiser.



A. Humidity controller
B. Temperature controlled chamber
C. Balance module
D. Humidity regulated module
E. Reference
F. Sample
G. Vapour humidifier
H. Flow control module
I. Dry gas

Figure 2.9.39.-1. – *Example of an apparatus for the determination of the water sorption (other designs are possible)*

Rates of water uptake

The rate and extent to which solids exposed to the atmosphere might either sorb or desorb water vapour can be a critical factor in the handling of solids. Even the simple act of weighing out samples of solid on an analytical balance and the exposure, therefore, of a thin layer of powder to the atmosphere for a few minutes can lead to significant error in, for example, the estimation of loss on drying values. It is well established that water-soluble solids exposed to relative humidities above that exhibited by a saturated solution of that solid will spontaneously dissolve via deliquescence and continue to dissolve over a long time period. The rate of water uptake in general depends on a number of parameters not found to be critical in equilibrium measurements because rates of sorption are primarily mass-transfer controlled with some contributions from heat-transfer mechanisms. Thus, factors such as vapour diffusion coefficients in air and in the solid, convective airflow, and the surface area and geometry of the solid bed and surrounding environment, can play an important role. Indeed, the method used to make measurements can often be the rate-determining factor because of these environmental and geometric factors.

DETERMINATION OF SORPTION-DESORPTION ISOTHERMS

Principle

The tendency to take up water vapour is best assessed by measuring sorption or desorption as a function of relative humidity, at constant temperature, and under conditions where sorption or desorption is essentially occurring independently of time, i.e. equilibrium. Relative humidity, *RH*, is defined by the following expression:

$$\frac{P_c}{P_0} \times 100$$

P_c = pressure of water vapour in the system;
P_0 = saturation pressure of water vapour under the same conditions.

The ratio P_c/P_0 is referred to as the relative pressure. Sorption or water uptake is best assessed starting with dried samples and subjecting them to a known relative humidity. Desorption is studied by beginning with a system already containing sorbed water and reducing the relative humidity. As the name indicates, the sorption-desorption isotherm is valid only for the reference temperature, hence a special isotherm exists for each temperature. Ordinarily, at equilibrium, moisture content at a particular relative humidity must be the same, whether determined from sorption or desorption measurements. However, it is common to see sorption-desorption hysteresis.

Methods

Samples may be stored in chambers at various relative humidities (Figure 2.9.39.-1). The mass gained or lost for each sample is then measured. The major advantage of this method is convenience, while the major disadvantages are the slow rate of reaching constant mass, particularly at high relative humidities, and the error introduced in opening and closing the chamber for weighing.

Dynamic gravimetric water sorption systems allow the on-line weighing of a sample in a controlled system to assess the interaction of the material with moisture at various programmable levels of relative humidity at a constant temperature. The major benefit of a controlled system is that isothermal conditions can be more reliably established and that the dynamic response of the sample to changing conditions can be monitored. Data points for the determination of the sorption isotherm (e.g. from 0 per cent to approximately 95 per cent *RH*, non condensing) are only taken after a sufficiently constant signal indicates that the sample has reached equilibrium at a given level of humidity. In some cases (e.g. deliquescence), the maximum time may be restricted although the equilibrium level is not reached. The apparatus must adequately control the temperature to ensure a good baseline stability as well as accurate control of

the relative humidity generation. The required relative humidities can be generated, e.g. by accurately mixing dry and saturated vapour gas with flow controllers.
The electrostatic behaviour of the powder must also be considered. The verification of the temperature and the relative humidity (controlled with, for example, a certified hygrometer, certified salt solutions or deliquescence points of certified salts over an adequate range), must be consistent with the instrument specification. The balance must provide a sufficient mass resolution and long term stability.

It is also possible to measure amounts of water uptake not detectable gravimetrically using volumetric techniques.
In some cases, direct analysis of water content by different methods such as determination of the boiling point, determination of water by distillation, loss on drying or gas chromatography may be advantageous. In the case of adsorption, to improve sensitivity, one can increase the specific surface area of the sample by reducing particle size or by using larger samples to increase the total area. It is important, however, that such comminution of the solid does not alter the surface structure of the solid or render it more amorphous or otherwise less ordered in crystallinity.
For absorption, where water uptake is independent of specific surface area, only increasing sample size will help. Increasing sample size, however, will increase the time to establish some type of equilibrium. To establish accurate values, it is important to get desolvation of the sample as thoroughly as possible. Higher temperatures and lower pressures (vacuum) facilitate this process; however, one must be aware of any adverse effects this might have on the solid such as dehydration, chemical degradation or sublimation. Using higher temperatures to induce desorption, as in a thermogravimetric apparatus, likewise must be carefully carried out because of these possible pitfalls.

Report and interpretation of the data
Sorption data are usually reported as a graph of the apparent mass change in per cent of the mass of the dry sample as a function of relative humidity or time. Sorption isotherms are reported both in tabular form and as a graph.
The measurement method must be traceable with the data.

Adsorption-desorption hysteresis can be interpreted, for example, in terms of the porosity of the sample, its state of agglomeration (capillary condensation), the formation of hydrates, polymorphic change, or liquefying of the sample. Certain types of systems, particularly those with microporous solids and amorphous solids, are capable of sorbing large amounts of water vapour. Here, the amount of water associated with the solid as relative humidity is decreased, is greater than the amount that originally sorbed as the relative humidity was increased. For microporous solids, vapour adsorption-desorption hysteresis is an equilibrium phenomenon associated with the process of capillary condensation. This takes place because of the high degree of irregular curvature of the micropores and the fact that they "fill" (adsorption) and "empty" (desorption) under different equilibrium conditions. For non-porous solids capable of absorbing water, hysteresis occurs because of a change in the degree of vapour-solid interaction due to a change in the equilibrium state of the solid, e.g. conformation of polymer chains, or because the time scale for structural equilibrium is longer than the time scale for water desorption. In measuring sorption-desorption isotherms, it is therefore important to establish that something close to an equilibrium state has been reached. Particularly with hydrophilic polymers at high relative humidities, the establishment of water sorption or desorption values independent of time is quite difficult, since one is usually dealing with a polymer plasticised into its "fluid" state, where the solid is undergoing significant change.

In the case of crystal hydrate formation, the plot of water uptake versus pressure or relative humidity will in these cases exhibit a sharp increase in uptake at a particular pressure and the amount of water taken up will usually exhibit a stoichiometric mole:mole ratio of water to solid. In some cases, however, crystal hydrates will not appear to undergo a phase change or the anhydrous form will appear amorphous. Consequently, water sorption or desorption may appear more like that seen with adsorption processes. X-ray crystallographic analysis and thermal analysis are particularly useful for the study of such systems.

For situations where water vapour adsorption occurs predominantly, it is very helpful to measure the specific surface area of the solid by an independent method and to express adsorption as mass of water sorbed per unit area of solid surface. This can be very useful in assessing the possible importance of water sorption in affecting solid properties. For example, 0.5 per cent *m/m* uptake of water could hardly cover the bare surface of 100 m^2/g, while for 1.0 m^2/g this amounts to 100 times more surface coverage. In the case of pharmaceutical solids which have a specific surface area in the range of 0.01 m^2/g to 10 m^2/g, what appears to be low water content could represent a significant amount of water for the available surface. Since the "dry surface area" is not a factor in absorption, sorption of water with amorphous or partially amorphous solids can be expressed on the basis of unit mass corrected for crystallinity, when the crystal form does not sorb significant amounts of water relative to the amorphous regions.

DETERMINATION OF THE WATER ACTIVITY

Principle
Water activity, A_w, is the ratio of vapour pressure of water in the product (P) to saturation pressure of water vapour (P_0) at the same temperature. It is numerically equal to 1/100 of the relative humidity (*RH*) generated by the product in a closed system. *RH* can be calculated from direct measurements of partial vapour pressure or dew point, or from indirect measurement by sensors whose physical or electric characteristics are altered by the *RH* to which they are exposed. Ignoring activity coefficients, the relationship between A_w and equilibrium relative humidity (*ERH*) are represented by the following equations:

$$A_w = \frac{P}{P_0}$$

$$ERH \text{ (per cent)} = A_w \times 100$$

Method
The water activity is determined by placing the sample in a small airtight cup inside which the equilibrium between the water in the solid and the headspace can be established. The volume of the headspace must be small in relation to the sample volume in order not to change the sorption state of sample during the test. The equilibration as a thermodynamic process takes time but may be accelerated by forced circulation within the cell. The acquired water activity value is only valid for the simultaneously determined temperature. This requires a precise temperature-measuring device as part of the equipment. Furthermore, the probe must be thermally insulated to guarantee a constant temperature during the test. The sensor measuring the humidity of the headspace air above the sample is a key

component. Theoretically, all types of hygrometers can be used, but for analytical purposes miniaturisation and robustness are a precondition. The A_w measurement may be conducted using the dew point/chilled mirror method[2]. A polished, chilled mirror is used as a condensing surface. The cooling system is electronically linked to a photoelectric cell into which light is reflected from the condensing mirror. An air stream, in equilibrium with the test sample, is directed at the mirror, which cools until condensation occurs on the mirror. The temperature at which this condensation begins is the dew point from which the *ERH* is determined. Commercially available instruments using the dew point/chilled mirror method or other technologies need to be evaluated for suitability, qualified, and calibrated when used to make water activity determinations. These instruments are typically calibrated over an adequate range, for example, using some saturated salt solutions at 25 °C such as those listed in Table 2.9.39.-1.

Table 2.9.39.-1. – *Standard saturated salt solutions*

Saturated salts solutions at 25 °C	*ERH* (per cent)	A_w
Potassium sulfate (K_2SO_4)	97.3	0.973
Barium chloride ($BaCl_2$)	90.2	0.902
Sodium chloride (NaCl)	75.3	0.753
Magnesium nitrate ($Mg(NO_3)_2$)	52.9	0.529
Magnesium chloride ($MgCl_2$)	32.8	0.328
Lithium chloride (LiCl)	11.2	0.112

[2] *AOAC International Official Method 978.18.*

Appendix X

A. Acetyl Value

(No Ph. Eur. method)

The acetyl value of a substance is the number of mg of potassium hydroxide required to neutralise the acetic acid liberated by the hydrolysis of 1 g of the acetylated substance.

Determine the *saponification value*, Appendix X G.

Acetylate by the following method. To 10 g in a 200-mL Kjeldahl flask add 20 mL of *acetic anhydride*. Support the flask on a sheet of heat resistant material in which a hole about 4 cm in diameter has been cut and heat with a small, naked flame, not more than 25 mm in height and which does not impinge on the bottom of the flask. Boil gently under a reflux air condenser for 2 hours, allow to cool, pour into 600 mL of *water* contained in a large beaker, add 0.2 g of *pumice powder* and boil for 30 minutes. Cool, transfer to a separating funnel and discard the lower layer. Wash the acetylated product with three or more 50-mL quantities of a warm, saturated solution of *sodium chloride* until the washings are no longer acidic to *litmus paper*. Finally shake with 20 mL of warm *water* and remove the aqueous layer as completely as possible. Pour the acetylated substance into a small dish, add 1 g of powdered *anhydrous sodium sulfate*, stir thoroughly and filter through a dry, pleated filter paper. Determine the *saponification value* of the acetylated substance.

Calculate the acetyl value from the expression $1335(b-a)/(1335-a)$ where a is the saponification value of the substance and b is the saponification value of the acetylated substance.

B. Acid Value

(Ph. Eur. method 2.5.1)

The acid value I_A is the number that expresses, in milligrams the quantity of potassium hydroxide required to neutralise the free acids present in 1 g of the substance.

Dissolve 10.00 g of the substance to be examined, or the quantity prescribed, (m g), in 50 mL of a mixture of equal volumes of *ethanol (96 per cent) R* and *light petroleum R3*, previously neutralised with *0.1 M potassium hydroxide* or *0.1 M sodium hydroxide*, unless otherwise specified, using 0.5 mL of *phenolphthalein solution R1* as indicator.

If necessary, heat to about 90 °C to dissolve the substance to be examined. When the substance to be examined has dissolved, titrate with *0.1 M potassium hydroxide* or *0.1 M sodium hydroxide* until the pink colour persists for at least 15 s (n mL of titrant). When heating has been applied to aid dissolution, maintain the temperature at about 90 °C during the titration.

$$I_A = \frac{5.611n}{m}$$

C. Ester Value

(Ph. Eur. method 2.5.2)

The ester value I_E is the number that expresses in milligrams the quantity of potassium hydroxide required to saponify the esters present in 1 g of the substance. It is calculated from the saponification value I_S and the acid value I_A:

$$I_E = I_S - I_A$$

D. Hydroxyl Value

(Ph. Eur. method 2.5.3)

The hydroxyl value I_{OH} is the number that expresses in milligrams the quantity of potassium hydroxide required to neutralise the acid combined by acylation in 1 g of the substance.

METHOD A

Introduce the quantity of the substance to be examined shown in Table 2.5.3.-1 (m g) into a 150 mL acetylation flask fitted with an air condenser, unless another quantity is prescribed in the monograph. Add the quantity of *acetic anhydride solution R1* stated in Table 2.5.3.-1 and attach the air condenser.

Table 2.5.3.-1

Presumed value I_{OH}	Quantity of sample (g)	Volume of acetylating reagent (mL)
10 - 100	2.0	5.0
100 - 150	1.5	5.0
150 - 200	1.0	5.0
200 - 250	0.75	5.0
250 - 300	0.60 or 1.20	5.0 or 10.0
300 - 350	1.0	10.0
350 - 700	0.75	15.0
700 - 950	0.5	15.0

Heat the flask in a water-bath for 1 h keeping the level of the water about 2.5 cm above the level of the liquid in the flask. Withdraw the flask and allow to cool. Add 5 mL of *water R* through the upper end of the condenser. If a cloudiness appears add sufficient *pyridine R* to clear it, noting the volume added. Shake the flask and replace in the water-bath for 10 min. Withdraw the flask and allow to cool. Rinse the condenser and the walls of the flask with 5 mL of *alcohol R*, previously neutralised to *phenolphthalein solution R1*. Titrate with 0.5 M alcoholic potassium hydroxide using 0.2 mL of *phenolphthalein solution R1* as indicator (n_1 mL of 0.5 M alcoholic potassium hydroxide). Carry out a blank test under the same conditions (n_2 mL of 0.5 M alcoholic potassium hydroxide).

$$I_{OH} = \frac{28.05(n_2 - n_1)}{m} + I_A$$

METHOD B

Introduce the prescribed quantity of the substance to be examined (m g) into a perfectly dry 5 mL conical flask fitted with a ground-glass or suitable plastic stopper and add 2.0 mL of *propionic anhydride reagent R*. Close the flask and shake gently to dissolve the substance. Allow to stand for 2 h unless otherwise prescribed. Remove the stopper and transfer the flask and its contents into a wide-mouthed 500 mL conical flask containing 25.0 mL of a 9 g/L solution of *aniline R* in *cyclohexane R* and 30 mL of *glacial acetic acid R*. Swirl the contents of the flask, allow to stand for 5 min, add 0.05 mL of *crystal violet solution R* and titrate with *0.1 M perchloric acid* until an emerald-green colour is obtained (n_1 mL of *0.1 M perchloric acid*). Carry out a blank test under the same conditions (n_2 mL of *0.1 M perchloric acid*).

$$I_{OH} = \frac{5.610(n_1 - n_2)}{m}$$

To take account of any water present, determine this (y per cent) by the semi-micro determination of water (*2.5.12*).

The hydroxyl value is then given by the equation:

$$I_{OH} = \text{(hydroxyl value as determined)} - 31.1y$$

E. Iodine Value

(Ph. Eur. method 2.5.4)

The iodine value I_I is the number that expresses in grams the quantity of halogen, calculated as iodine, that can be fixed in the prescribed conditions by 100 g of the substance.

When the monograph does not specify the method to be used, method A is applied. Any change from method A to method B is validated.

METHOD A

Unless otherwise prescribed, use the following quantities (Table 2.5.4.-1) for the determination.

Table 2.5.4.-1

Presumed value I_I	Quantity of sample (g)
less than 20	1.0
20 - 60	0.5 - 0.25
60 - 100	0.25 - 0.15
more than 100	0.15 - 0.10

Introduce the prescribed quantity of the substance to be examined (m g) into a 250 mL flask fitted with a ground-glass stopper and previously dried or rinsed with *glacial acetic acid R*, and dissolve it in 15 mL of *chloroform R* unless otherwise prescribed. Add very slowly 25.0 mL of *iodine bromide solution R*. Close the flask and keep it in the dark for 30 min unless otherwise prescribed, shaking frequently. Add 10 mL of a 100 g/L solution of *potassium iodide R* and 100 mL of *water R*. Titrate with *0.1 M sodium thiosulfate*, shaking vigorously until the yellow colour is almost discharged. Add 5 mL of *starch solution R* and continue the titration adding the *0.1 M sodium thiosulfate* dropwise until the colour is discharged (n_1 mL of *0.1 M sodium thiosulfate*). Carry out a blank test under the same conditions (n_2 mL of *0.1 M sodium thiosulfate*).

$$I_I = \frac{1.269\ (n_2 - n_1)}{m}$$

METHOD B

Unless otherwise prescribed, use the following quantities (Table 2.5.4.-2) for the determination.

Table 2.5.4.-2

Presumed value I_I	Mass (g) (corresponding to an excess of 150 per cent ICl)	Mass (g) (corresponding to an excess of 100 per cent ICl)	Iodine chloride solution (mL)
<3	10	10	25
3	8.4613	10.5760	25
5	5.0770	6.3460	25
10	2.5384	3.1730	20
20	0.8461	1.5865	20
40	0.6346	0.7935	20
60	0.4321	0.5288	20
80	0.3173	0.3966	20
100	0.2538	0.3173	20
120	0.2115	0.2644	20
140	0.1813	0.2266	20
160	0.1587	0.1983	20
180	0.1410	0.1762	20
200	0.1269	0.1586	20

The mass of the sample is such that there will be an excess of *iodine chloride solution R* of 50 per cent to 60 per cent of the amount added, i.e. 100 per cent to 150 per cent of the amount absorbed.

Introduce the prescribed quantity of the substance to be examined (*m* g) into a 250 mL flask fitted with a ground-glass stopper and previously rinsed with *glacial acetic acid R* or dried, and dissolve it in 15 mL of a mixture of equal volumes of *cyclohexane R* and *glacial acetic acid R*, unless otherwise prescribed. If necessary, melt the substance before dissolution (melting point greater than 50 °C). Add very slowly the volume of *iodine chloride solution R* stated in Table 2.5.4.-2. Close the flask and keep it in the dark for 30 min, unless otherwise prescribed, shaking frequently. Add 10 mL of a 100 g/L solution of *potassium iodide R* and 100 mL of *water R*. Titrate with *0.1 M sodium thiosulfate*, shaking vigorously until the yellow colour is almost discharged. Add 5 mL of *starch solution R* and continue the titration adding the *0.1 M sodium thiosulfate* dropwise until the colour is discharged (n_1 mL of *0.1 M sodium thiosulfate*). Carry out a blank test under the same conditions (n_2 mL of *0.1 M sodium thiosulfate*).

$$I_I = \frac{1.269\ (n_2 - n_1)}{m}$$

Iodine Monochloride Method

(No Ph. Eur. method)

When the use of iodine flasks is prescribed, use flasks with a nominal capacity of 250 mL and complying with British Standard 2735:1956 (Specification for iodine flasks), unless otherwise specified.

Dissolve the specified quantity of the substance being examined in 10 mL of *dichloromethane* in a dry iodine flask. Add 20 mL of *iodine monochloride solution*, insert the stopper, previously moistened with *dilute potassium iodide solution*, and allow to stand in the dark at 15° to 25° for 30 minutes. Place 15 mL of *dilute potassium iodide solution* in the top cup, carefully remove the stopper, rinse the stopper and the sides of the flask with 100 mL of *water*, shake and titrate with 0.1M *sodium thiosulfate VS* using *starch mucilage*, added towards the end of the titration, as indicator. At the same time carry out the operation in exactly the same manner, but without the substance being examined.

Calculate the iodine value from the expression 1.269 *v/w* where *v* is the difference, in mL, between the titrations and *w* is the weight, in g, of the substance taken.

The approximate weight, in g, of the substance to be taken may be calculated by dividing 20 by the highest expected iodine value. If more than half of the available halogen is absorbed, the test must be repeated, using a smaller quantity of the substance.

F. Peroxide Value

(Ph. Eur. method 2.5.5)

The peroxide value I_P is the number that expresses in milliequivalents of active oxygen the quantity of peroxide contained in 1000 g of the substance, as determined by the methods described below.

When the monograph does not specify the method to be used, method A is applied. Any change from method A to method B is validated.

METHOD A

Place 5.00 g of the substance to be examined (*m* g) in a 250 mL conical flask fitted with a ground-glass stopper. Add 30 mL of a mixture of 2 volumes of *chloroform R* and 3 volumes of *glacial acetic acid R*. Shake to dissolve the substance and add 0.5 mL of *saturated potassium iodide solution R*. Shake for exactly 1 min then add 30 mL of *water R*. Titrate with *0.01 M sodium thiosulfate*, adding the titrant slowly with continuous vigorous shaking, until the yellow colour is almost discharged. Add 5 mL of *starch solution R* and continue the titration, shaking vigorously, until the colour is discharged (n_1 mL of *0.01 M sodium thiosulfate*). Carry out a blank test under the same conditions (n_2 mL of *0.01 M sodium thiosulfate*). The volume of *0.01 M sodium thiosulfate* used in the blank titration must not exceed 0.1 mL.

$$I_p = \frac{10(n_1 - n_2)}{m}$$

METHOD B

Carry out the operations avoiding exposure to actinic light.

Place 50 mL of a mixture of 2 volumes of *trimethylpentane R* and 3 volumes of *glacial acetic acid R* in a conical flask and replace the stopper. Swirl the flask until the substance to be examined (*m* g; see Table 2.5.5.-1) has dissolved. Using a suitable volumetric pipette, add 0.5 mL of *saturated potassium iodide solution R* and replace the stopper. Allow the solution to stand for 60 ± 1 s, thoroughly shaking the solution continuously, then add 30 mL of *water R*.

Table 2.5.5.-1

Expected peroxide value I_p	Mass of substance to be examined (g)
0 to 12	5.00 to 2.00
12 to 20	2.00 to 1.20
20 to 30	1.20 to 0.80
30 to 50	0.800 to 0.500
50 to 90	0.500 to 0.300

Titrate the solution with *0.01 M sodium thiosulfate* (V_1 mL), adding it gradually and with constant, vigorous shaking, until the yellow iodine colour has almost disappeared. Add about 0.5 mL of *starch solution R1* and continue the titration, with constant shaking especially near the end-point, to liberate all of the iodine from the solvent layer. Add the sodium thiosulfate solution dropwise until the blue colour just disappears.

Depending on the volume of *0.01 M sodium thiosulfate* used, it may be necessary to titrate with *0.1 M sodium thiosulfate*.

NOTE There is a 15 s to 30 s delay in neutralising the starch indicator for peroxide values of 70 and greater, due to the tendency of trimethylpentane to float on the surface of the aqueous medium and the time necessary to adequately mix the solvent and the aqueous titrant, thus liberating the last traces of iodine. It is recommended to use *0.1 M sodium thiosulfate* for peroxide values greater than 150. A small amount (0.5 per cent to 1.0 per cent *m/m*) of high HLB emulsifier (for example polysorbate 60) may be added to the mixture to retard the phase separation and decrease the time lag in the liberation of iodine.

Carry out a blank determination (V_0 mL). If the result of the blank determination exceeds 0.1 mL of titration reagent, replace the reagents and repeat the determination.

$$I_p = \frac{1000(V_1 - V_0)c}{m}$$

c = concentration of the sodium thiosulfate solution, in moles per litre.

G. Saponification Value

The saponification value is the number of mg of potassium hydroxide required to neutralise the free acids and to saponify the esters in 1 g of the substance.

Use Method I unless otherwise specified in the monograph.

Method I

(No Ph. Eur. method)

Dissolve 35 to 40 g of *potassium hydroxide* in 20 mL of *water* and add sufficient *ethanol (96%)* to produce 1000 mL. Allow to stand overnight and pour off the clear liquid.

Weigh 2 g of the substance into a 200-mL flask, add 25.0 mL of the ethanolic solution of potassium hydroxide and boil under a reflux condenser for 1 hour, rotating the contents frequently. While the solution is still hot, titrate the excess of alkali with 0.5M *hydrochloric acid VS* using 1 mL of *phenolphthalein solution R1* as indicator. Repeat the operation without the substance being examined.

Calculate the saponification value from the expression 28.05 *v/w* where *v* is the difference, in mL, between the titrations and *w* is the weight, in g, of substance taken.

Method II

(Ph. Eur. method 2.5.6)

The saponification value I_S is the number that expresses in milligrams the quantity of potassium hydroxide required to neutralise the free acids and to saponify the esters present in 1 g of the substance.

Unless otherwise prescribed, use the quantities indicated in Table 2.5.6.-1 for the determination.

Table 2.5.6.-1

Presumed value I_S	Quantity of sample (g)
<3	20
3 to 10	12 to 15
10 to 40	8 to 12
40 to 60	5 to 8
60 to 100	3 to 5
100 to 200	2.5 to 3
200 to 300	1 to 2
300 to 400	0.5 to 1

Introduce the prescribed quantity of the substance to be examined (*m* g) into a 250 mL borosilicate glass flask fitted with a reflux condenser. Add 25.0 mL of 0.5 M alcoholic potassium hydroxide and a few glass beads. Attach the condenser and heat under reflux for 30 min, unless otherwise prescribed. Add 1 mL of *phenolphthalein solution R1* and titrate immediately (while still hot) with *0.5 M hydrochloric acid* (n_1 mL of *0.5 M hydrochloric acid*). Carry out a blank test under the same conditions (n_2 mL of *0.5 M hydrochloric acid*).

$$I_S = \frac{28.05(n_2 - n_1)}{m}$$

H. Unsaponifiable Matter

The unsaponifiable matter is the percentage content, w/w, of material not volatile at 100° to 105° that is obtained by extraction with an organic solvent from the saponified substance being examined.

Use Method I unless otherwise specified in the monograph.
Use ungreased ground-glass glassware for each method.

Method I

(No Ph. Eur. method)

To 2.0 to 2.5 g of the substance being examined in a 250-mL flask add 25 mL of 0.5M *ethanolic potassium hydroxide* and boil under a reflux condenser in a water-bath for 1 hour, swirling the contents frequently. Wash the contents of the flask into a separating funnel with the aid of 50 mL of *water* and, while the liquid is still slightly warm, extract by shaking vigorously with three 50-mL quantities of *peroxide-free ether*, rinsing the flask with the first quantity of ether. Mix the ether solutions in a separating funnel containing 20 mL of *water*. (If the ether solutions contain solid suspended matter, filter them into the separating funnel through a fat-free filter paper and wash the filter paper with *peroxide-free ether*.) Gently rotate the separating funnel for a few minutes without violent shaking, allow the liquids to separate and discard the aqueous layer. Wash the ether solution by shaking vigorously with two 20-mL quantities of *water* and then treat with three 20-mL quantities of 0.5M *potassium hydroxide*, shaking vigorously on each occasion, each treatment being followed by washing with 20 mL of *water*. Finally wash with successive 20-mL quantities of *water* until the aqueous layer is no longer alkaline to *phenolphthalein solution R1*. Transfer the ether extract to a weighed flask, rinsing the separating funnel with *peroxide-free ether*, distil the ether and add 3 mL of *acetone* to the flask. With the aid of a gentle current of air, remove the solvent completely from the flask, which is almost entirely immersed in boiling water and preferably held obliquely and rotated. Dry to constant weight at a temperature not exceeding 80° and dissolve the contents of the flask in 10 mL of freshly boiled *ethanol (96%)*, previously neutralised to *phenolphthalein solution R1*. Titrate with 0.1M *ethanolic sodium hydroxide VS* using *phenolphthalein solution R1* as indicator.

If the volume of 0.1M *ethanolic sodium hydroxide VS* required does not exceed 0.1 mL, the amount of residue weighed is to be taken as the unsaponifiable matter. Calculate the unsaponifiable matter as a percentage of the substance being examined.

If the volume of 0.1M *ethanolic sodium hydroxide VS* required exceeds 0.1 mL, the amount of residue weighed cannot be taken as the unsaponifiable matter and the test must be repeated.

Method II

(Ph. Eur. method 2.5.7)

The term "unsaponifiable matter" is applied to the substances non-volatile at 100-105 °C obtained by extraction with an organic solvent from the substance to be examined after it has been saponified. The result is calculated as per cent *m/m*.

Use ungreased ground-glass glassware.

Introduce the prescribed quantity of the substance to be examined (*m* g) into a 250 mL flask fitted with a reflux condenser. Add 50 mL of *2 M alcoholic potassium hydroxide R* and heat on a water-bath for 1 h, swirling frequently. Cool to a temperature below 25 °C and transfer the contents of the

flask to a separating funnel with the aid of 100 mL of *water R*. Shake the liquid carefully with 3 quantities, each of 100 mL, of *peroxide-free ether R*. Combine the ether layers in another separating funnel containing 40 mL of *water R*, shake gently for a few minutes, allow to separate and reject the aqueous phase. Wash the ether phase with 2 quantities, each of 40 mL, of *water R* then wash successively with 40 mL of a 30 g/L solution of *potassium hydroxide R* and 40 mL of *water R*; repeat this procedure 3 times. Wash the ether phase several times, each with 40 mL of *water R*, until the aqueous phase is no longer alkaline to phenolphthalein. Transfer the ether phase to a tared flask, washing the separating funnel with *peroxide-free ether R*.

Distil off the ether with suitable precautions and add 6 mL of *acetone R* to the residue. Carefully remove the solvent in a current of air. Dry to constant mass at 100-105 °C. Allow to cool in a desiccator and weigh (*a* g).

$$\text{Unsaponifiable matter} = \frac{100a}{m} \text{ per cent}$$

Dissolve the residue in 20 mL of *alcohol R*, previously neutralised to *phenolphthalein solution R* and titrate with *0.1 M ethanolic sodium hydroxide*. If the volume of *0.1 M ethanolic sodium hydroxide* used is greater than 0.2 mL, the separation of the layers has been incomplete; the residue weighed cannot be considered as "unsaponifiable matter". In case of doubt, the test must be repeated.

J. Determination of Cineole

(Ph. Eur. method 2.8.11)

Weigh 3.00 g of the oil, recently dried with *anhydrous sodium sulfate R*, into a dry test-tube and add 2.10 g of melted *cresol R*. Place the tube in the apparatus for the determination of freezing point (*2.2.18*) and allow to cool, stirring continuously. When crystallisation takes place there is a small rise in temperature. Note the highest temperature reached (t_1).

Remelt the mixture on a water-bath at a temperature that does not exceed t_1 by more than 5 °C and place the tube in the apparatus, maintained at a temperature 5 °C below t_1. When crystallisation takes place, or when the temperature of the mixture has fallen 3 °C below t_1, stir continuously. Note the highest temperature at which the mixture crystallises (t_2). Repeat the operation until 2 highest values obtained for t_2 do not differ by more than 0.2 °C. If supercooling occurs, induce crystallisation by adding a small crystal of the complex consisting of 3.00 g of *cineole R* and 2.10 g of melted *cresol R*. If t_2 is below 27.4 °C, repeat the determination after the addition of 5.10 g of the complex.

The content of cineole corresponding to the highest temperature observed (t_2) is given in Table 2.8.11.-1. If 5.10 g of the complex has been added, calculate the cineole content per cent *m/m* from the expression:

$$2(A - 50)$$

where A is the value found in Table 2.8.11.-1.

The content of cineole, corresponding to the highest temperature observed (t_2), is obtained, where necessary, by interpolation.

Table 2.8.11.-1

t_2 °C	cineole per cent *m/m*	t_2 °C	cineole per cent *m/m*	t_2 °C	cineole per cent *m/m*	t_2 °C	cineole per cent *m/m*
24	45.5	32	56.0	40	67.0	48	82.0
25	47.0	33	57.0	41	68.5	49	84.0
26	48.5	34	58.5	42	70.0	50	86.0
27	49.5	35	60.0	43	72.5	51	88.5
28	50.5	36	61.0	44	74.0	52	91.0
29	52.0	37	62.5	45	76.0	53	93.5
30	53.5	38	63.5	46	78.0	54	96.0
31	54.5	39	65.0	47	80.0	55	99.0

K. Determination of Aldehydes

(No Ph. Eur. method)

To 1 g of the oil in a glass-stoppered tube (approximately 150 mm × 25 mm) add 5 mL of *toluene* and 15 mL of *alcoholic hydroxylamine solution*, shake vigorously and titrate immediately with 0.5M *potassium hydroxide in ethanol (60%) VS* until the red colour changes to yellow. Continue shaking and neutralising until the full yellow colour of the indicator is permanent in the lower layer after shaking vigorously for 2 minutes and allowing to separate; the reaction is complete in about 15 minutes. This procedure gives an approximate value for the aldehyde content of the oil.

Repeat this procedure, using as the colour standard for the end point of the titration the titrated liquid of the first determination with the addition of 0.5 mL of 0.5M *potassium hydroxide in ethanol (60%) VS*. Calculate the content of aldehydes from the second determination, using the equivalent given in the monograph.

L. Oxidising Substances

(Ph. Eur. method 2.5.30)

Transfer 4.0 g to a glass-stoppered, 125 mL conical flask and add 50.0 mL of *water R*. Insert the stopper and swirl for 5 min. Transfer to a glass-stoppered 50 mL centrifuge tube and centrifuge. Transfer 30.0 mL of the clear supernatant to a glass-stoppered 125 mL conical flask. Add 1 mL of *glacial acetic acid R* and 0.5 g to 1.0 g of *potassium iodide R*. Insert the stopper, swirl, and allow to stand for 25 min to 30 min in the dark. Add 1 mL of *starch solution R* and titrate with *0.002 M sodium thiosulfate* until the starch-iodine colour disappears. Carry out a blank determination. Not more than 1.4 mL of *0.002 M sodium thiosulfate* is required (0.002 per cent, calculated as H_2O_2).

1 mL of *0.002 M sodium thiosulfate* is equivalent to 34 µg of oxidising substances, calculated as hydrogen peroxide.

M. Essential Oils

Fatty Oils and Resinified Essential Oils in Essential Oils

(Ph. Eur. method 2.8.7)

Allow 1 drop of the essential oil to fall onto filter paper. The drop evaporates completely within 24 h without leaving any translucent or greasy spot.

Foreign Esters in Essential Oils
(Ph. Eur. method 2.8.6)

Heat 1 mL of the essential oil for 2 min on a water-bath with 3.0 mL of a freshly prepared 100 g/L solution of *potassium hydroxide R* in *alcohol R*. No crystals are formed within 30 min, even after cooling.

Odour and Taste of Essential Oils
(Ph. Eur. method 2.8.8)

Mix 3 drops of the essential oil with 5 mL of *alcohol (90 per cent V/V) R* and stir in 10 g of powdered *sucrose R*. The odour and taste are similar to that of the plant or parts of the plant from which the essential oil has been obtained.

Residue on Evaporation of Essential Oils
(Ph. Eur. method 2.8.9)

The residue on evaporation of an essential oil is the percentage by mass of the oil which remains after evaporation on a water-bath under the conditions specified below.

Apparatus The apparatus (see Figure 2.8.9.-1) consists of:
- water-bath with a cover having holes of 70 mm diameter;
- evaporating dish of heat-resistant glass which is inert to the contents;
- desiccator.

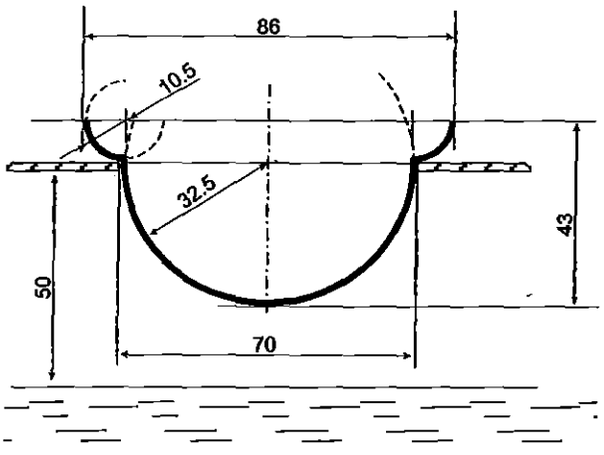


Figure 2.8.9.-1
Dimensions in millimetres

Method Weigh the evaporating dish after having heated it on the water-bath for 1 h and cooled it in the desiccator. Weigh into the evaporating dish 5.00 g of the essential oil, unless otherwise prescribed. Evaporate the oil by heating on a water-bath in a draught-free atmosphere for the prescribed time. Allow to cool in the desiccator and weigh.

During the test, the level of water in the bath is maintained about 50 mm beneath the level of the cover.

Solubility in Alcohol of Essential Oils
(Ph. Eur. method 2.8.10)

Place 1.0 mL of the essential oil in a 25 mL or 30 mL glass-stoppered cylinder. Place in a constant temperature device, maintained at a temperature of 20 ± 0.2 °C. Using a burette of at least 20 mL capacity, add the alcohol of the strength prescribed in the monograph by increments of 0.1 mL until solution is complete and then continue adding by increments of 0.5 mL to a total of 20 mL, shaking frequently and vigorously. Record the volume of alcohol added when a clear solution has been obtained and, if the solution becomes cloudy or opalescent before 20 mL of alcohol has been added, record the volume added when the cloudiness or opalescence appears and, where applicable, the volume added when the cloudiness or opalescence disappears.

If a clear solution has not been obtained when 20 mL of alcohol of the prescribed strength has been added, repeat the test using the next highest concentration of alcohol.

An essential oil is said to be "soluble in *n* volumes and more of alcohol of given strength *t*" when the clear solution in *n* volumes remains clear when compared with the undiluted oil after further addition of alcohol of the same strength up to a total of 20 volumes of alcohol.

An essential oil is said to be "soluble in *n* volumes of alcohol of given strength *t*, becoming cloudy when diluted" when the clear solution in *n* volumes becomes cloudy in n_1 volumes (n_1 less than 20) and stays so after further gradual addition of alcohol of the same strength up to a total of 20 volumes of alcohol.

An essential oil is said to be "soluble in *n* volumes of alcohol of given strength *t* with cloudiness between n_1 and n_2 volumes" when the clear solution in *n* volumes becomes cloudy in n_1 volumes (n_1 less than 20) and stays so after further gradual addition of alcohol of the same strength up to a total of n_2 volumes of alcohol and then becomes clear (n_2 less than 20).

An essential oil is said to be "soluble with opalescence" when the alcoholic solution shows a bluish tinge, similar to that of a standard of opalescence freshly prepared as follows: mix 0.5 mL of *silver nitrate solution R2* and 0.05 mL of *nitric acid R*; add 50 mL of a 12 mg/L solution of *sodium chloride R*; mix and allow to stand protected from light for 5 min.

Water in Essential Oils
(Ph. Eur. method 2.8.5)

Mix 10 drops of the essential oil with 1 mL of *carbon disulfide R*. The solution remains clear on standing.

N. Fixed Oils

Alkaline Impurities in Fatty Oils
(Ph. Eur. method 2.4.19)

In a test-tube mix 10 mL of recently distilled *acetone R* and 0.3 mL of *water R* and add 0.05 mL of a 0.4 g/L solution of *bromophenol blue R* in *alcohol R*. Neutralise the solution if necessary with *0.01 M hydrochloric acid* or *0.01 M sodium hydroxide*. Add 10 mL of the oil to be examined, shake and allow to stand. Not more than 0.1 mL of *0.01 M hydrochloric acid* is required to change the colour of the upper layer to yellow.

Identification of Fixed Oils by Thin-layer Chromatography
(Ph. Eur. method 2.3.2)

METHOD A

Thin-layer chromatography (*2.2.27*).

Test solution Unless otherwise prescribed, dissolve about 20 mg (1 drop) of the fatty oil in 3 mL of *methylene chloride R*.

Reference solution Dissolve about 20 mg (1 drop) of *maize oil R* in 3 mL of *methylene chloride R*.

Plate A suitable octadecylsilyl silica gel for high-performance thin-layer chromatography as the coating substance.

Mobile phase:
— *mobile phase A*: *ether R*;
— *mobile phase B*: *methylene chloride R*, *glacial acetic acid R*, *acetone R* (20:40:50 *V/V/V*).

Application 1 µL.

Development Twice over a path of 0.5 cm with mobile phase A, then twice over a path of 8 cm with mobile phase B.

Drying In air.

Detection Spray with a 100 g/L solution of *phosphomolybdic acid R* in *ethanol (96 per cent) R*. Heat the plate at 120 °C for about 3 min and examine in daylight.

The chromatogram obtained typically shows spots comparable to those in Figure 2.3.2.-1.

METHOD B

Thin-layer chromatography (*2.2.27*).

Test solution Unless otherwise prescribed, dissolve about 20 mg (1 drop) of the fatty oil in 3 mL of *methylene chloride R*.

Reference solution Dissolve about 20 mg (1 drop) of *maize oil R* in 3 mL of *methylene chloride R*.

Plate A suitable octadecylsilyl silica gel for high-performance thin-layer chromatography as the coating substance.

Mobile phase *methylene chloride R*, *glacial acetic acid R*, *acetone R* (20:40:50 *V/V/V*).

Application 1 µL as bands of 8 mm. A suitable automated apparatus may be used.

Development Over a path of 7 cm.

Drying In air.

Detection Treat with a 100 g/L solution of *phosphomolybdic acid R* in *ethanol (96 per cent) R*. Heat the plate at 120 °C for 3 min and examine in daylight.

The chromatogram obtained typically shows zones comparable to those in Figure 2.3.2.-2.

Test for Foreign Oils by Thin-layer Chromatography

(Ph. Eur. method 2.4.21)

Examine by thin-layer chromatography (*2.2.27*) using *kieselguhr G R* as the coating substance. Impregnate a plate by placing it in a chromatographic tank containing the necessary quantity of a mixture of 10 volumes of *liquid paraffin R* and 90 volumes of *light petroleum R* so that the plate dips about 5 mm beneath the surface of the liquid. When the impregnation mixture has risen by at least 12 cm from the lower edge of the plate, remove the plate and allow the solvent to evaporate for 5 min. Carry out the chromatography in the same direction as the impregnation.

Preparation of the mixture of fatty acids Heat 2 g of the oil with 30 mL of 0.5 M alcoholic potassium hydroxide under a reflux condenser for 45 min. Add 50 mL of *water R*, allow to cool, transfer to a separating funnel and extract with three quantities, each of 50 mL, of *ether R*. Discard the ether extracts, acidify the aqueous layer with *hydrochloric acid R* and extract with three quantities, each of 50 mL, of *ether R*. Combine the ether extracts and wash with three quantities, each of 10 mL, of *water R*; discard the washings, dry the ether over *anhydrous sodium sulfate R* and filter. Evaporate the ether on a water-bath. Use the residue to prepare the test solution. The fatty acids may also be obtained from the soap solution prepared during the determination of the unsaponifiable matter.

Test solution Dissolve 40 mg of the mixture of fatty acids obtained from the substance to be examined in 4 mL of *chloroform R*.

Reference solution Dissolve 40 mg of the mixture of fatty acids obtained from a mixture of 19 volumes of *maize oil R* and 1 volume of *rapeseed oil R* in 4 mL of *chloroform R*.

Apply to the plate 3 µL of each solution. Develop over a path of 8 cm using a mixture of 10 volumes of *water R* and 90 volumes of *glacial acetic acid R*. Dry the plate at 110 °C for 10 min. Allow to cool and, unless otherwise prescribed, place the plate in a chromatographic chamber, with a tightly fitting lid, that has previously been saturated with iodine vapour by placing *iodine R* in an evaporating dish at the bottom of the chamber. After some time brown or yellowish-brown spots become visible. Remove the plate and allow to stand for a few minutes. When the brown background colour has disappeared, spray with *starch solution R*. Blue spots appear which may become brown on drying and again become blue after spraying with *water R*. The chromatogram obtained with the test solution always shows a spot with an R_F of about 0.5 (oleic acid) and a spot with an R_F of about 0.65 (linoleic acid) corresponding to the spots in the chromatogram obtained with the reference solution. With some oils a spot with an R_F of about 0.75 may be present (linolenic acid). By comparison with the spot in the chromatogram obtained with the reference solution, verify the absence in the chromatogram obtained with the test solution of a spot with an R_F of about 0.25 (erucic acid).

Test for Foreign Oils by Gas Chromatography

(Ph. Eur. method 2.4.22)

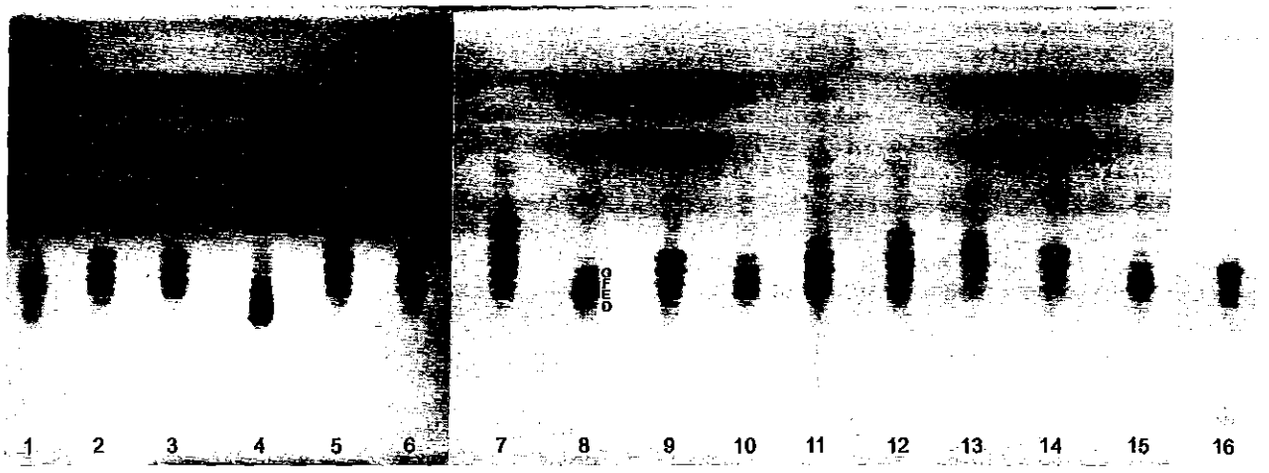
The test for foreign oils is carried out on the methyl esters of the fatty acids contained in the oil to be examined by gas chromatography (*2.2.28*).

METHOD A

This method is not applicable to oils that contain glycerides of fatty acids with an epoxy-, hydroepoxy-, hydroperoxy-, cyclopropyl or cyclopropenyl group, or those that contain a large proportion of fatty acids of chain length less than 8 carbon atoms or to oils with an acid value greater than 2.0.

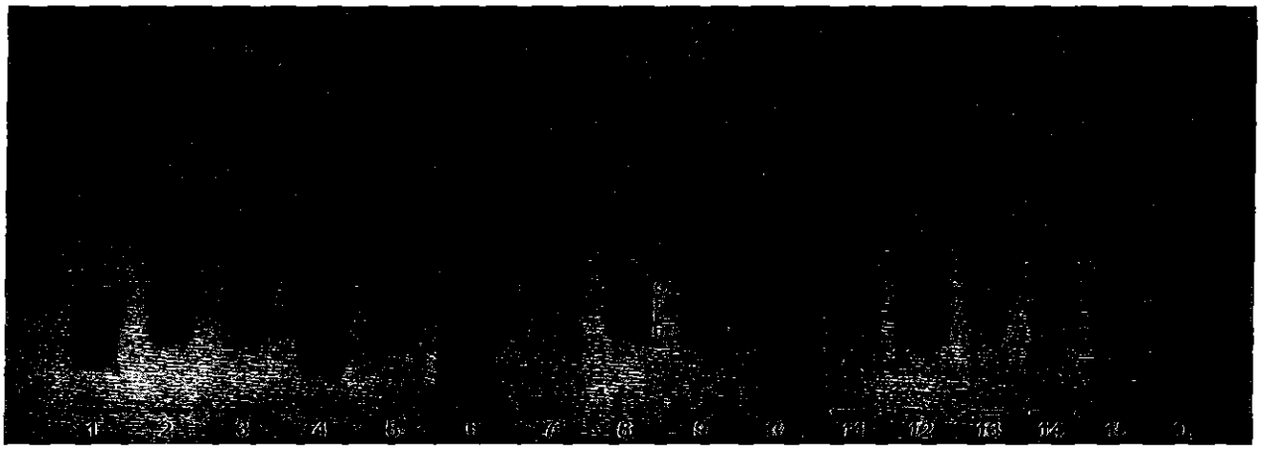
Test solution When prescribed in the monograph, dry the oil to be examined before the methylation step. Weigh 1.0 g of the oil into a 25 mL round-bottomed flask with a ground-glass neck fitted with a reflux condenser and a gas port into the flask. Add 10 mL of *anhydrous methanol R* and 0.2 mL of a 60 g/L solution of *potassium hydroxide R* in *methanol R*. Attach the reflux condenser, pass *nitrogen R* through the mixture at a rate of about 50 mL/min, shake and heat to boiling. When the solution is clear (usually after about 10 min), continue heating for a further 5 min. Cool the flask under running water and transfer the contents to a separating funnel. Rinse the flask with 5 mL of *heptane R* and transfer the rinsings to the separating funnel and shake. Add 10 mL of a 200 g/L solution of *sodium chloride R* and shake vigorously. Allow to separate and transfer the organic layer to a vial containing *anhydrous sodium sulfate R*. Allow to stand, then filter.

Reference solution (a) Prepare 0.50 g of the mixture of calibrating substances with the composition described in one of the 2.4.22 tables, as prescribed in the individual monograph (if the monograph does not mention a specific solution, use the composition described in Table 2.4.22.-1).



1. arachis oil
2. sesame oil
3. maize oil
4. rapeseed oil
5. soya-bean oil
6. rapeseed oil (erucic acid-free)
7. linseed oil
8. olive oil
9. sunflower oil
10. almond oil
11. wheat-germ oil
12. borage oil
13. evening primrose oil
14. safflower oil (type I)
15. safflower oil (type II)
16. hydrogenated arachis oil

Figure 2.3.2.-1. – *Chromatograms for the identification of fatty oils (method A)*



1. arachis oil
2. sesame oil
3. maize oil
4. rapeseed oil
5. soya-bean oil
6. rapeseed oil (erucic acid-free)
7. linseed oil
8. olive oil
9. sunflower oil
10. almond oil
11. wheat-germ oil
12. borage oil
13. evening primrose oil
14. safflower oil (type I)
15. safflower oil (type II)
16. hydrogenated arachis oil

Figure 2.3.2.-2. – *Chromatograms for the identification of fatty oils (method B)*

Dissolve in *heptane R* and dilute to 50.0 mL with the same solvent.

Reference solution (b) Dilute 1.0 mL of reference solution (a) to 10.0 mL with *heptane R*.

Reference solution (c) Prepare 0.50 g of a mixture of fatty acid methyl esters that corresponds in composition to the mixture of fatty acids indicated in the monograph of the substance to be examined. Dissolve in *heptane R* and dilute to 50.0 mL with the same solvent. Commercially available mixtures of fatty acid methyl esters may also be used.

Column:
- *material*: fused silica, glass or quartz;
- *size*: *l* = 10-30 m, Ø = 0.2-0.8 mm;
- *stationary phase*: *macrogol 20 000 R* (film thickness 0.1-0.5 µm) or another suitable stationary phase.

Carrier gas *helium for chromatography R* or *hydrogen for chromatography R*.

Flow rate 1.3 mL/min (for a column Ø = 0.32 mm).

Split ratio 1:100 or less, according to the internal diameter of the column used (1:50 when Ø = 0.32 mm).

Temperature:
- *column*: in isothermal conditions, 160-200 °C, according to the length and type of column used (200 °C for a column 30 m long and coated with a layer of *macrogol 20 000 R*); if a linear temperature programming is necessary, raise the temperature of the column at a rate of 3 °C/min from 170 °C to 230 °C, for example;
- *injection port*: 250 °C;
- *detector*: 250 °C.

Detection Flame ionisation.

Injection 1 µL.

System suitability When using the mixture of calibrating substances in Table 2.4.22.-1 or Table 2.4.22.-3:
- *resolution*: minimum 1.8 between the peaks due to methyl oleate and methyl stearate in the chromatogram obtained with reference solution (a);
- *signal-to-noise ratio*: minimum 5 for the peak due to methyl myristate in the chromatogram obtained with reference solution (b);
- *number of theoretical plates*: minimum 30 000, calculated for the peak due to methyl stearate in the chromatogram obtained with reference solution (a).

System suitability When using the mixture of calibrating substances in Table 2.4.22.-2:
- *resolution*: minimum 4.0 between the peaks due to methyl caprylate and methyl decanoate in the chromatogram obtained with reference solution (a);
- *signal-to-noise ratio*: minimum 5 for the peak due to methyl caproate in the chromatogram obtained with reference solution (b);
- *number of theoretical plates*: minimum 15 000, calculated for the peak due to methyl decanoate in the chromatogram obtained with reference solution (a).

ASSESSMENT OF CHROMATOGRAMS

Avoid working conditions tending to give masked peaks (presence of constituents with small differences between retention times, for example linolenic acid and arachidic acid).

Qualitative analysis

Identify the peaks in the chromatogram obtained with reference solution (c) (isothermal operating conditions or linear temperature programming).

When using isothermal operating conditions, the peaks may also be identified by drawing calibration curves using the chromatogram obtained with reference solution (a) and the information given in Tables 2.4.22.-1, 2.4.22.-2 or 2.4.22.-3.

Table 2.4.22.-1. – *Mixture of calibrating substances (for gas chromatography with capillary column and split inlet system, it is recommended that the component with the longest chain length of the mixture to be examined be added to the calibration mixture, when the qualitative analysis is done using calibration curves)*

Mixture of the following substances	Composition (per cent *m/m*)
Methyl laurate R	5
Methyl myristate R	5
Methyl palmitate R	10
Methyl stearate R	20
Methyl arachidate R	40
Methyl oleate R	20

Table 2.4.22.-2. – *Mixture of calibrating substances (for gas chromatography with capillary column and split inlet system, it is recommended that the component with the longest chain length of the mixture to be examined be added to the calibration mixture, when the qualitative analysis is done using calibration curves)*

Mixture of the following substances	Composition (per cent *m/m*)
Methyl caproate R	10
Methyl caprylate R	10
Methyl decanoate R	20
Methyl laurate R	20
Methyl myristate R	40

Table 2.4.22.-3. – *Mixture of calibrating substances (for gas chromatography with capillary column and split inlet system, it is recommended that the component with the longest chain length of the mixture to be examined be added to the calibration mixture, when the qualitative analysis is done using calibration curves)*

Mixture of the following substances	Composition (per cent *m/m*)
Methyl myristate R	5
Methyl palmitate R	10
Methyl stearate R	15
Methyl arachidate R	20
Methyl oleate R	20
Methyl eicosenoate R	10
Methyl behenate R	10
Methyl lignocerate R	10

Measure the reduced retention time (t'_R) of each peak in the chromatogram obtained with reference solution (a). t'_R is the retention time measured from the solvent peak and not from the time of injection. Plot the straight line:

$$log_{10}(t'_R) = f(\text{equivalent chain length})$$

The logarithms of t'_R of unsaturated acids are situated on this line at points corresponding to non-integer values of carbon atoms known as 'equivalent chain lengths'; the equivalent chain length is the length of the theoretical saturated chain that would have the same t'_R as the fatty acid to be identified. For example, linoleic acid has the same t'_R as the theoretical saturated fatty acid having 18.8 carbon atoms.

Identify the peaks in the chromatogram obtained with the test solution by means of the straight line and the reduced retention times. Equivalent chain lengths are given in Table 2.4.22.-4.

Table 2.4.22.-4. – *Equivalent chain lengths (this value, which is to be calculated using calibration curves, is given as an example for a column of macrogol 20 000 R)*

Fatty acid	Equivalent chain length
Caproic acid	6.0
Caprylic acid	8.0
Capric acid	10.0
Lauric acid	12.0
Myristic acid	14.0
Palmitic acid	16.0
Palmitoleic acid	16.3
Margaric acid	17.0
Stearic acid	18.0
Oleic acid	18.3
Linoleic acid	18.8
Gamma-linolenic acid	19.0
Alpha-linolenic acid	19.2
Arachidic acid	20.0
Eicosenoic acid (gondoic acid)	20.2
Arachidonic acid	21.2
Behenic acid	22.0
Erucic acid	22.2
12-Oxostearic acid	22.7
Ricinoleic acid	23.9
12-Hydroxystearic acid	23.9
Lignoceric acid	24.0
Nervonic acid	24.2

Quantitative analysis

In general, the normalisation procedure is used in which the sum of the areas of the peaks in the chromatogram, except

that of the solvent, is set at 100 per cent. The content of a constituent is calculated by determining the area of the corresponding peak as a percentage of the sum of the areas of all the peaks. Disregard any peak with an area less than 0.05 per cent of the total area.

In certain cases, for example in the presence of fatty acids with 12 or less carbon atoms, correction factors can be prescribed in the individual monograph to convert peak areas in per cent *m/m*.

METHOD B

This method is not applicable to oils that contain glycerides of fatty acids with an epoxy-, hydroepoxy-, hydroperoxy-, cyclopropyl or cyclopropenyl group or to oils with an acid value greater than 2.0.

Test solution Introduce 0.100 g of the substance to be examined into a 10 mL centrifuge tube with a screw cap. Dissolve with 1 mL of *heptane R* and 1 mL of *dimethyl carbonate R* and mix vigorously under gentle heating (50-60 °C). Add, while still warm, 1 mL of a 12 g/L solution of *sodium R* in *anhydrous methanol R*, prepared with the necessary precautions, and mix vigorously for about 5 min. Add 3 mL of *distilled water R* and mix vigorously for about 30 s. Centrifuge for 15 min at 1500 *g*. Inject 1 µL of the organic phase.

Reference solutions and assessment of chromatograms Where there is no specific prescription in the individual monograph, proceed as described under Method A.

Column:
- *material*: fused silica;
- *size*: *l* = 30 m, Ø = 0.25 mm;
- *stationary phase*: *macrogol 20 000 R* (film thickness 0.25 µm).

Carrier gas *helium for chromatography R.*

Flow rate 0.9 mL/min.

Split ratio 1:100.

Temperature:

	Time (min)	Temperature (°C)
Column	0 - 15	100
	15 - 36	100 → 225
	36 - 61	225
Injection port		250
Detector		250

Detection Flame ionisation.

Injection 1 µL.

METHOD C

This method is not applicable to oils that contain glycerides of fatty acids with epoxy-, hydroepoxy-, hydroperoxy-, aldehyde, ketone, cyclopropyl and cyclopropenyl groups, and conjugated polyunsaturated and acetylenic compounds because of partial or complete destruction of these groups.

Test solution Dissolve 0.10 g of the substance to be examined in 2 mL of a 20 g/L solution of *sodium hydroxide R* in *methanol R* in a 25 mL conical flask and boil under a reflux condenser for 30 min. Add 2.0 mL of *boron trifluoride-methanol solution R* through the condenser and boil for 30 min. Add 4 mL of *heptane R* through the condenser and boil for 5 min. Cool and add 10.0 mL of *saturated sodium chloride solution R*, shake for about 15 s and add a quantity of *saturated sodium chloride solution R* such that the upper phase is brought into the neck of the flask. Collect 2 mL of the upper phase, wash with 3 quantities, each of 2 mL, of *water R* and dry over *anhydrous sodium sulfate R*.

Reference solutions, chromatographic procedure and assessment of chromatograms Where there is no specific prescription in the individual monograph, proceed as described under Method A.

O. Anisidine Value

(Ph. Eur. method 2.5.36)

The anisidine value is defined as 100 times the optical density measured in a 1 cm cell of a solution containing 1 g of the substance to be examined in 100 mL of a mixture of solvents and reagents according to the following method.

Carry out the operations as rapidly as possible, avoiding exposure to actinic light.

Test solution (a) Dissolve 0.500 g of the substance to be examined in *trimethylpentane R* and dilute to 25.0 mL with the same solvent.

Test solution (b) To 5.0 mL of test solution (a) add 1.0 mL of a 2.5 g/L solution of *p-anisidine R* in *glacial acetic acid R*, shake and store protected from light.

Reference solution To 5.0 mL of *trimethylpentane R* add 1.0 mL of a 2.5 g/L solution of *p-anisidine R* in *glacial acetic acid R*, shake and store protected from light.

Measure the absorbance (*2.2.25*) of test solution (a) at the maximum at 350 nm using *trimethylpentane R* as the compensation liquid. Measure the absorbance of test solution (b) at 350 nm exactly 10 min after its preparation, using the reference solution as the compensation liquid.

Calculate the anisidine value from the expression:

$$\frac{25 \times (1.2A_1 - A_2)}{m}$$

A_1 = absorbance of test solution (b) at 350 nm,
A_2 = absorbance of test solution (a) at 350 nm,
m = mass of the substance to be examined in test solution (a), in grams.

P. Oils Rich in Omega-3-acids

1. Composition of Fatty Acids

(Ph. Eur. method 2.4.29)

The assay may be used for quantitative determination of the eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and total omega-3-acids content in products from fish oil containing omega-3 acids in different concentrations. The method is applicable to triglycerides or ethyl esters. The former includes fish oils/fish-liver oils and omega-3-concentrates in triglyceride form. The results are expressed as triglycerides or ethyl esters, respectively.

EPA AND DHA

Gas chromatography (*2.2.28*). *Carry out the operations as rapidly as possible, avoiding exposure to actinic light, oxidising agents, oxidation catalysts (for example, copper and iron) and air.*

The assay is carried out on the methyl esters (after derivatisation of triglycerides - see step B below) or ethyl esters of (*all-Z*)-eicosa-5,8,11,14,17-pentaenoic acid (EPA; 20:5 n-3) and (*all-Z*)-docosa-4,7,10,13,16,19-hexaenoic acid (DHA; 22:6 n-3) in the substance to be examined.

Internal standard *methyl tricosanoate R.*

Test solutions Prepare all test solutions in duplicate.

Step A

— *Test solution (a).* Dissolve a quantity of the sample to be examined according to Table 2.4.29.-1 and 70.0 mg of the internal standard in a 50 mg/L solution of *butylhydroxytoluene R* in *trimethylpentane R* and dilute to 10.0 mL with the same solution. Gentle heating (up to 60 °C) may be applied to dissolve the internal standard. Ethyl esters are now ready for analysis. For triglycerides continue as described in step B.

Table 2.4.29.-1

Approximate sum EPA + DHA (per cent)	Mass of sample to be examined (g)
30 - 50	0.4 - 0.5
50 - 70	0.3
70 - 90	0.25

— *Test solution (b).* Dissolve a quantity of the sample to be examined according to Table 2.4.29.-1 in a 50 mg/mL solution of *butylhydroxytoluene R* in *trimethylpentane R* and dilute to 10.0 mL with the same solution. Ethyl esters are now ready for analysis. For triglycerides continue as described in step B.

Step B

Introduce 2.0 mL of test solutions (a) and (b) obtained in step A into separate quartz tubes and evaporate the solvent with a gentle current of *nitrogen R.* Add 1.5 mL of a 20 g/L solution of *sodium hydroxide R* in *methanol R,* cover with *nitrogen R,* cap tightly with a polytetrafluoroethylene-lined cap, mix and heat on a water-bath for 7 min. Allow to cool. Add 2 mL of *boron trichloride-methanol solution R,* cover with *nitrogen R,* cap tightly, mix and heat on a water-bath for 30 min. Cool to 40-50 °C, add 1 mL of *trimethylpentane R,* cap and shake vigorously for at least 30 s. Immediately add 5 mL of a *saturated sodium chloride solution R,* cover with *nitrogen R,* cap and shake thoroughly for at least 15 s. Transfer the upper layer to a separate tube. Shake the methanol layer once more with 1 mL of *trimethylpentane R.* Wash the combined trimethylpentane extracts with 2 quantities, each of 1 mL, of *water R* and dry over *anhydrous sodium sulfate R.*

Reference solutions Prepare reference solutions (a_1) and (a_2) in duplicate; reference solution (c) only has to be prepared for triglycerides, and only if tetracos-15-enoic acid methyl ester is not clearly observed in the chromatogram obtained with test solution (a).

— *Reference solution (a_1).* Dissolve 70.0 mg of the internal standard and 90.0 mg of *eicosapentaenoic acid ethyl ester CRS* in a 50 mg/L solution of *butylhydroxytoluene R* in *trimethylpentane R* and dilute to 10.0 mL with the same solution. Gentle heating (up to 60 °C) may be applied to dissolve the internal standard.

— *Reference solution (a_2).* Dissolve 60.0 mg of *docosahexaenoic acid ethyl ester CRS* and 70.0 mg of the internal standard in a 50 mg/L solution of *butylhydroxytoluene R* in *trimethylpentane R* and dilute to 10.0 mL with the same solution. Gentle heating (up to 60 °C) may be applied to dissolve the internal standard.

For ethyl ester samples, reference solutions (a_1) and (a_2) are ready for analysis. For analysis of triglycerides, continue with step B in the same manner as for test solutions (a) and (b).

— *Reference solution (b).* Dissolve 0.300 g of *methyl arachidate R,* 0.300 g of *methyl behenate R,* 0.300 g of *methyl palmitate R* and 0.300 g of *methyl stearate R* in a 50 mg/L solution of *butylhydroxytoluene R* in *trimethylpentane R* and dilute to 10.0 mL with the same solution.

— *Reference solution (c).* Dissolve a mixture containing 55.0 mg of *docosahexaenoic acid methyl ester R* and 5.0 mg of *tetracos-15-enoic acid methyl ester R* in a 50 mg/L solution of *butylhydroxytoluene R* in *trimethylpentane R* and dilute to 10.0 mL with the same solution.

Column:

— *material:* fused silica;
— *dimensions:* l = at least 25 m, Ø = 0.25 mm;
— *stationary phase:* *macrogol 20 000 R* (film thickness 0.2 µm).

Carrier gas *hydrogen for chromatography R* or *helium for chromatography R.*

Flow rate 1 mL/min.

Split ratio 1:200, alternatively splitless with temperature control (sample solutions need to be diluted 1/200 with a 50 mg/L solution of *butylhydroxytoluene R* in *trimethylpentane R* before injection).

If necessary, adapt the split ratio and/or sample dilution to obtain a symmetry factor of 0.8-1.5 for the peaks due to the methyl or ethyl esters of eicosapentaenoic acid and docosahexaenoic acid, while at the same time observing that for test solution (b) any peaks due to the corresponding esters of linolenic acid (C18:3; n-3), stearidonic acid (C18:4; n-3), eicosatetraenoic acid (C20:4; n-3), heneicosapentaenoic acid (C21:5; n-3), and docosapentaenoic acid (C22:5; n-3) are clearly detectable. If both requirements are unachievable, then the clear detection of the corresponding esters mentioned above for test solution (b) takes precedence.

If necessary, adapt the split ratio and/or sample dilution to obtain a symmetry factor of 0.8-1.5 for the peaks due to the components of reference solution (b).

Temperature:

	Split injection		Splitless injection	
	Time (min)	Temperature (°C)	Time (min)	Temperature (°C)
Column	0 - 2	170	0 - 2	90
	2 - 25.7	170 → 240	2 - 4.7	90 → 170
	25.7 - 28	240	4.7 - 28	170 → 240
			28 - 30	240
Injection port		250		90 - 250*
Detector		270		270

*90 °C for on-column injection

Detection Flame ionisation.

Injection 1 µL.

System suitability:

— in the chromatogram obtained with reference solution (b), multiply the area of the peaks due to methyl palmitate, methyl stearate, methyl arachidate and methyl behenate by the corresponding response factors in Table 2.4.29.-2; normalise the corrected areas of the peaks of the fatty acid methyl esters to a sum of 100 per cent; the normalised area percentage of each fatty acid methyl ester is to be within ± 1.0 percentage units of the corresponding weight percentage;

Table 2.4.29.-2

Fatty acid methyl ester	Theoretical response factor
Methyl palmitate	1.049
Methyl stearate	1.029
Methyl arachidate	1.013
Methyl behenate	1.000

— *resolution*:
  — *ethyl esters*: minimum 1.2 between the peaks due to methyl tricosanoate and heneicosapentaenoic acid ethyl ester in the chromatogram obtained with test solution (a);
  — *triglycerides*: minimum 1.2 between the peaks due to docosahexaenoic acid methyl ester and tetracos-15-enoic acid methyl ester in the chromatogram obtained with test solution (a) or with reference solution (c);
— in the chromatogram obtained with test solution (a), the peaks due to methyl tricosanoate and any heneicosapentaenoic acid methyl ester present when compared with the chromatogram obtained with test solution (b) are clearly separated.

Calculate the percentage content of EPA and DHA using the following expression and taking into account the assigned value of the reference substances:

$$\frac{A_x \times m_1}{A_1 \times m_2} \times Rf \times C \times 100$$

Rf = response factor for EPA and DHA as given by the expression:

$$\frac{A_{x,3} \times m_{x,r}}{A_{x,r} \times m_{x,3}}$$

m_1 = mass of the internal standard in test solution (a), in milligrams;
m_2 = mass of the sample to be examined in test solution (a), in milligrams;
$m_{x,3}$ = mass of the internal standard in reference solution (a_1) (EPA determination), or in reference solution (a_2) (DHA determination), in milligrams;
$m_{x,r}$ = mass of *eicosapentaenoic acid ethyl ester CRS* in reference solution (a_1) or *docosahexaenoic acid ethyl ester CRS* in reference solution (a_2), in milligrams;
A_x = area of the peak due to eicosapentaenoic acid ester or docosahexaenoic acid ester in the chromatogram obtained with test solution (a);
$A_{x,r}$ = area of the peak due to eicosapentaenoic acid ester in the chromatogram obtained with reference solution (a_1) or docosahexaenoic acid ester in the chromatogram obtained with reference solution (a_2);
A_1 = area of the peak due to the internal standard in the chromatogram obtained with test solution (a);
$A_{x,3}$ = area of the peak due to the internal standard in the chromatogram obtained with reference solution (a_1) (EPA determination) or with reference solution (a_2) (DHA determination);
C = conversion factor between ethyl ester and triglycerides:

Ethyl esters: $C = 1.00$;
Triglycerides: $C = 0.954$ for EPA;
$C = 0.957$ for DHA.

TOTAL OMEGA-3 ACIDS

From the assay for EPA and DHA, calculate the percentage content of the total omega-3 acids using the following expression and identifying the peaks from the chromatograms:

$$EPA + DHA + \frac{A_{n-3}\,(EPA + DHA)}{A_{EPA} + A_{DHA}}$$

EPA = percentage content of EPA;
DHA = percentage content of DHA;
A_{n-3} = sum of the areas of the peaks due to linolenic acid (C18:3; n-3), stearidonic acid (C18:4; n-3), eicosatetraenoic acid (C20:4; n-3), heneicosapentaenoic acid (C21:5; n-3), and docosapentaenoic acid (C22:5; n-3) in the chromatogram obtained with test solution (b);
A_{EPA} = area of the peak due to EPA ester in the chromatogram obtained with test solution (b);
A_{DHA} = area of the peak due to DHA ester in the chromatogram obtained with test solution (b).

2. Total Cholesterol in Oils Rich in Omega-3-Acids

(Ph. Eur. method 2.4.32)

This method may be used for the quantitative determination of the sum of free and esterified cholesterol in products of fish oils rich in omega-3 acids (as ethyl esters or triglycerides).

Gas chromatography (*2.2.28*).

Internal standard stock solution Dissolve 0.15 g of *(5α)-cholestane R* in *heptane R* and dilute to 50.0 mL with the same solvent. The solution may be stored in a deep-freeze for up to 6 months.

Internal standard working solution. Prepare the solution immediately before use Dilute 1.0 mL of the internal standard stock solution to 10.0 mL with *heptane R*.

Cholesterol stock solution Dissolve 50.0 mg of *cholesterol R* in *heptane R* and dilute to 100.0 mL with the same solvent. The solution may be stored in a deep-freeze for up to 6 months.

Cholesterol working solution. Prepare the solution immediately before use Dilute 1.0 mL of the cholesterol stock solution to 10.0 mL with *heptane R*.

Cholesterol and α-tocopherol stock solution Dissolve 50.0 mg of *cholesterol R* and 50.0 mg of *α-tocopherol R* in *heptane R* and dilute to 100.0 mL with the same solvent. The solution may be stored at room temperature for up to 3 months.

Reference solution. Prepare the solution on the day of use Dilute 1.0 mL of the cholesterol and α-tocopherol stock solution to 100.0 mL with *heptane R*.

Calibration solutions See Table 2.4.32.-1. *Prepare the solutions on the day of use.* Dilute each solution to 20.0 mL with a 10 per cent *V/V* solution of *ethyl acetate R* in *heptane R*.

For high levels of cholesterol (3.0-20.0 mg/g), use all 5 calibration solutions.

For low levels of cholesterol (0.2-3.0 mg/g), use the following calibration solutions: 0.2 mg/g, 1.0 mg/g and 3.0 mg/g.

Test solution Weigh 0.100 g of the substance to be examined into a 15 mL quartz tube (for fish oils and cod-liver oils, shake the oil to be examined vigorously in a suitable container, allow to stand for 10-15 min and while maintaining the container upright, remove a sample from the middle layer of the oil for weighing). Add 1.0 mL of the internal standard working solution. Evaporate the solvent on a heating block at 50 °C under a gentle stream of *nitrogen R*. Add 0.5 mL of a 50 per cent *m/m* solution of *potassium hydroxide R* and 3.0 mL of *ethanol (96 per cent) R*. Fill the tube with *nitrogen R*, cap and homogenise. Heat on the heating block at 100 °C for 1 h. Cool for about 10 min, add 6.0 mL of *distilled water R* and homogenise. Condition a 20 mL solid phase extraction (SPE) column containing 1 g of *end-capped octadecylsilyl silica gel for chromatography R*

Table 2.4.32.-1. – *Preparation of calibration solutions*

Calibration solutions		Cholesterol stock solution (mL)	Cholesterol working solution (mL)	Internal standard working solution (mL)
Cholesterol concentration (mg/mL)	Cholesterol concentration (mg/g)[a]			
0.1	20.0	4.0		1.0
0.05	10.0	2.0		1.0
0.015	3.0	0.60		1.0
0.005	1.0		2.0	1.0
0.001	0.2		0.40	1.0

(a)Based on a sample weight of 0.100 g of the substance to be examined.

(particles with a diameter of 55 µm and a pore size of 7 nm) with 5 mL of a 50 per cent *V/V* solution of *ethanol (96 per cent) R* in *distilled water R*. Transfer 5.0 mL of the saponified sample to the SPE column ensuring that the column does not dry out. Wash the column with 5.0 mL of a 50 per cent *V/V* solution of *ethanol (96 per cent) R* in *distilled water R*. Elute the column using 20.0 mL of a 10 per cent *V/V* solution of *ethyl acetate R* in *heptane R*. Collect the eluate and use it as the test solution.

Column:
- *material*: fused silica;
- *size*: *l* = 15 m, Ø = 0.25 mm;
- *stationary phase*: *phenyl(5)methyl(95)polysiloxane R* (film thickness 0.25 µm).

Carrier gas *helium for chromatography R.*

Pressure 48 kPa (corresponding to a flow rate of about 0.6 mL/min at 200 °C and about 0.4 mL/min at 330 °C).

Split ratio 1:5.

Temperature:

	Time (min)	Temperature (°C)
Column	0 - 1	200
	1 - 7.5	200 → 330
	7.5 - 10	330
Injection port		250
Detector		340

Prior to analysis, heat the column at 340 °C for at least 30 min using the indicated pressure.

Detection Flame ionisation.

Injection 1 µL.

Retention time (5α)-cholestane = about 7.5 min; cholesterol = about 9 min.

Plot the calibration curve. The x-axis represents the nominal concentration of cholesterol in milligrams per gram of the substance to be examined in each calibration solution. The y-axis represents the ratio of the area of the peak due to cholesterol to the area of the peak due to (5α)-cholestane in the chromatogram obtained with each calibration solution. Calculate the slope (*S*) and the intercept with the y-axis (*Y*).

System suitability:
- *resolution*: minimum 1.5 between the peaks due to cholesterol and α-tocopherol in the chromatogram obtained with the reference solution;
- the coefficient of determination (r^2) of the calibration curve is not less than 0.995.

Calculate the content of total cholesterol, expressed in milligrams of cholesterol per gram of substance to be examined, using the following expression:

$$\frac{\frac{A_1}{A_2} - Y}{m_1 \times S} \times 0.100$$

A_1 = area of the peak due to cholesterol in the chromatogram obtained with the test solution;
A_2 = area of the peak due to (5α)-cholestane in the chromatogram obtained with the test solution;
m_1 = mass of the substance to be examined in the test solution, in grams;
Y = intercept of the calibration curve with the y-axis;
S = slope of the calibration curve, in grams per milligram.

Q. Sterols in Fatty Oils

(Ph. Eur. method 2.4.23)

When the monograph does not specify the method to be used, method A is applied. Any change from method A to method B must be validated.

METHOD A

Separation of the sterol fraction (TLC)

Prepare the unsaponifiable matter and then isolate the sterol fraction of the fatty oil by thin-layer chromatography (*2.2.27*), using a *TLC silica gel plate R* with a 0.2 mm to 0.5 mm layer.

Test solution (a) In a 150 mL flask fitted with a reflux condenser, place a volume of a 2 g/L solution of *betulin R* in *methylene chloride R* containing betulin corresponding to about 10 per cent of the sterol content of the sample used for the determination (e.g. in the case of olive oil add 500 µL, in the case of other vegetable oils add 1500 µL of the betulin solution). If the monograph requires the percentage content of the individual sterols in the sterol fraction, the addition of betulin may be omitted. Evaporate to dryness under a current of *nitrogen R*. Add 5.00 g (*m*) of the substance to be examined. Add 50 mL of *2 M alcoholic potassium hydroxide R* and heat on a water-bath for 1 h, swirling frequently. Cool to a temperature below 25 °C and transfer the contents of the flask to a separating funnel with 100 mL of *water R*. Shake the liquid carefully with 3 quantities, each of 100 mL, of *peroxide-free ether R*. Combine the ether layers in another separating funnel containing 40 mL of *water R*, shake gently for a few minutes, allow to separate and reject the aqueous phase. Wash the ether phase with several quantities, each of 40 mL, of *water R*, until the aqueous phase is no longer alkaline to phenolphthalein. Transfer the ether phase to a tared flask, washing the separating funnel with *peroxide-free ether R*. Distil off the ether with suitable precautions and add 6 mL of *acetone R* to the residue. Carefully remove the

solvent in a current of *nitrogen R*. Dry to constant mass at 100-105 °C. Allow to cool in a desiccator and weigh. Transfer the residue to a small test tube with *methylene chloride R*. Evaporate under a stream of *nitrogen R* to a volume of about 1 mL. Depending on the unsaponifiable content of the oil, adapt the final concentration of the solution to 25-50 mg/mL.

Test solution (b) Treat 5.00 g of *rapeseed oil R* as prescribed for the substance to be examined, beginning at the words "Add 50 mL of *2 M alcoholic potassium hydroxide R*".

Test solution (c) Treat 5.00 g of *sunflower oil R* as prescribed for the substance to be examined, beginning at the words "Add 50 mL of *2 M alcoholic potassium hydroxide R*".

Reference solution Dissolve 25 mg of *cholesterol R* and 10 mg of *betulin R* in 1 mL of *methylene chloride R*.

Use a separate plate for each test solution. Apply as a band of 10 mm, at 20 mm from the base and 10 mm from the left edge, 10 µL of the reference solution and as bands of 150 mm, at 20 mm from the base, 0.5 mL of test solutions (a), (b) or (c). Develop over a path of 17 cm using a mixture of 35 volumes of *ether R* and 65 volumes of *hexane R*. Dry the plates in a current of *nitrogen R*. Spray the plates with a 2 g/L solution of *dichlorofluorescein R* in *anhydrous ethanol R* and examine in ultraviolet light at 254 nm. The chromatogram obtained with the reference solution shows bands due to cholesterol and betulin. The chromatograms obtained with the test solutions show bands with similar R_F values due to sterols. From each of the chromatograms, remove an area of coating corresponding to the area occupied by the sterol bands and additionally the area of the zones 2-3 mm above and below the visible zones corresponding to the reference solution. Place separately in three 50 mL flasks. To each flask add 15 mL of *methylene chloride R* and heat under reflux with stirring, for 15 min. Filter each solution through a sintered-glass filter (40) (*2.1.2*) or suitable filter paper and wash each filter with 3 quantities, each of 15 mL, of *methylene chloride R*. Place the combined filtrate and washings from each filter separately in 3 flasks, evaporate under a stream of *nitrogen R* to 5-10 mL. Transfer to a small test tube and evaporate to dryness under a stream of *nitrogen R*.

Determination of the sterols (GC)

Gas chromatography (*2.2.28*). *Carry out the operations protected from humidity and prepare the solutions immediately before use.*

Test solution To the sterols separated from the substance to be examined by thin-layer chromatography add a freshly prepared mixture of 0.04 mL of *chlorotrimethylsilane R*, 0.1 mL of *hexamethyldisilazane R* and 0.5 mL of *anhydrous pyridine R*. Allow to stand for at least 5 min and use the liquid phase.

Reference solution (a) To 9 parts of the sterols separated from *rapeseed oil R* by thin-layer chromatography add 1 part of *cholesterol R*. To the mixture add a freshly prepared mixture of 0.04 mL of *chlorotrimethylsilane R*, 0.1 mL of *hexamethyldisilazane R* and 0.5 mL of *anhydrous pyridine R*. Allow to stand for at least 5 min and use the liquid phase.

Reference solution (b) To the sterols separated from *sunflower oil R* by thin-layer chromatography add a freshly prepared mixture of 0.04 mL of *chlorotrimethylsilane R*, 0.1 mL of *hexamethyldisilazane R* and 0.5 mL of *anhydrous pyridine R*. Allow to stand for at least 5 min and use the liquid phase.

Column:
- *material*: fused silica;
- *size*: *l* = 20-30 m, Ø = 0.25-0.32 mm;
- *stationary phase*: *phenyl(5)methyl(95)polysiloxane R* or *cyanopropyl(7)phenyl(7)methyl(86)polysiloxane R* (film thickness 0.25 µm).

Carrier gas *hydrogen for chromatography R* or *helium for chromatography R*.

Linear velocity 30-50 cm/s (hydrogen) or 20-35 cm/s (helium).

Split ratio 1:50 (hydrogen) or 1:100 (helium).

Temperature:
- *column*: 260 °C;
- *injection port*: 280 °C;
- *detector*: 290 °C.

Detection Flame ionisation.

Injection 1 µL.

Identification of peaks The chromatogram obtained with reference solution (a) shows 4 principal peaks corresponding to cholesterol, brassicasterol, campesterol and β-sitosterol and the chromatogram obtained with reference solution (b) shows 4 principal peaks corresponding to campesterol, stigmasterol, β-sitosterol and Δ7-stigmastenol. The relative retentions of the sterols with reference to β-sitosterol (main peak) are given in Table 2.4.23.-1.

Table 2.4.23.-1. – *Relative retentions of sterols with reference to β-sitosterol for 2 different columns*

	Cyanopropyl (7)(phenyl) (7)(methyl)(86) polysiloxane	Phenyl(5)methyl (95)polysiloxane
Cholesterol	0.64	0.63
Brassicasterol	0.70	0.71
24-Methylenecholesterol	0.79	0.80
Campesterol	0.82	0.81
Campestanol	0.83	0.82
Stigmasterol	0.87	0.87
Δ7-Campesterol	0.93	0.92
Δ5,23-Stigmastadienol	0.95	0.95
Clerosterol	0.96	0.96
β-Sitosterol	1	1
Sitostanol	1.01	1.02
Δ5-Avenasterol	1.03	1.03
Δ5,24-Stigmastadienol	1.09	1.08
Δ7-Stigmastenol(1)	1.13	1.12
Δ7-Avenasterol	1.18	1.16
Betulin	1.4	1.4

(1) This sterol may also be referred to as Δ7-stigmasterol in literature.

The peak due to the internal standard (betulin) must be clearly separated from the peaks due to the sterols to be determined.

For the chromatogram obtained with the test solution, identify the peaks and calculate the percentage content of each sterol in the sterol fraction of the substance to be examined using the following expression:

$$\frac{A}{S} \times 100$$

A = area of the peak due to the component to be determined;
S = sum of the areas of the peaks due to the components indicated in Table 2.4.23.-1; disregard the peak due to betulin.

If required in the monograph, calculate the content of each sterol in milligrams per 100 grams of the substance to be examined using the following expression:

$$\frac{A \times m' \times 100}{A' \times m}$$

A = area of the peak due to the component to be determined;
A' = area of the peak due to betulin;
m = mass of the sample of the substance to be examined, in grams;
m' = mass of *bendin R* added, in milligrams.

METHOD B

Preparation of the unsaponifiable matter

Prepare the unsaponifiable matter according to the method stated in the test for unsaponifiable matter of the monograph on the substance to be examined. Failing this, prepare the unsaponifiable matter according to the method described in chapter *2.5.7. Unsaponifiable matter*. After the final neutralisation step, evaporate the ethanol, then add 6 mL of *acetone R* and evaporate the solvent. Dry the residue at 100-105 °C. It is not necessary to dry to constant mass.

Simultaneously prepare under the same conditions the unsaponifiable matter of *sunflower oil R*. This will in particular serve to locate the sterol fraction to be collected.

Separation of the sterol fraction (LC)

Liquid chromatography (*2.2.29*).

Test solution Take up the residue with 3 quantities, each of 4 mL, of the solvent used during the preparation of the unsaponifiable matter (generally *ether R* or *light petroleum R*) and transfer to a 15 mL tube. Evaporate to dryness under a current of *nitrogen R*. Dissolve the residue in a volume of mobile phase sufficient to obtain a solution with an approximate concentration of 40 mg/mL. Add a few drops of *2-propanol R1* to improve the solubility (3 drops are normally sufficient to ensure complete solubilisation). Filter through a membrane filter (nominal pore size 0.45 µm).

Reference solution Proceed as described for the test solution with the unsaponifiable matter obtained with *sunflower oil R*.

Precolumn:
— *size*: l = 5 mm, Ø = 4.6 mm;
— *stationary phase*: *silica gel for chromatography R* (5 µm) with a pore size of 6 nm.

Column:
— *size*: l = 0.25 m, Ø = 4.6 mm;
— *stationary phase*: *silica gel for chromatography R* (5 µm) with a pore size of 6 nm.

Mobile phase *2-propanol R1, hexane R* (1:99 *V/V*).

Flow rate 1 mL/min.

Detection Spectrophotometer at 210 nm.

Injection 50 µL.

Identification of the peaks due to sterols The sterol fraction elutes at the end of the chromatogram. Locate the fraction to be collected using the chromatogram obtained with the reference solution, which shows 2 principal peaks eluting approximately between 23 min and 32 min. Collect the fraction at the detector outlet in a 15 mL tube with a screw cap. Evaporate the solvent under a current of *nitrogen R*.

Determination of the sterols (GC)

Gas chromatography (*2.2.28*).

Test solution Dissolve the residue of the sterol fraction obtained with the test solution in the previous LC step in 0.2 mL of *anhydrous pyridine R* and 0.2 mL of a mixture of 1 volume of *chlorotrimethylsilane R* and 99 volumes of *N,O-bis(trimethylsilyl)trifluoroacetamide R*. Stopper the tube tightly and heat at 80 °C for 20 min. Allow to cool and use the liquid phase.

Reference solution Dissolve the residue of the sterol fraction obtained with the reference solution in the previous LC step in 0.2 mL of *anhydrous pyridine R* and 0.2 mL of a mixture of 1 volume of *chlorotrimethylsilane R* and 99 volumes of *N,O-bis(trimethylsilyl)trifluoroacetamide R*. Stopper the tube tightly and heat at 80 °C for 20 min. Allow to cool and use the liquid phase.

A standard of cholesterol (*cholesterol R*) may also be used, alone or as a mixture with the sterol fraction of sunflower oil. Proceed with derivatisation as described for the test solution.

Column:
— *material*: fused silica;
— *size*: l = 30 m, Ø = 0.25 mm;
— *stationary phase*: *phenyl(5)methyl(95)polysiloxane R* (film thickness 0.25 µm).

Carrier gas *helium for chromatography R*.

Flow rate 2.6 mL/min.

Split ratio 1:25.

Temperature:

	Time (min)	Temperature (°C)
Column	0 - 38	260
	38 - 44	260 → 290
	44 - 49	290
Injection port		290
Detector		290

Detection Flame ionisation.

Injection 1-3 µL (depending on the expected amount of sterols in the substance to be examined).

Identification of peaks Use the chromatogram obtained with the reference solution to identify the peaks due to campesterol, stigmasterol, β-sitosterol and Δ7-stigmastenol. Identify the peaks due to the sterols in the chromatogram obtained with the test solution using the chromatogram obtained with the reference solution and the relative retentions with reference to β-sitosterol (main peak) given in Table 2.4.23.-1.

System suitability Reference solution:
— *resolution*: minimum 4.0 between the peaks due to campesterol and stigmasterol.

Calculate the percentage content of each sterol in the sterol fraction of the substance to be examined using the following expression:

$$\frac{A}{S} \times 100$$

A = area of the peak due to the component to be determined;
S = sum of the areas of the peaks due to the components indicated in Table 2.4.23.-1, except betulin.

Appendix XI

A. Total Solids

(No Ph. Eur. method)

The term 'total solids' is applied to the residue obtained when the prescribed amount of the preparation is dried to constant weight under the conditions specified below.

APPARATUS

A shallow, flat-bottomed, flanged dish, about 75 mm in diameter and about 25 mm deep, made of nickel or other suitable metal of high heat conductivity and low specific heat and which is not affected by boiling water. Suitable dishes are described in British Standard 1742:1951 (Methods for the chemical analysis of condensed milk).

METHOD

Place the quantity stated in the monograph in a tared dish, evaporate at as low a temperature as possible until the ethanol is removed and heat on a water-bath until the residue is apparently dry. Transfer to an oven operating without a fan and dry to constant weight at 105° unless otherwise stated in the monograph. It may be necessary, for residues of a hygroscopic nature, to use a dish provided with a well-fitting cover and to cool in a desiccator.

B1. Ethanol-soluble Extractive

(No Ph. Eur. method)

Macerate 5 g of the air-dried drug, coarsely powdered, with 100 mL of *ethanol* of the specified strength in a closed flask for 24 hours, shaking frequently during the first 6 hours and then allowing to stand for 18 hours. Filter rapidly, taking precautions against loss of ethanol, evaporate 20 mL of the filtrate to dryness in a tared, flat-bottomed, shallow dish and dry at 105° to constant weight. Calculate the percentage of ethanol-soluble extractive with reference to the air-dried drug.

B2. Water-soluble Extractive

(No Ph. Eur. method)

To 5 g of the powdered drug (710) add 200 mL of boiling *water*. Allow to stand for 10 minutes, shaking occasionally. Allow to cool, dilute to 200 mL with *water* and filter. Evaporate 20 mL of the filtrate to dryness on a water-bath. Dry the residue in an oven at 100° to 105° for 15 minutes.

C. Swelling Index

(Ph. Eur. method 2.8.4)

The swelling index is the volume in millilitres occupied by 1 gram of a drug, including any adhering mucilage, after it has swollen in an aqueous liquid for 4 h.

In a 25 mL ground-glass stoppered cylinder graduated over a height of 125 ± 5 mm in 0.5 mL divisions, place 1.0 g of the drug, whole or of the degree of comminution prescribed in the monograph. Unless otherwise prescribed, moisten the drug with 1.0 mL of *alcohol R*, add 25 mL of *water R* and close the cylinder. Shake vigorously every 10 min for 1 h. Allow to stand for 3 h. At 90 min after the beginning of the test, release any large volumes of liquid retained in the layer of the drug and any particles of the drug floating at the surface of the liquid by rotating the cylinder about a vertical axis. Measure the volume occupied by the drug, including any adhering mucilage. Carry out 3 tests at the same time.

The swelling index is given by the mean of the 3 tests.

D. Foreign Matter

(Ph. Eur. method 2.8.2)

Herbal drugs should be free from moulds, insects and other animal contamination.

Foreign matter is material consisting of any or all of the following:

1) *foreign organs*: matter coming from the source plant but not defined as the herbal drug;

2) *foreign elements*: matter not coming from the source plant and of either vegetable or mineral origin.

DRIED PLANTS

Sampling and sample preparation

Apply general chapter *2.8.20. Herbal drugs: sampling and sample preparation*.

Determination of foreign matter

Weigh 100-500 g of the sample, or the minimum quantity prescribed in the individual monograph, and spread it out in a thin layer. Examine for foreign matter by inspection with the unaided eye or by use of a lens (6 ×). Separate foreign matter, weigh it and calculate the percentage present.

FRESH PLANTS

Where general chapter *2.8.20. Herbal drugs: sampling and sample preparation* cannot be applied, use one of the following methods, as appropriate: use method A when the test can be carried out on the whole batch; use method B when the test cannot be carried on the whole batch.

METHOD A

Sampling and sample preparation

Carry out the test on the whole batch.

Determination of foreign matter

Spread the batch out in a thin layer and examine for foreign matter by inspection with the unaided eye or by use of a lens (6 ×). Separate foreign matter, weigh it and calculate the percentage present.

METHOD B

Sampling and sample preparation

If it is not possible to inspect the whole batch, proceed as follows.

Bulk sample Prepare the bulk sample as described in general chapter *2.8.20. Herbal drugs: sampling and sample preparation*.

Test sample Use the bulk sample or, where the bulk sample is greater than 1 kg, reduce it to a mass of 500-1000 g by a suitable method which retains the representative nature of the bulk sample.

Determination of foreign matter

Use the sample, or the minimum quantity prescribed in the individual monograph, and spread it out in a thin layer. Examine for foreign matter by inspection with the unaided eye or by use of a lens (6 ×). Separate foreign matter, weigh it and calculate the percentage present.

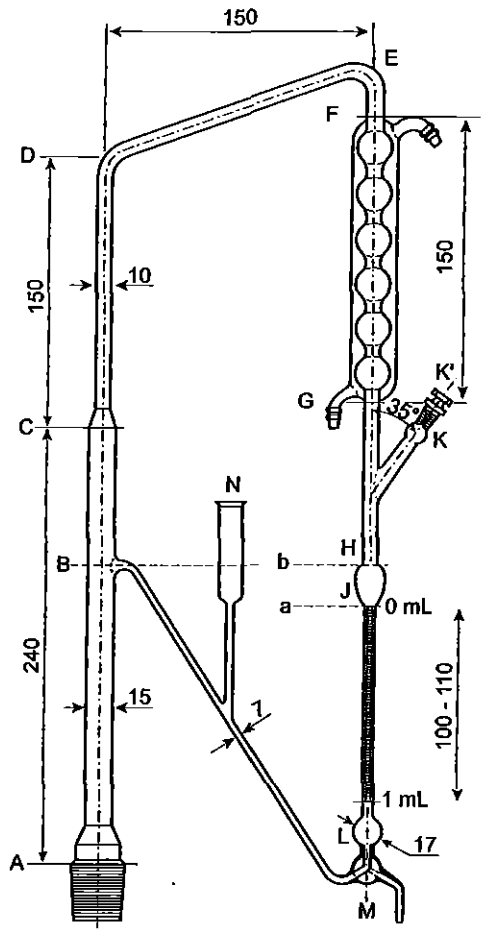


Figure 2.8.12.-1. – *Apparatus for the determination of essential oils in herbal drugs*

Dimensions in millimetres

E. Essential Oils in Herbal Drugs

(Ph. Eur. method 2.8.12)

PRINCIPLE

The determination of essential oils in herbal drugs is carried out by steam distillation in a special apparatus under the conditions described below. The distillate is collected in the graduated tube using the solvent prescribed in the monograph (usually *xylene R*, *trimethylpentane R* or *1,2,4-trimethylbenzene R*) to take up the essential oil; the aqueous phase is automatically returned to the distillation flask.

EQUIPMENT

The equipment typically consists of:

- a suitable round-bottomed flask with a short, ground-glass neck that has an internal diameter of about 29 mm at the wide end;
- a condenser assembly (see Figure 2.8.12.-1) that closely fits the flask, the different parts being fused into 1 piece and made of glass with a low coefficient of thermal expansion:
  - a vented stopper (*K'*) with an opening about 1 mm in diameter, and a tube (*K*), the wide end of which is made of ground-glass and has an internal diameter of 10 mm;
  - a pear-shaped swelling (*J*) with a capacity of 3 mL;
  - a tube (*JL*) graduated in 0.01 mL;
  - a bulb-shaped swelling (*L*) with a capacity of about 2 mL;
  - a 3-way tap (*M*);
  - a junction (*B*) situated at a level 20 mm higher than the uppermost graduation of tube *JL*;
- a suitable heating device, allowing fine temperature control;
- a vertical support with a horizontal ring covered with insulating material.

PROCEDURE

Use a thoroughly cleaned apparatus. Carry out the determination according to the nature of the herbal drug to be examined. Place the prescribed volume of distillation liquid in the flask, add a few pieces of porous porcelain and attach the condenser assembly. Introduce *water R* through the filling funnel (*N*) until it reaches level *B*. Remove the stopper (*K'*) and introduce the prescribed quantity of the solvent indicated in the monograph using a pipette with its tip placed at the bottom of tube *K*. Re-insert the stopper (*K'*) and ensure that the vent is unobstructed. Heat the liquid in the flask to boiling and adjust the distillation rate to 2-3 mL/min, unless otherwise prescribed.

Determination of the rate of distillation

During distillation, determine the rate of distillation by lowering the level of the water by means of tap *M* until the meniscus reaches the level of the lower mark (*a*) (see Figure 2.8.12.-2). Close tap *M* and measure the time taken for the liquid to reach the upper mark (*b*). Modify the heat to obtain the target distillation rate. If the distillation rate is still not within the prescribed range, repeat the operation. If the distillation conditions are not changed, it is sufficient to determine the distillation rate at regular intervals rather than before each test.

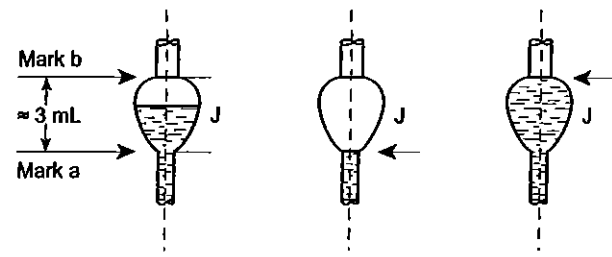


Figure 2.8.12.-2

Determination of the solvent volume after blank distillation

If using *xylene R* or *trimethylpentane R*, distil for 30 min. Ensure that tubes *BM* and *JM* are connected via tap *M* during distillation. Stop heating and wait at least 10 min before reading the volume of solvent in the graduated tube.

If using *1,2,4-trimethylbenzene R*, the 30 min blank distillation step is not necessary. Stop heating after adjusting the distillation rate and wait at least 10 min before reading the volume of solvent in the graduated tube.

Determination of the essential oil in the herbal drug

Introduce the prescribed quantity of the herbal drug into the flask and continue distillation as described above for the time and at the rate prescribed. Stop heating, read the volume of liquid collected in the graduated tube after 10 min and subtract the volume of the solvent previously noted. The difference represents the quantity of essential oil in the sample. Calculate the result in millilitres per kilogram of herbal drug.

Recovery of the mixture of solvent and essential oil
When the essential oil is to be used for other analytical purposes, the water-free mixture of solvent and essential oil may be recovered as follows: remove the stopper (*K'*) and introduce 0.1 mL of a 1 g/L solution of *sodium fluoresceinate R* and 0.5 mL of *water R*. Run the mixture of solvent and essential oil into the bulb-shaped swelling (*L*) by opening tap *M*, allow to stand for 5 min and lower the level of the mixture slowly until it just reaches the level of tap *M*. Open tap *M* clockwise so that the water flows out of the connecting tube (*BM*). Wash the tube with *acetone R* introduced through the filling funnel (*N*). Turn tap *M* anti-clockwise in order to recover the mixture of solvent and essential oil in an appropriate flask.

F. Continuous Extraction of Drugs

(No Ph. Eur. method)

Where the process of *maceration* or *percolation* is specified in a monograph, carry out the following procedures with any modification indicated in the monograph.

MACERATION
Place the solid materials with the whole of the menstruum in a closed vessel and allow to stand for 7 days, shaking occasionally. Strain, press the marc and mix the liquids obtained. Clarify by subsidence or filtration.

PERCOLATION
Moisten the solid materials with a sufficient quantity of the menstruum, allow to stand for 4 hours in a well-closed vessel, pack in a percolator and add sufficient of the menstruum to saturate the materials. When the liquid begins to drop from the percolator, close the outlet, add sufficient of the menstruum to leave a layer above the drug and allow to macerate for 24 hours. Allow percolation to proceed slowly until the percolate measures about three-quarters of the required volume. Press the marc, mix the expressed liquid with the percolate and add sufficient of the menstruum to produce the required volume. Clarify by subsidence or filtration.

Continuous extraction of a drug for the purpose of an assay consists of percolating the drug with the solvent stated in the monograph at a temperature approximately that of the boiling point of the solvent.

The apparatus described below, or any similar apparatus, may be used, provided that it permits the uniform percolation of the drug and the regular flow of the vapour of the solvent around the percolator.

The apparatus is shown in Fig. 11F–1. A is an outer tube of stout glass; the wider part is about 18 cm long and has an internal diameter of 4.8 to 5 cm; the lower end C is about 5 cm long and has an external diameter of about 1.6 cm. B is a straight glass tube open at both ends, about 9 cm long and with an external diameter of about 3.8 cm; over its lower, flanged end is tied firmly a piece of calico or other suitable material. D is a glass coil which supports the margin of the tube B and prevents it from resting in contact with the outer tube A. The lower end C of the outer tube A is fitted by a cork or ground-glass joint to the distillation flask E, in which a suitable quantity of the solvent has been placed. The drug to be extracted, previously moistened with the solvent or subjected to any preliminary treatment required, is introduced into the inner tube B, which is supported so that the percolate drops into the outer tube. A pad of absorbent cotton G is placed on the top of the drug, the inner tube is lowered into position and the outer tube connected by means of a suitable cork or ground-glass joint with the tube of a reflux condenser F. The flask is heated and the extraction continued as directed.

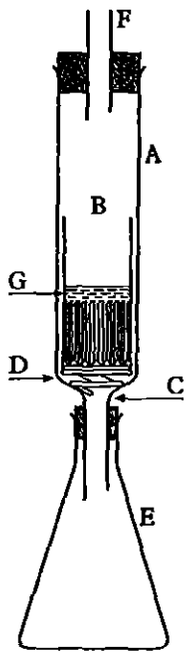


Fig. 11F–1 Apparatus for the Continuous Extraction of Drugs

G. Complete Extraction of Alkaloids

(No Ph. Eur. method)

Complete extraction is indicated by the following tests.

EXTRACTION WITH AN AQUEOUS OR ALCOHOLIC LIQUID
After extracting at least three times with the liquid, add to 0.1 to 0.2 mL of the next portion, after acidifying with 2M *hydrochloric acid* if necessary, 0.05 mL of *potassium tetraiodomercurate solution* or, for solanaceous alkaloids, 0.05 mL of *potassium iodobismuthate solution R1*.
No precipitate or turbidity is produced.

EXTRACTION WITH AN IMMISCIBLE SOLVENT
After extracting at least three times with the solvent, add to 1 to 2 mL of the next portion 1 to 2 mL of 0.1M *hydrochloric acid*, remove the organic solvent by evaporation, transfer the aqueous residue to a test tube and add 0.05 mL of *potassium tetraiodomercurate solution* or, for solanaceous alkaloids, 0.05 mL of *potassium iodobismuthate solution R1* or, for emetine, 0.05 mL of *iodinated potassium iodide solution*.
Not more than a very faint opalescence is produced.

CONTINUOUS EXTRACTION
After percolating for at least 2 hours, collect 1 to 2 mL of the effluent and carry out the procedure described under 'Extraction with an aqueous or alcoholic liquid' or 'Extraction with an immiscible solvent', as appropriate.

H. Stomata

(Ph. Eur. method 2.8.3)

STOMATA

There are several types of stomata (see Figure 2.8.3.-1), distinguished by the form and arrangement of the surrounding cells:

(1) The *anomocytic* (irregular-celled) type: the stoma is surrounded by a varying number of cells in no way differing from those of the epidermis generally,

(2) The *anisocytic* (unequal-celled) type: the stoma is usually surrounded by 3 subsidiary cells, of which one is markedly smaller than the others,

(3) The *diacytic* (cross-celled) type: the stoma is accompanied by 2 subsidiary cells, whose common wall is at right angles to the guard cells,

(4) The *paracytic* (parallel-celled) type: the stoma has on each side one or more subsidiary cells parallel to the long axis of the pore and guard cells.

STOMATAL INDEX

$$\text{Stomatal Index} = \frac{100 \times S}{E + S}$$

S = the number of stomata in a given area of leaf,
E = the number of epidermal cells (including trichomes) in the same area of leaf.

For each sample of leaf, make not fewer than 10 determinations and calculate the mean.

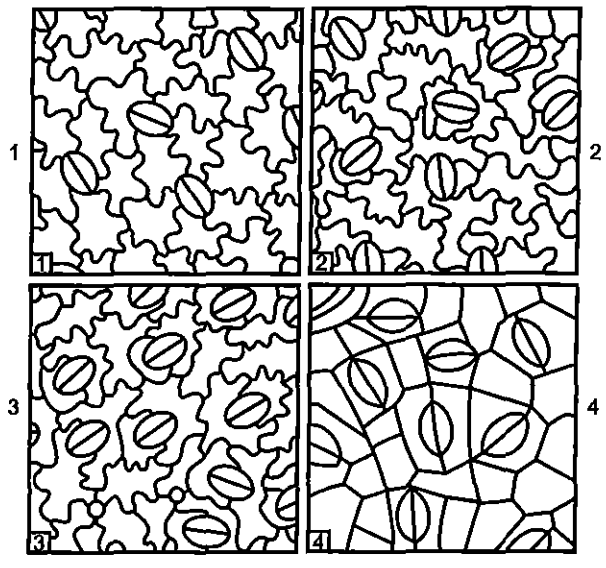


Figure 2.8.3.-1

J. Ash

Use Method I unless otherwise directed in the monograph.

Method I

(No Ph. Eur. method)

FOR HERBAL DRUGS

Incinerate 2 to 3 g of the ground drug in a tared platinum or silica dish at a temperature not exceeding 450° until free from carbon, cool and weigh. If a carbon-free ash cannot be obtained in this way, exhaust the charred mass with hot *water*, collect the residue on an ashless filter paper, incinerate the residue and filter paper, add the filtrate, evaporate to dryness and ignite at a temperature not exceeding 450°. Calculate the percentage of ash with reference to the air-dried drug.

FOR OTHER SUBSTANCES

Carry out the above method using 1 g, unless otherwise stated. Calculate the percentage of ash.

Method II

(Ph. Eur. method 2.4.16)

Heat a silica or platinum crucible to redness for 30 min, allow to cool in a desiccator and weigh. Unless otherwise prescribed, evenly distribute 1.00 g of the substance or the powdered herbal drug to be examined in the crucible. Dry at 100 °C to 105 °C for 1 h and ignite to constant mass in a muffle furnace at 600 °C ± 25 °C, allowing the crucible to cool in a desiccator after each ignition. Flames should not be produced at any time during the procedure. If after prolonged ignition the ash still contains black particles, take up with hot water, filter through an ashless filter paper and ignite the residue and the filter paper. Combine the filtrate with the ash, carefully evaporate to dryness and ignite to constant mass.

K. Acid-insoluble Ash

Use Method I unless otherwise directed in the monograph.

Method I

(No Ph. Eur. method)

Boil the *ash* for 5 minutes with 25 ml of 2M *hydrochloric acid*, collect the insoluble matter in a sintered-glass crucible or on an ashless filter paper, wash with hot *water* and ignite. Calculate the percentage of acid-insoluble ash with reference to the air-dried drug.

Method II

(Ph. Eur. method 2.8.1)

Ash insoluble in hydrochloric acid is the residue obtained after extracting the sulfated or total ash with hydrochloric acid, calculated with reference to 100 g of drug.

To the crucible containing the residue from the determination of sulfated or total ash, add 15 mL of *water R* and 10 mL of *hydrochloric acid R*, cover with a watch-glass, boil the mixture gently for 10 min and allow to cool. Filter through an ashless filter, wash the residue with hot *water R* until the filtrate is neutral, dry, ignite to dull redness, allow to cool in a desiccator and weigh. Reheat until the difference between 2 consecutive weighings is not more than 1 mg.

L. Pesticide Residues

(Ph. Eur. method 2.8.13)

Definition

For the purposes of the Pharmacopoeia, a pesticide is any substance or mixture of substances intended for preventing, destroying or controlling any pest, unwanted species of plants or animals causing harm during or otherwise interfering with the production, processing, storage, transport or marketing of herbal drugs. The item includes substances intended for use as growth-regulators, defoliants or desiccants and any substance applied to crops, either before or after harvest, to

protect the commodity from deterioration during storage and transport. Pesticide residues can be present and are controlled in herbal drugs and herbal drug preparations.

Limits

Unless otherwise indicated in the monograph, the herbal drug to be examined at least complies with the limits indicated in Table 2.8.13.-1. The limits applying to pesticides that are not listed in Table 2.8.13.-1 and whose presence is suspected for any reason comply with the limits (levels) cross-referred to by Regulation (EC) No. 396/2005, including annexes and successive updates. Limits for pesticides that are not listed in Table 2.8.13.-1 or in European Union texts are calculated using the following expression:

$$\frac{ADI \times M}{MDD_{HD} \times 100}$$

ADI = acceptable daily intake, as published by FAO-WHO, in milligrams per kilogram of body mass;
M = body mass in kilograms (60 kg);
MDD_{HD} = daily dose of the herbal drug, in kilograms.

The limits for pesticides in herbal drug preparations are calculated using the following expressions:

If $DER \leq 10$: $MRL_{HD} \times DER$

If $DER > 10$: $\frac{ADI \times M}{MDD_{HP} \times 100}$

MRL_{HD} = maximum residue limit of the pesticide in the herbal drug as given in Table 2.8.13.-1 or in EU texts or calculated using the expression mentioned above;
DER = drug/extract ratio, i.e. the ratio between the quantity of herbal drug used in the manufacture of a herbal drug preparation and the quantity of herbal drug preparation obtained;
MDD_{HP} = daily dose of the herbal drug preparation, in kilograms.

The competent authority may grant total or partial exemption from the test when the complete history (nature and quantity of the pesticides used, date of each treatment during cultivation and after the harvest) of the treatment of the batch is known and can be checked precisely according to good agricultural and collection practice (GACP).

Table 2.8.13.-1

Substance	Limit (mg/kg)
Acephate	0.1
Alachlor	0.05
Aldrin and dieldrin (sum of)	0.05
Azinphos-ethyl	0.1
Azinphos-methyl	1
Bromophos-ethyl	0.05
Bromophos-methyl	0.05
Brompropylate	3
Chlordane (sum of *cis-*, *trans* - and oxychlordane)	0.05
Chlorfenvinphos	0.5
Chlorpyriphos-ethyl	0.2
Chlorpyriphos-methyl	0.1
Chlorthal-dimethyl	0.01
Cyfluthrin (sum of)	0.1
λ-Cyhalothrin	1
Cypermethrin and isomers (sum of)	1
DDT (sum of *o,p'*-DDE, *p,p'*-DDE, *o,p'*-DDT, *p,p'*-DDT, *o, p'*-TDE and *p,p'*-TDE)	1
Deltamethrin	0.5
Diazinon	0.5
Dichlofluanid	0.1
Dichlorvos	1
Dicofol	0.5
Dimethoate and omethoate (sum of)	0.1
Dithiocarbamates (expressed as CS2)	2
Endosulfan (sum of isomers and endosulfan sulfate)	3
Endrin	0.05
Ethion	2
Etrimphos	0.05
Fenchlorophos (sum of fenchlorophos and fenchlorophos-oxon)	0.1
Fenitrothion	0.5
Fenpropathrin	0.03
Fensulfothion (sum of fensulfothion, fensulfothion-oxon, fensulfothion-oxonsulfon and fensulfothion-sulfon)	0.05
Fenthion (sum of fenthion, fenthion-oxon, fenthion-oxon-sulfon, fenthion-oxon-sulfoxid, fenthion-sulfon and fenthion-sulfoxid)	0.05
Fenvalerate	1.5
Flucytrinate	0.05
τ-Fluvalinate	0.05
Fonophos	0.05
Heptachlor (sum of heptachlor, *cis*-heptachlorepoxide and *trans*-heptachlorepoxide)	0.05
Hexachlorbenzene	0.1
Hexachlorocyclohexane (sum of isomers α-, β-, δ- and ε)	0.3
Lindan (γ-hexachlorocyclohexane)	0.6
Malathion and malaoxon (sum of)	1
Mecarbam	0.05
Methacriphos	0.05
Methamidophos	0.05
Methidathion	0.2
Methoxychlor	0.05
Mirex	0.01
Monocrotophos	0.1
Parathion-ethyl and paraoxon-ethyl (sum of)	0.5
Parathion-methyl and paraoxon-methyl (sum of)	0.2
Pendimethalin	0.5
Pentachloranisol	0.01
Permethrin and isomers (sum of)	1
Phosalone	0.1
Phosmet	0.05
Piperonyl butoxide	3
Pirimiphos-ethyl	0.05
Pirimiphos-methyl (sum of pirimiphos-methyl and *N*-desethyl-pirimiphos-methyl)	4
Procymidone	0.1
Profenophos	0.1
Prothiophos	0.05
Pyrethrum (sum of cinerin I, cinerin II, jasmolin I, jasmolin II, pyrethrin I and pyrethrin II)	3
Quinalphos	0.05
Quintozene (sum of quintozene, pentachloraniline and methyl penthachlorphenyl sulfide)	1
S-421	0.02
Tecnazene	0.05
Tetradifon	0.3
Vinclozolin	0.4

Sampling of herbal drugs

Sampling is done according to general chapter *2.8.20. Herbal drugs: sampling and sample preparation.*

Qualitative and quantitative analysis of pesticide residues

The analytical procedures used are validated (e.g. according to Document No. SANCO/10232/2006 or any subsequent

revisions of this document). In particular, they satisfy the following criteria:

- — the chosen method, especially the purification steps, is suitable for the combination pesticide residue/substance to be examined, and not susceptible to interference from co-extractives;
- — natural occurrence of some constituents is considered in the interpretation of results (e.g. disulfide from crucifers);
- — the concentration of test and reference solutions and the setting of the apparatus are such that the responses used for quantification of the pesticide residues are within the dynamic range of the detector; test solutions containing pesticide residues at a level outside the dynamic range, may be diluted within the calibration range, provided that the concentration of the matrix in the solution is adjusted in the case where the calibration solutions must be matrix-matched;
- — between 70 per cent to 110 per cent of each pesticide is recovered;
- — repeatability of the method: RSD is not greater than the values indicated in Table 2.8.13.-2;
- — reproducibility of the method: RSD is not greater than the values indicated in Table 2.8.13.-2.

Table 2.8.13.-2

Concentration range of the pesticide (mg/kg)	Repeatability (RSD) (per cent)	Reproducibility (RSD) (per cent)
0.001 - 0.01	30	60
> 0.01 - 0.1	20	40
> 0.1 - 1	15	30
> 1	10	20

M. Tannins in Herbal Drugs

(Ph. Eur. method 2.8.14)

Carry out all the extraction and dilution operations protected from light.

In the case of a herbal drug or a dry extract, to the stated amount of the powdered drug (180) (*2.9.12*) or the extract in a 250 mL round-bottomed flask add 150 mL of *water R*. Heat on a water-bath for 30 min. Cool under running water and transfer quantitatively to a 250 mL volumetric flask. Rinse the round-bottomed flask and collect the washings in the volumetric flask, then dilute to 250.0 mL with *water R*. Allow the solids to settle and filter the liquid through a filter paper 125 mm in diameter. Discard the first 50 mL of the filtrate.

In the case of a liquid extract or a tincture, dilute the stated amount of the liquid extract or tincture to 250.0 mL with *water R*. Filter the mixture through a filter paper 125 mm in diameter. Discard the first 50 mL of the filtrate.

Total polyphenols Dilute 5.0 mL of the filtrate to 25.0 mL with *water R*. Mix 2.0 mL of this solution with 1.0 mL of *phosphomolybdotungstic reagent R* and 10.0 mL of *water R* and dilute to 25.0 mL with a 290 g/L solution of *sodium carbonate R*. After 30 min measure the absorbance (*2.2.25*) at 760 nm (A_1), using *water R* as the compensation liquid.

Polyphenols not adsorbed by hide powder To 10.0 mL of the filtrate, add 0.10 g of *hide powder CRS* and shake vigorously for 60 min. Filter and dilute 5.0 mL of the filtrate to 25.0 mL with *water R*. Mix 2.0 mL of this solution with 1.0 mL of *phosphomolybdotungstic reagent R* and 10.0 mL of *water R* and dilute to 25.0 mL with a 290 g/L solution of *sodium carbonate R*. After 30 min measure the absorbance (*2.2.25*) at 760 nm (A_2), using *water R* as the compensation liquid.

Standard Dissolve immediately before use 50.0 mg of *pyrogallol R* in *water R* and dilute to 100.0 mL with the same solvent. Dilute 5.0 mL of the solution to 100.0 mL with *water R*. Mix 2.0 mL of this solution with 1.0 mL of *phosphomolybdotungstic reagent R* and 10.0 mL of *water R* and dilute to 25.0 mL with a 290 g/L solution of *sodium carbonate R*. After 30 min measure the absorbance (*2.2.25*) at 760 nm (A_3), using *water R* as the compensation liquid.

Calculate the percentage content of tannins expressed as pyrogallol from the expression:

$$\frac{62.5(A_1 - A_2)m_2}{A_3 \times m_1}$$

m_1 = mass of the sample to be examined, in grams;
m_2 = mass of pyrogallol, in grams.

N. Bitterness Value

(Ph. Eur. method 2.8.15)

The bitterness value is the reciprocal of the dilution of a compound, a liquid or an extract that still has a bitter taste. It is determined by comparison with quinine hydrochloride, the bitterness value of which is set at 200 000.

Determination of the correction factor

A taste panel comprising at least 6 persons is recommended. The mouth must be rinsed with *water R* before tasting.

To correct for individual differences in tasting bitterness amongst the panel members it is necessary to determine a correction factor for each panel member.

Stock solution Dissolve 0.100 g of *quinine hydrochloride R* in *water R* and dilute to 100.0 mL with the same solvent. Dilute 1.0 mL of this solution to 100.0 mL with *water R*.

Reference solutions Prepare a series of dilutions by placing in a first tube 3.6 mL of the stock solution and increasing the volume by 0.2 mL in each subsequent tube to a total of 5.8 mL; dilute the contents of each tube to 10.0 mL with *water R*.

Determine as follows the dilution with the lowest concentration that still has a bitter taste. Take 10.0 mL of the weakest solution into the mouth and pass it from side to side over the back of the tongue for 30 s. If the solution is not found to be bitter, spit it out and wait for 1 min. Rinse the mouth with *water R*. After 10 min, use the next dilution in order of increasing concentration.

Calculate the correction factor k for each panel member from the expression:

$$k = \frac{n}{5.00}$$

n = number of millilitres of the stock solution in the dilution of lowest concentration that is judged to be bitter.

Persons who are unable to taste any bitterness when using the reference solution prepared from 5.8 mL of stock solution have to be excluded from the panel.

Sample preparation

If necessary, reduce the sample to a powder (710) (*2.9.12*). To 1.0 g of sample add 100 mL of boiling *water R*. Heat on a water-bath for 30 min, stirring continuously. Allow to cool and dilute to 100 mL with *water R*. Shake vigorously and filter, discarding the first 2 mL of the filtrate. The filtrate is labelled C-1 and has a dilution factor (DF) of 100.

If liquids have to be examined, 1 mL of the liquid is diluted with a suitable solvent to 100 mL and designated C-1.

Determination of the bitterness value

Test solutions:
10.0 mL of C-1 is diluted with *water R* to 100 mL: C-2 (DF = 1000)
10.0 mL of C-2 is diluted with *water R* to 100 mL: C-3 (DF = 10 000)
20.0 mL of C-3 is diluted with *water R* to 100 mL: C-3A (DF = 50 000)
10.0 mL of C-3 is diluted with *water R* to 100 mL: C-4 (DF = 100 000)

Starting with dilution C-4 each panel member determines the dilution which still has a bitter taste. This solution is designated D. Note the DF of solution D is Y.

Starting with solution D prepare the following sequence of dilutions:

Solution D (mL)	1.2	1.5	2.0	3.0	6.0	8.0
water R (mL)	8.8	8.5	8.0	7.0	4.0	2.0

Determine the number of millilitres of solution D which, when diluted to 10.0 mL with *water R*, still has a bitter taste (X).

Calculate the bitterness value for each panel member from the expression:

$$\left(\frac{Y \times k}{X \times 0.1}\right)$$

Calculate the bitterness value of the sample to be examined as the average value for all panel members.

O. Foam Index

(Ph. Eur. method 2.8.24)

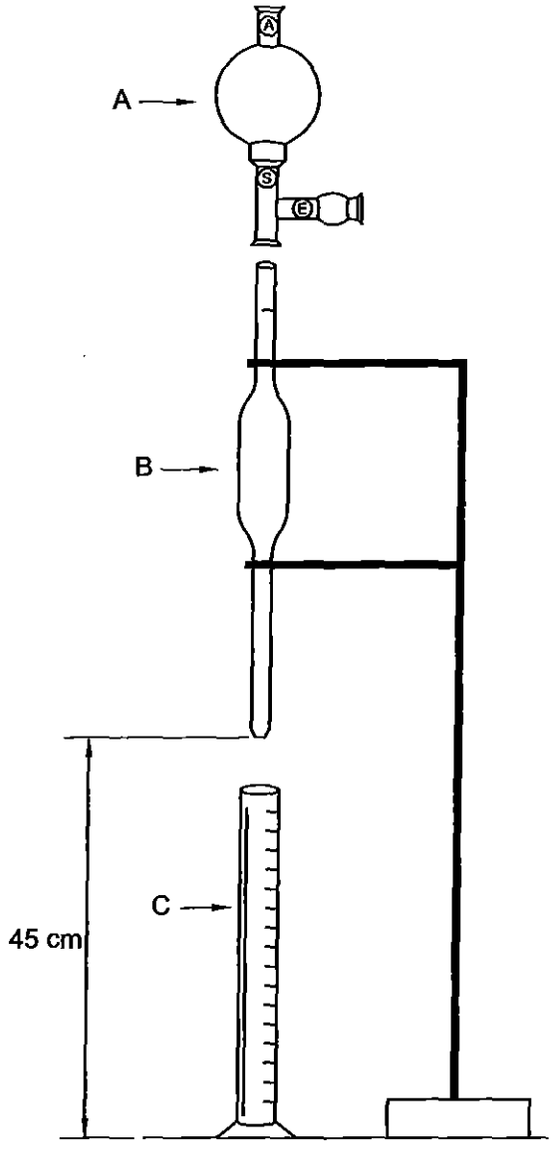
PRINCIPLE

The foam index is determined by measuring the height of the foam produced by the equivalent of 1 g of herbal drug or herbal drug preparation under the stated test conditions.

EQUIPMENT

The equipment (see Figure 2.8.24.-1) typically consists of:
— a pipette stand;
— a 50 mL class A pipette with a rubber filler bulb;
— a 250 mL glass measuring cylinder with 2 mL graduations and an internal diameter of 38 ± 3 mm.

Mount the pipette on its stand so that the tip will be located 45 cm from the base of the cylinder placed under it. Position the cylinder so that the pipette tip is aligned with its centre. Before performing the test, fill the pipette and cylinder with water to check that the liquid dispensed from the pipette runs into the centre of the measuring cylinder. Mark the location of the measuring cylinder.



A. rubber pipette filler
B. 50 mL class A pipette
C. 250 mL glass measuring cylinder with 2 mL graduations and an internal diameter of 38 ± 3 mm

Figure 2.8.24.-1. – *Apparatus suitable for measuring the foam index*

PROCEDURE

Preparation of the test solution

Introduce the quantity of powdered herbal drug (355) (*2.9.12*) or herbal drug preparation prescribed in the monograph into a 250 mL beaker. Add 50 mL of *distilled water R* and allow to stand for 30 min, mixing 3-5 times with a spatula during this period to disperse the powder without producing any foam. Rinse the spatula and the internal walls of the beaker with a further 50 mL of *distilled water R* to ensure that as much of the herbal drug or herbal drug preparation as possible is contained within the liquid. Allow to stand undisturbed for 30 min. Filter through a filter paper 125 mm in diameter. Use the filtrate as the test solution.

Method

Mount the pipette on the stand. Take up 50.0 mL of the test solution using the pipette. Rinse the measuring cylinder with *distilled water R* to wet its walls, then introduce the remaining

volume of test solution, taking care not to produce any foam. Position the measuring cylinder on its mark under the pipette. Open pipette filler valve E to allow the solution to run from the pipette into the measuring cylinder. Record the maximum height of the foam before it starts to collapse. Repeat the test twice, carefully rinsing the pipette and the measuring cylinder with *distilled water R* between tests.

Results

Determine the foam index (I_F) using the following equation:

$$I_F = \frac{H}{m}$$

H = height of the resulting foam, in centimetres;
m = mass of the herbal drug or herbal drug preparation used to prepare the test solution, in grams.

Record the average of 3 determinations as the result.

This result represents the height of the foam for a test solution with a theoretical concentration of 1 g in 100 mL.

P. Dry Residue of Extracts

(Ph. Eur. method 2.8.16)

In a flat-bottomed dish about 50 mm in diameter and about 30 mm in height, introduce rapidly 2.00 g or 2.0 mL of the extract to be examined. Evaporate to dryness on a water-bath and dry in an oven at 100-105 °C for 3 h. Allow to cool in a desiccator over *diphosphorus pentoxide R* or *anhydrous silica gel R* and weigh. Calculate the result as a mass percentage or in grams per litre.

Q. Loss on Drying of Extracts

(Ph. Eur. method 2.8.17)

In a flat-bottomed dish about 50 mm in diameter and about 30 mm in height, weigh rapidly 0.50 g of the extract to be examined, finely powdered. Dry in an oven at 100-105 °C for 3 h. Allow to cool in a desiccator over *diphosphorus pentoxide R* or *anhydrous silica gel R* and weigh. Calculate the result as a mass percentage.

R. Test for Aristolochic Acids in Herbal Drugs

Use Method R1 unless otherwise directed in the monograph.

R1. Test for Aristolochic Acids in Herbal Drugs

(Ph. Eur. method 2.8.21)

CAUTION: aristolochic acids are very toxic and carcinogenic. Perform manipulations in a fume cupboard whenever possible. Take particular precautions, such as use of a glove box, when the substance is in dry form because of its electrostatic properties and the tendency to disperse through the working areas.

Methods A and B are intended to be cross-referenced in monographs on herbal drugs that, according to chemotaxonomic knowledge, are expected to be free from aristolochic acids, but that may be subject to adulteration or substitution with plant material containing aristolochic acids. Methods A and B are intended to be used in the screening of herbal drugs for aristolochic acids at the stated limits and will usually be complemented by macroscopic and/or microscopic tests to exclude plant material containing aristolochic acids.

Method C will not be used in specific monographs but is provided as a method to confirm the presence of aristolochic acid I at levels equal to or greater than 2 ppm. It may be applied if chromatographic data suggests the presence of aristolochic acid I.

These methods are not designed for inclusion as assay methods in monographs on those drugs that produce aristolochic acids as secondary metabolites; for these, a more sensitive, validated method is required.

METHOD A: SCREENING TEST FOR ARISTOLOCHIC ACIDS

Thin-layer chromatography *(2.2.27)*.

Solvent mixture *anhydrous formic acid R, water R, methanol R* (1:9:40 *V/V/V*).

Test solution To 1.0 g of the powdered herbal drug (710) *(2.9.12)* add 10.0 mL of the solvent mixture, sonicate for 10 min and centrifuge.

Reference solution (a) Disperse an amount of *aristolochia HRS* corresponding to 0.10 mg of aristolochic acid I in 20.0 mL of the solvent mixture, sonicate for 10 min and centrifuge.

Reference solution (b) Dilute 1.0 mL of reference solution (a) to 25.0 mL with *methanol R*.

Plate *TLC silica gel F_{254} plate R* (2-10 µm).

Mobile phase *anhydrous formic acid R, water R, ethyl acetate R, toluene R* (3:3:30:60 *V/V/V/V*); use the upper layer.

Application 20 µL as bands of 8 mm.

Development Over a path of 6 cm.

Drying In a current of cold air for 5 min.

Detection Spray with a 100 g/L solution of *stannous chloride R* in *dilute hydrochloric acid R* until the plate is slightly wet, heat at 100 °C for 1 min and examine in ultraviolet light at 365 nm.

System suitability:

— the chromatogram obtained with reference solution (a) shows 2 greenish-blue zones due to aristolochic acids I and II between R_F = 0.35 and R_F = 0.55, which may not be completely separated;
— the chromatogram obtained with reference solution (b) shows at least 1 of these zones (corresponding to 2 ppm of aristolochic acid I).

Results In the chromatogram obtained with the test solution no zone is similar in position and fluorescence to any of the zones due to aristolochic acids in the chromatogram obtained with reference solution (a).

If the chromatogram obtained with the test solution shows any zones similar in position and fluorescence to any of the zones due to aristolochic acids I and II in the chromatogram obtained with reference solution (a), apply method B.

METHOD B: LIMIT TEST FOR ARISTOLOCHIC ACID I

Liquid chromatography *(2.2.29)*.

Solvent mixture *acetonitrile R, water R* (50:50 *V/V*).

Test solution Weigh 2.0 g of the powdered herbal drug (710) *(2.9.12)* into a 250 mL, brown, screw-cap bottle and add 100.0 mL of the solvent mixture. Stir for 30 min at about 300 r/min and filter through a membrane filter (nominal pore size 0.45 µm).

Reference solution (a) Dissolve the contents of a vial of *aristolochic acid I CRS* in the solvent mixture to obtain a concentration of 0.04 µg/mL of aristolochic acid I.

Reference solution (b) Dissolve the contents of a vial of *aristolochic acid for system suitability CRS* (containing aristolochic acids I and II) in the solvent mixture and dilute to 20.0 mL with the solvent mixture.

Column:
- *size:* l = 0.15 m, Ø = 2.1 mm;
- *stationary phase: octadecylsilyl silica gel for chromatography R* (3.5 µm);
- *temperature:* 40 °C.

Mobile phase:
- *mobile phase A: trifluoroacetic acid R, water R* (0.1:99.9 *V/V*);
- *mobile phase B: trifluoroacetic acid R, acetonitrile R* (0.1:99.9 *V/V*);

Time (min)	Mobile phase A (per cent *V/V*)	Mobile phase B (per cent *V/V*)
0 - 25	85 → 35	15 → 65
25 - 30	35 → 0	65 → 100
30 - 31	0 → 85	100 → 15

Flow rate 0.3 mL/min.

Detection Spectrophotometer at 390 nm.

Injection 25 µL.

System suitability:
- *resolution:* minimum 3.0 between the peaks due to aristolochic acids I and II in the chromatogram obtained with reference solution (b);
- *signal-to-noise ratio:* minimum 10 for the peak due to aristolochic acid I in the chromatogram obtained with reference solution (a).

Limit:
- the sample complies with the test if the chromatogram obtained with the test solution shows no peak with the same retention time as the peak due to aristolochic acid I in the chromatogram obtained with reference solution (a) (2 ppm).

METHOD C: CONFIRMATORY TEST FOR ARISTOLOCHIC ACID I

Liquid chromatography (*2.2.29*) coupled with mass spectrometry (*2.2.43*).

Solvent mixture *acetonitrile R, water R* (50:50 *V/V*).

Test solution Weigh 2.0 g of the powdered herbal drug (710) (*2.9.12*) into a 250 mL, brown, screw-cap bottle and add 100.0 mL of the solvent mixture. Sonicate for 30 min and filter through a membrane filter (nominal pore size 0.45 µm).

Reference solution (a) Dissolve the contents of a vial of *aristolochic acid I CRS* in the solvent mixture to obtain a concentration of 0.04 µg/mL of aristolochic acid I.

Reference solution (b) Prepare a solution according to the instructions supplied with *aristolochic acid I CRS* to obtain a concentration of 0.45 µg of aristolochic acid I in 10.0 mL of the test solution.

Column:
- *size:* l = 0.15 m, Ø = 2.1 mm;
- *stationary phase: octadecylsilyl silica gel for chromatography R* (3.5 µm);
- *temperature:* 40 °C.

Mobile phase:
- *mobile phase A: anhydrous formic acid R*, 1 g/L solution of *ammonium acetate R* in *water R* (0.1:99.9 *V/V*);
- *mobile phase B: anhydrous formic acid R*, 1 g/L solution of *ammonium acetate R* in *methanol R* (0.1:99.9 *V/V*);

Time (min)	Mobile phase A (per cent *V/V*)	Mobile phase B (per cent *V/V*)
0 - 15	70 → 0	30 → 100
15 - 16	0	100
16 - 17	0 → 70	100 → 30

Flow rate 0.4 mL/min.

Injection 20 µL; inject reference solution (a) twice, the test solution twice, reference solution (a) twice, then reference solution (b) twice.

Detection Mass detector as described below under A or B. Adjust the flow rate, the temperature and the detector settings so as to comply with the system suitability criterion.

A. Ion-trap mass spectrometer equipped with an electrospray ionisation (ESI) interface and MS^n analyser.

Set the mass spectrometer parameters for the MS^3 mode as follows:

Mode	Parent (*m/z*)	Isolation width (*m/z*)	Relative collision energy (per cent)
MS^2	359 $[M + NH_4]^+$	2.0	30
MS^3	298	2.0	35

- full scan of product ions: from *m/z* 80 to *m/z* 370;
- product ions to be monitored: *m/z* 252, *m/z* 268 and *m/z* 281.

System suitability:
- *signal-to-noise ratio:* minimum 100 for the monitored product ions in the chromatogram obtained with reference solution (a);
- *matrix interference test:* the average of the 2 ratios of reference solution (b) is inside the ± 40 per cent interval of the average of the 2 ratios of reference solution (a); otherwise it is necessary to adjust the detector settings.

Results Evaluate the average ratios (252/268 and 281/268) of the relative intensity of the 3 product ions of aristolochic acid I in the test solution; evaluate the average of the 2 ratios of the signals at the retention time of aristolochic acid I in reference solution (a); if the average of the 2 ratios of the test solution is within the ± 40 per cent interval of the average of the 2 ratios of reference solution (a), aristolochic acid I is present in the test solution.

B. Triple-quadrupole mass spectrometer equipped with an ESI interface and MS^n analyser.

Set the mass spectrometer parameters for the MS^2 mode as follows:
- precursor ion: *m/z* 359 $[M + NH_4]^+$;
- product ions to be monitored: *m/z* 265, *m/z* 281 and *m/z* 296.

System suitability:
- *signal-to-noise ratio:* minimum 100 for the monitored product ions in the chromatogram obtained with reference solution (a);
- *matrix interference test:* the average of the 2 ratios of reference solution (b) is inside the ± 40 per cent

interval of the average of the 2 ratios of reference solution (a); otherwise it is necessary to adjust the detector settings.

Results Evaluate the average ratios (265/281 and 296/281) of the relative intensity of the 3 product ions of aristolochic acid I in the test solution; evaluate the average of the 2 ratios of the signals at the retention time of aristolochic acid I in reference solution (a); if the average of the 2 ratios of the test solution is within the ± 40 per cent interval of the average of the 2 ratios of reference solution (a), aristolochic acid I is present in the test solution.

R2. Test for Aristolochic Acids I and II in Herbal Drugs

(No Ph. Eur. Method)

CAUTION *Aristolochic acids have been shown to be highly toxic and carcinogenic. Extraordinary care should be taken in any procedure in which they are used.*

In line with the prohibition of the use of *Aristolochia* species in unlicensed herbal medicines in the United Kingdom, a test for absence of aristolochic acids I and II in herbal drugs has been included in the British Pharmacopoeia. The limit of detection has been shown to be 0.00078 mg/mL (approximately 1 ppm of aristolochic acids I and II). It is advised that the limit of detection for the system-in-use is determined by the analyst. Aristolochic acids I and II are not confined to the genus *Aristolochia*. The acids are also reported as present in certain species of *Asarum*.

Sample preparation

Unless otherwise specified in the monograph, weigh 2 g of the ground herbal drug into a centrifuge tube. Add 10 mL of 0.1M *sodium hydroxide*, shake for at least 2 hours and centrifuge the mixture for 10 minutes at approximately 4000 revolutions per minute. Filter the supernatant layer if visible particles remain in the suspension. Pass a 1.0 mL portion of the sample solution with the aid of vacuum through a solid-phase extraction column of 1 mL capacity and containing 30 mg of *divinylbenzene and vinylpyrrolidone copolymer for chromatography* (30 µm) (Waters Oasis HLB, 30 mg/mL or Phenomenex StrataX, 30 mg/mL is suitable) previously washed with 1 mL of *methanol*, followed by 1 mL of *water*. Wash the column with 1 mL of 0.1M *sodium hydroxide* followed by 1 mL of a mixture containing 2 volumes of *glacial acetic acid*, 28 volumes of *water* and 70 volumes of *methanol*. Elute the sample with 0.25 mL of a mixture containing 98% of *methanol* and 2% of *concentrated ammonia*. Evaporate the extract to dryness at 40° under a stream of *nitrogen* and dissolve in 0.25 mL of *methanol*. If a larger sample volume is required then a larger capacity solid-phase extraction column may be used.

Use this as solution (1) for the identification method described below.

Identification

Carry out the method for *liquid chromatography*, Appendix III D, using the following solutions. Prepare solution (1) as described above under Sample preparation. Solution (2) contains 0.01% w/v of *aristolochic acid BPCRS* in *methanol*.

The chromatographic procedure may be carried out using (a) a stainless steel column (25 cm × 4.6 mm) packed with *base-deactivated octadecylsilyl silica gel for chromatography* (4 µm) (Genesis C18 is suitable) maintaining the column temperature at 30°, (b) a mixture of 45 volumes of 0.1% v/v of *orthophosphoric acid* and 55 volumes of *acetonitrile* as the mobile phase with a flow rate of 1.3 mL per minute and (c) a detection wavelength of 225 nm. The identity of any peaks suspected to be due to arisotolochic acids I and II may be clarified by use of the UV spectrum recorded with a diode array detector.

Inject 10 µL of solution (2) and allow the chromatography to proceed for 10 minutes. The test is not valid unless the *resolution factor* between the peaks corresponding to aristolochic acid II (retention time about 6 minutes) and aristolochic acid I (retention time about 7 minutes) is at least 3.0. Inject solution (2) six times. The relative standard deviation of the areas of the peaks is at most 1.5%.

Inject 10 µL of solution (1) and allow the chromatography to proceed for 30 minutes. In the chromatogram obtained with solution (1) the peaks due to aristolochic acid I and aristolochic acid II are absent.

S. Determination of Mycotoxins in Herbal Drugs

1. Determination of Aflatoxin B_1 in Herbal Drugs

(Ph. Eur. method 2.8.18)

CAUTION: aflatoxins are very toxic and carcinogenic. Perform manipulations in a fume cupboard whenever possible. Take particular precautions, such as use of a glove box, when toxins are in dry form because of their electrostatic properties and the tendency to disperse through the working areas. Decontamination procedures for laboratory wastes of aflatoxins were developed by the International Agency for Research on Cancer (IARC).

Aflatoxins are naturally occurring mycotoxins produced mainly by *Aspergillus flavus* and *Aspergillus parasiticus*. These fungi are common and widespread in nature and are most often found when certain grains are grown under conditions of stress such as drought. The mould occurs in soil, decaying vegetation, hay, and grains undergoing microbial spoilage, and invades all types of organic substrates whenever and wherever the conditions are favourable for its growth. Favourable conditions include high moisture content and high temperature. At least 13 different types of aflatoxin are produced in nature and most of these are known to be highly toxic and carcinogenic. Aflatoxin B_1 is considered the most toxic. Herbal drugs that are subject to contamination by aflatoxins are tested by a validated method.

Unless otherwise indicated in the monograph, herbal drugs contain not more than 2 µg/kg of aflatoxin B_1.

The competent authority may also require compliance with a limit of 4 µg/kg for the sum of aflatoxins B_1, B_2, G_1 and G_2.

The method described below is cited as an example of a method that has been shown to be suitable for devil's claw root, ginger and senna pods. Its suitability for other herbal drugs must be demonstrated or another validated method used.

METHOD

Liquid chromatography (*2.2.29*).

Aflatoxins are subject to light degradation. Carry out the determination protected from daylight by using UV-absorbing foil on windows in combination with subdued light, or curtains or blinds in combination with artificial light (fluorescent tubes are acceptable). Protect aflatoxin-containing solutions from daylight.

Rinse glassware before use with a 10 per cent *V/V* solution of *sulfuric acid R* and then rinse carefully with *distilled water R* until no more acid is present.

Test solution Use an immunoaffinity column containing antibodies against aflatoxin B_1 with a capacity of not less than 100 ng of aflatoxin B_1 and which gives a recovery of not less than 80 per cent when a solution of 5 ng of aflatoxin B_1 in a mixture of 12.5 mL of *methanol R* and 87.5 mL of *water R* is passed through. Allow the immunoaffinity column to reach room temperature. To 5.00 g of the powdered drug (500) (*2.9.12*) add 100 mL of a mixture of 30 volumes of *water R* and 70 volumes of *methanol R* and extract by sonication for 30 min. Filter through folded filter paper. Pipette 10.0 mL of the clear filtrate into a 150 mL conical flask. Add 70 mL of *water R*. Pass 40 mL through the immunoaffinity column at a flow rate of 3 mL/min (not exceeding 5 mL/min). Wash the column with 2 volumes, each of 10 mL, of *water R* at a flow rate not exceeding 5 mL/min and dry by applying a slight vacuum for 5-10 s or by passing air through the immunoaffinity column by means of a syringe for 10 s. Apply 0.5 mL of *methanol R* to the column and allow to pass through by gravity. Collect the eluate in a 5 mL volumetric flask. After 1 min, apply a 2nd portion of 0.5 mL of *methanol R*. After a further 1 min, apply a 3rd portion of 0.5 mL of *methanol R*. Collect most of the applied elution solvent by pressing air through or applying vacuum to the column. Dilute to 5 mL with *water R* and shake well. If the solution is clear it can be used directly for analysis. Otherwise, pass it through a disposable filter unit prior to injection. Use a disposable filter unit (e.g. 0.45 µm pore size polytetrafluoroethylene filter) that has been shown not to cause loss of aflatoxin by retention.

Aflatoxin B_1 primary stock solution Dissolve *aflatoxin B_1 R* in a mixture of 2 volumes of *acetonitrile R* and 98 volumes of *toluene R* to give a 10 µg/mL solution. To determine the exact concentration of aflatoxin B_1 in the stock solution, record the absorption curve (*2.2.25*) between 330 nm and 370 nm in quartz cells.

Calculate the aflatoxin B_1 mass concentration, in micrograms per millilitre, using the following expression:

$$\frac{A \times M \times 100}{\varepsilon \times l}$$

A	=	absorbance determined at the maximum of the absorption curve;
M	=	molar mass of aflatoxin B_1 (312 g/mol);
ε	=	molar absorptivity of aflatoxin B_1 in the toluene-acetonitrile mixture (1930 m^2/mol);
l	=	optical path length of the cell (1 cm).

Aflatoxin B_1 secondary stock solution Prepare a secondary stock solution containing 100 ng/mL aflatoxin B_1 by diluting aflatoxin B_1 primary stock solution with a mixture of 2 volumes of *acetonitrile R* and 98 volumes of *toluene R*. Wrap the flask tightly in aluminium foil and store it below 4 °C. Before use, do not remove the aluminium foil until the contents have reached room temperature. If the solution has to be stored for a long period (for example, 1 month), weigh the flask and record the mass before and after each use of the solution.

Aflatoxin B_1 standard solutions Place the volumes of aflatoxin secondary stock solution indicated in Table 2.8.18.-1 in separate 250 mL volumetric flasks. Pass a stream of nitrogen through at room temperature until the solvent has just evaporated. To each flask, add 75 mL of *methanol R*, allow the aflatoxin B_1 to dissolve and dilute to 250 mL with *water R*.

Table 2.8.18.-1. – *Aflatoxin B_1 standard solutions*

Standard solution	Volume of secondary stock solution (µL)	Final concentration of standard solution (ng/mL)
1	125	0.05
2	250	0.1
3	500	0.2
4	750	0.3
5	1000	0.4

Calibration curve Prepare the calibration curve using aflatoxin B_1 standard solutions 1 to 5, which cover a range equivalent to 1-8 µg/kg of aflatoxin B_1 in the herbal drug. Check the plot for linearity. If the content of aflatoxin B_1 in the sample to be examined is outside of the calibration range, the test solution must be diluted to an aflatoxin content that is appropriate for the established calibration curve.

Column:
- *size: l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase: octadecylsilyl silica gel for chromatography R* (5 µm).

Mobile phase:
- *mobile phase A* (for post-column derivatisation with photochemical reactor or pyridinium bromide): *acetonitrile R, methanol R, water R* (2:3:6 *V/V/V*);
- *mobile phase B* (for post-column derivatisation with electrochemically derived bromine): add 0.12 g of *potassium bromide R* and 350 µL of dilute nitric acid R1 per litre of mobile phase A.

Flow rate 1 mL/min.

Detection Fluorescence detector with a 360 nm excitation filter and a 420 nm cut-off emission filter, or equivalent. Recommended settings for adjustable detectors are 365 nm (excitation wavelength) and 435 nm (emission wavelength).

Injection 500 µL.

Post-column derivatisation with pyridinium hydrobromide perbromide (PBPB):
- pulseless pump;
- T-piece with zero dead volume;
- polytetrafluoroethylene reaction tube, l = 0.45 m, Ø = 0.5 mm;
- mobile phase A;
- post-column derivatisation reagent: dissolve 50 mg of *pyridinium hydrobromide perbromide R* in 1000 mL of *water R* (store protected from light and use within 4 days);
- flow rate of the derivatisation reagent: 0.4 mL/min.

Post-column derivatisation with photochemical reactor (PHRED)
- reactor unit with one 254 nm low pressure mercury UV bulb (minimum 8 W);
- polished support plate;
- knitted reactor coil: polytetrafluoroethylene tubing knitted tightly around the UV bulb, l = 25 m, Ø = 0.25 mm, nominal void volume 1.25 mL;
- exposure time: 2 min;
- mobile phase A.

Post-column derivatisation with electrochemically generated bromine (KOBRA):
- KOBRA-cell: electrochemical cell that generates a reactive form of bromine for derivatisation of aflatoxins, resulting in enhanced fluorescence; available from various commercial suppliers;
- Derivation direct-current supply in series with the KOBRA-cell, providing a constant current of about 100 µA;

— polytetrafluoroethylene reaction tube, l = 0.12 m, Ø = 0.25 mm;
— mobile phase B.

Elution order Aflatoxin G_2, aflatoxin G_1, aflatoxin B_2, aflatoxin B_1.

Calculation Calculate the calibration curve $y = ax + b$, with aflatoxin B_1 concentration (ng/mL) on the x-axis and the signal (S) on the y-axis. The aflatoxin B_1 concentration (C) in a measured solution is equal to $\frac{S-b}{a}$.

Calculate the aflatoxin B_1 content of the herbal drug, in nanograms per gram, using the following expression:

$$\frac{V_1 \times V_2 \times C}{m \times V_i}$$

m	=	mass of the herbal drug taken for analysis, in grams;
V_1	=	volume of the solvent used for extraction, in millilitres;
V_i	=	aliquot taken for immunoaffinity clean-up, in millilitres;
V_2	=	final volume of solution after elution from the immunoaffinity column and dilution, in millilitres;
C	=	measured aflatoxin B_1 concentration of the test solution, in nanograms per millilitre.

The presence of aflatoxin B_1 may be confirmed by recording the chromatogram without post-column derivatisation, which leads to a large decrease (greater than 10-fold) in the response due to aflatoxin B_1.

2. Determination of Ochratoxin A in Herbal Drugs

(Ph. Eur. method 2.8.22)

CAUTION: ochratoxin A is nephrotoxic and nephrocarcinogenic. Perform manipulations in a fume cupboard. Take particular precautions, such as use of a glove box, when toxins are in dry form because of their electrostatic properties and the tendency to disperse through the working areas. Decontamination procedures for laboratory glassware containing ochratoxin A are necessary (see appendix).

Herbal drugs that are subject to contamination by ochratoxin A are tested by a validated method.

The method described below is cited as an example of a method that has been shown to be suitable for liquorice extract and liquorice root. Its suitability for other herbal drugs must be demonstrated or another validated method used.

METHOD

Liquid chromatography (*2.2.29*).

Use brown glassware that is free from detergent residues. If necessary rinse glassware before use with a 10 per cent *V/V* solution of *sulfuric acid R* and then rinse carefully with *distilled water R* until no more acid is present.

Solution A Mix 80 volumes of *water R*, previously adjusted to pH 2.3 with *anhydrous formic acid R*, and 20 volumes of *acetonitrile R*.

Test solution Use an immunoaffinity column containing antibodies against ochratoxin A with a capacity of not less than 100 ng of ochratoxin A and which gives a recovery of not less than 70 per cent. Allow the immunoaffinity column to reach room temperature.

To 2.00 g of the powdered drug (250) (*2.9.12*) add 80 mL of a 30 g/L solution of *sodium hydrogen carbonate R* and extract by sonication for 30 min (change water of ultrasonic bath after 15 min). Cool to room temperature and dilute to 100.0 mL (V_1) with the same solution. Centrifuge. Mix thoroughly 5.0 mL (V_i) of the clear supernatant with 30 mL *buffer solution pH 7.4 R* and pass the whole solution through the immunoaffinity column at a flow rate of 3 mL/min (do not exceed 5 mL/min). Wash the column first with 10 mL *buffer solution pH 7.4 R* then with 2 quantities, each of 10 mL, of *water R* at a flow rate not exceeding 5 mL/min and dry by applying a slight vacuum for 5-10 s or by passing air through the immunoaffinity column by means of a syringe for 10 s. Apply 0.5 mL of *methanol R* to the column and allow to pass through by gravity.

Collect the eluate in a 4 mL glass vial. After 30 s, apply a 2nd quantity of 0.5 mL of *methanol R* and allow to pass through the column by gravity into the same glass vial. After a further 30 s, repeat with a 3rd portion of 0.5 mL of *methanol R*. Collect any solvent retained on the column by pressing air through or applying vacuum to the column. Evaporate the combined eluates completely to dryness using a thermal block with a nitrogen blanket (40 °C). Dissolve the residue in 0.5 mL (V_2) of solution A. If the solution is clear it can be used directly for analysis. Otherwise, pass it through a disposable filter unit prior to injection. Use a disposable filter unit (e.g. 0.45 µm pore size polytetrafluoroethylene filter) that has been shown not to cause loss of ochratoxin A by retention.

Ochratoxin A primary stock solution Dilute 1.0 mL of *ochratoxin A solution R* to 100.0 mL with *methanol R* and shake thoroughly.

Ochratoxin A secondary stock solution Dilute 10.0 mL of ochratoxin A primary stock solution to 100.0 mL with *methanol R* and shake thoroughly.

Ochratoxin A standard solutions Place the volumes of ochratoxin A primary stock solution or ochratoxin A secondary stock solution indicated in Table 2.8.22.-1 into separate flasks and make up to 50.0 mL with solution A.

Table 2.8.22.-1. – *Ochratoxin A standard solutions*

Standard solution	Volume of ochratoxin A primary stock solution (µL)	Final concentration of ochratoxin A in standard solution (ng/mL)
1	5000	50
2	2500	25
3	1000	10
4	500	5
5	250	2.5
Standard solution	**Volume of ochratoxin A secondary stock solution (µL)**	**Final concentration of ochratoxin A in standard solution (ng/mL)**
6	500	0.5
7	100	0.1

Calibration curve Prepare the calibration curve using ochratoxin A standard solutions 1 to 7, which cover a range equivalent to 0.5-250 µg/kg of ochratoxin A in the herbal drug. Check the plot for linearity. If the content of ochratoxin A in the sample to be examined is outside of the calibration range, the test solution must be diluted to an ochratoxin A content that is appropriate for the established calibration curve.

Column:
— *size*: l = 0.15 m, Ø = 4.6 mm;
— *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm);
— *temperature*: 45 °C.

Mobile phase:
— *mobile phase A*: *water R* adjusted to pH 2.3 with *phosphoric acid R*;

— *mobile phase B: acetonitrile R*;

Time (min)	Mobile phase A (per cent *V/V*)	Mobile phase B (per cent *V/V*)
0 - 30	80 → 40	20 → 60
30 - 35	40 → 20	60 → 80
35 - 37	20	80
37 - 40	20 → 80	80 → 20

Flow rate 0.8 mL/min.

Detection Fluorescence detector; recommended settings for adjustable detectors are 330 nm (excitation wavelength) and 460 nm (emission wavelength).

Injection 20 µL.

Calculation Calculate the calibration curve $y = ax + b$, with ochratoxin A concentration (in nanograms per millilitre) on the x-axis and the signal (S) on the y-axis. The ochratoxin A concentration (C) in a measured solution is equal to $\frac{S-b}{a}$.

Calculate the ochratoxin A content of the herbal drug, in nanograms per gram, using the following expression:

$$\frac{V_1 \times V_2 \times C}{m \times V_i}$$

m = mass of the herbal drug used to prepare the test solution, in grams;
V_1 = volume of dilution, in millilitres;
V_i = aliquot taken for immunoaffinity clean-up, in millilitres;
V_2 = volume in which the residue is taken up, in millilitres;
C = measured ochratoxin A concentration of the test solution, in nanograms per millilitre.

APPENDIX: DECONTAMINATION PROCEDURES FOR LABORATORY GLASSWARE

Rinse glassware with *methanol R* and decontaminate by immersion in *strong sodium hypochlorite solution R* for at least 2 h, then wash thoroughly with water.

T. Herbal Drugs: Sampling and Sample Preparation

(Ph. Eur. method 2.8.20)

In order to reduce the effect of sampling in qualitative and quantitative analysis, it is necessary to ensure that the composition of the sample used is representative of the batch of material being examined. The following procedures are the minimum considered applicable for herbal drugs. *NOTE: other procedures may be used if they can be demonstrated to produce representative batch samples.*

BULK SAMPLE

Where external examination of containers, markings and labels of a batch indicate that it can be considered to be homogeneous, sample the number of randomly selected containers indicated below. Where a batch cannot be considered to be homogeneous, divide it into sub-batches that are as homogeneous as possible, then sample each sub-batch as a homogeneous batch using, as a minimum, the number of randomly selected containers indicated below.

Number of containers in batch (N)	Number of containers to be sampled (n)
1 - 3	all
> 3	$n^* = \sqrt{N} + 1$

* round n up to the next integer

Take one sample from each container to be sampled. The sample is taken from the upper, middle or lower section of the container, such that the samples taken are representative of different parts of the containers. In the case of large bales or bags, samples must be taken from a depth of at least 10 cm. The mass of the material taken from each container is such that the total mass of the bulk sample complies with the following values.

Mass of herbal drug in the batch (kg)	Minimum mass of samples as a percentage of the mass of the batch of herbal drug
< 50	1.00*
50 - 100	0.50
> 100 - 250	0.25
> 250 - 500	0.20
> 500 - 1000	0.18
> 1000 - 2500	0.15
> 2500 - 5000	0.10
> 5000 - 10 000	0.08
> 10 000 - 25 000	0.05

NOTE: if the mass of the batch is greater than 25 000 kg, it is divided into sub-batches, and the procedure is applied to each sub-batch as though it were a homogeneous batch.

* subject to a minimum total mass of 125 g for the bulk sample; if this minimum requirement represents more than 10.0 per cent of the mass of herbal drug in the batch, the whole batch may be used as the sample.

Prepare the bulk sample by combining and thoroughly mixing the samples taken from each of the randomly selected containers (see Table 2.8.20.-1).

TEST SAMPLE

Unless otherwise prescribed in the monograph, prepare the test sample as follows.

Reduce the size of the bulk sample by quartering (see Note below) or by any other method that produces a homogeneous sample, making sure that each retained portion remains representative of the whole, until the minimum retained quantity complies with the following conditions.

Type of herbal drug	Minimum weight of test sample
Roots, rhizomes, bark, herbs	500 g or mass of whole sample if bulk sample is less than 500 g
Leaves, flowers, seeds, fruits	250 g or mass of whole sample if bulk sample is less than 250 g
Broken or fragmented drugs (where average mass of the pieces is less than 0.5 g)	125 g

NOTE: quartering consists of placing the bulk sample, thoroughly mixed, as a level and square-shaped heap and dividing it diagonally into 4 equal parts. 2 opposite quarters are retained and carefully remixed. The process is repeated as necessary until the required minimum mass is obtained for the test sample.

Mill the test sample in a single pass through a 1 mm screen or the screen size specified in the monograph. The use of a milling machine is recommended.

Pass the milled sample through a 1 mm standard sieve or the sieve specified in the monograph. The residue retained on the sieve must not be more than 10 per cent of the total

Table 2.8.20.-1. – *Operation of the sampling procedure in order to obtain the prescribed bulk sample*

Mass of herbal drug in container (kg)	0.5			1			5		
Total mass of herbal drug in the batch (kg)	No. of containers in batch	No. of containers to be sampled	Total mass of samples (g)	No. of containers in batch	No. of containers to be sampled	Total mass of samples (g)	No. of containers in batch	No. of containers to be sampled	Total mass of samples (g)
0.5	1	1	125	–	–	–	–	–	–
1	2	2	125	1	1	125	–	–	–
5	10	5	125	5	4	125	1	1	125
10	20	6	125	10	5	125	2	2	125
25	–	–	–	25	6	250	5	4	250
100	–	–	–	100	11	500	20	6	500
250	–	–	–	–	–	–	50	9	625
500	–	–	–	–	–	–	100	11	1000
Mass of herbal drug in container (kg)	**25**			**125**			**500**		
Total mass of herbal drug in the batch (kg)	**No. of containers in batch**	**No. of containers to be sampled**	**Total mass of samples (g)**	**No. of containers in batch**	**No. of containers to be sampled**	**Total mass of samples (g)**	**No. of containers in batch**	**No. of containers to be sampled**	**Total mass of samples (g)**
25	1	1	250	–	–	–	–	–	–
100	4	3	500	–	–	–	–	–	–
250	10	5	625	2	2	625	–	–	–
500	20	6	1000	4	3	1000	1	1	1000
1000	40	8	1800	8	4	1800	2	2	1800
2000	80	10	3000	16	5	3000	4	3	3000
3000	120	12	3000	24	6	3000	6	4	3000
5000	200	16	5000	40	8	5000	10	5	5000
10 000	400	21	8000	80	10	8000	20	6	8000
25 000	800	30	12 500	160	14	12 500	40	8	12 500

mass of the milled sample, of which not more than 2 per cent of the total mass of the milled sample may be of a particle size greater than 1.5 mm or 1.5 times the specified particle size in the monograph. If these conditions are met, the sample and residue are to be well mixed to form the test sample for analysis.

In those cases where these requirements are not met, the test sample for analysis is composed of the 2 parts measured separately. Therefore, the quantity required for each analysis is derived by weighing proportional quantities of the powder and the residue.

NOTE: for determination of microscopic characters, a portion of the milled test sample is re-milled through a 0.355 mm screen.

U. Microscopic Examination of Herbal Drugs

(Ph. Eur. method 2.8.23)

The microscopic examination of herbal drugs is carried out on the powdered drug (355) (*2.9.12*) unless otherwise prescribed in the monograph.

Chloral hydrate solution R is the most commonly prescribed reagent. However, certain features are not visible or not easily seen after mounting in this reagent. In this case, other reagents are used, for example, a 50 per cent *V/V* solution of *glycerol R*, which makes it possible to visualise starch granules. It may also be necessary to prescribe specific reagents in a monograph, for example: *lactic reagent R* which is used to show the presence of various features, 10 per cent *V/V* alcoholic solution of *phloroglucinol R* and *hydrochloric acid R*, which are used to identify the presence of lignin in cells or tissues, *ruthenium red solution R*, which is used to show the presence of mucilage in cells or *glycerol R* used to show the presence of starch and inulin.

Examination under polarised light (between crossed nicol prisms) is used to identify starch granules (black cross phenomenon), calcium oxalate crystals (refringence) or lignified structures.

MOUNTING IN CHLORAL HYDRATE SOLUTION

Place 2-3 drops of *chloral hydrate solution R* on a glass microscope slide. Disperse a very small quantity of the powdered drug in the liquid and cover the preparation with a cover slip. Heat the preparation very gently to boiling on a hot plate or a micro gas burner. Maintain gentle boiling for a short time. Make sure that the quantity of mounting fluid is sufficient. If necessary, add more fluid using a tapered glass pipette. Allow to cool and then examine under a microscope. Repeat the heating until the starch granules and the water-soluble contents of the cells are no longer visible. Examine under a microscope.

Chloral hydrate tends to crystallise as long needles. To avoid this, proceed as follows: after heating, remove the cover slip; to the preparation add 1 drop of a 10 per cent *V/V* mixture of *chloral hydrate solution R* in *glycerol R*; place a clean cover slip on the preparation; examine under a microscope.

MOUNTING IN A 50 PER CENT *V/V* SOLUTION OF GLYCEROL

Place 2 drops of a 50 per cent *V/V* solution of *glycerol R* on a glass microscope slide. Disperse a very small quantity of the powdered drug in the liquid and cover the preparation with a cover slip. Examine under a microscope.

MOUNTING IN A 10 PER CENT *V/V* ALCOHOLIC SOLUTION OF PHLOROGLUCINOL AND HYDROCHLORIC ACID

Place a very small quantity of the powdered drug on a glass microscope slide. Add 1-2 drops of a 10 per cent *V/V* alcoholic solution of *phloroglucinol R*. Mix and allow the solvent to evaporate almost completely. Add 1-2 drops of *hydrochloric acid R* and cover the preparation with a cover slip. Examine immediately under a microscope. The red colour indicates the presence of lignin.

MOUNTING IN LACTIC REAGENT

Place 2-3 drops of *lactic reagent R* on a glass microscope slide. Disperse a very small quantity of the powdered drug in the liquid and cover the preparation with a cover slip. Heat the preparation very gently to boiling. Maintain gentle boiling for a short time. Make sure that the quantity of mounting fluid is sufficient. If necessary, add more fluid using a tapered glass pipette. Allow to cool and then examine under a microscope. Lignified structures stain bright yellow; structures containing cellulose remain colourless. Starch granules stain more or less violet; certain secretions (e.g., essential oils, resins, oleoresins) stain orange and cork stains red.

MOUNTING IN RUTHENIUM RED SOLUTION

Place 2 drops of *ruthenium red solution R* on a glass microscope slide. Disperse a very small quantity of the powdered drug in the liquid and cover the preparation with a cover slip. After about 1 minute, allow a drop of *distilled water R* to be taken up between the slide and the cover slip. Examine under a microscope. The mucilage stains violet red.

V. Deoxyribonucleic Acid (DNA) Based Identification Techniques for Herbal Drugs

1. INTRODUCTION

Deoxyribonucleic Acid (DNA) barcoding has been widely used as a molecular method for the identification of herbal drugs. This method uses short regions of DNA with species specific sequences as barcodes for recognition. Within the British Pharmacopoeia, selected barcode sequences will be the basis for any molecular identification technique. Where a DNA-based method is specified within a monograph as an identification method, the identified region and its species specific sequence will be published as part of the monograph. An example of a barcoding protocol is given below and contains the methodology and the sequence of the region identified as specific to *Ocimum tenuiflorum*.

DNA barcoding of plant material is achieved by a multistage procedure involving DNA extraction, polymerase chain reactions (PCRs) and Sanger sequencing. DNA-based identification techniques for herbal drugs utilise PCR in the following two applications:

(a) to sequence a target region of DNA that contains key characters for species identification,

(b) the use of a species specific PCR for a target DNA sequence and subsequent detection of the amplicon.

The principles and quality requirements for these two applications are described. Alternate methods may be used if they comply with the quality requirements described below.

2. SCOPE OF METHOD

To establish the requirements for DNA extraction from herbal drug material and the use of this extracted DNA for either:

(a) amplification and sequencing of a target region of the extracted DNA for the purposes of species identification; or,

(b) amplicon production and detection techniques to demonstrate the presence of a defined DNA sequence as indicated in (a) and (b) under INTRODUCTION.

3. PRINCIPLE OF THE METHOD

DNA sequences for the identification of herbal drugs can be detected by either direct DNA sequencing or species specific amplification. Both techniques rely on the complementary base pairing of DNA. PCR must be utilised to amplify the target region.

KEY STAGES OF PCR ARE:

- — double stranded DNA is denatured at high temperature to form single stranded DNA;
- — two primers bind specifically to regions flanking the sequence to be amplified, one on either strand of DNA;
- — a thermo-stable polymerase enzyme constructs the complementary DNA sequence starting from the primer binding positions;
- — the three processes described above are repeated as required by the protocol, (typically between 30 and 40 times) to result in the formation of millions of copies of the sequence between the primer binding positions; this is the amplicon;
- — the amplicon is detected by various means and can be used as the template for Sanger sequencing.

KEY STAGES OF SANGER SEQUENCING ARE:

- — the amplicons are denatured at high temperature to form single stranded DNA amplicons;

— one primer is used (this may be one used in the original PCR) and binds specifically to one end of the single stranded amplicons;
— a thermo-stable polymerase enzyme constructs the complementary DNA sequence, starting from the primer binding position;
— at random positions the construction of the complementary DNA sequence is halted due to the incorporation of a fluorescently labelled di-deoxynucleotide (ddNTP);
— amplicons are formed of every possible length, each with a fluorescently labelled ddNTP terminal end;
— amplicons are separated by capillary electrophoresis according to their length, and the fluorescent label recorded using a laser;
— the nucleotide identity at each base position of the amplicon is ascertained;
— this process is repeated with another primer (this may be one used in the original PCR) that binds specifically to the opposite end of the single stranded amplicons.

4. LABORATORY SET-UP REQUIREMENTS

Because of the high risk of contamination of the working materials with environmental, user or different sample DNA, segregation of working areas by time and/or space is an effective method of reducing this risk. The movement of people and materials between working areas should be kept to a minimum and be monitored. All areas should be suitably decontaminated before and after each use and safeguards introduced to prevent cross contamination of samples.

Recommended working areas include:
— ***DNA extraction*** for the use of reagents and equipment used to extract DNA from plant materials;
— ***Pre-PCR*** exclusively for the use of reagents, equipment and materials that have not encountered amplicons;
— ***PCR*** equipment positioned in a dedicated area for the amplification process which occurs in a closed system;
— ***post-PCR*** dedicated to amplicon detection methods, as for instance gel electrophoresis and UV transillumination.

5. DNA EXTRACTION

Pure DNA must be isolated from the herbal drug to be tested. Several methods are available for this purpose, including those based on commercial kits. Representative samples must be taken from each batch to be tested to ensure consistency, Appendix XI T. *Herbal Drugs: Sampling and Sample Preparations*. DNA extraction using 200 mg of herbal drug as a superfine powder is suitable for the test. DNA extraction should also be performed on herbal drug mixed with *trnH-psbA* BP Nucleic Acid Reference Material (BPNARM). The efficiency of the extraction shall be confirmed by PCR amplification of the *trnH-psbA* BPNARM and herbal drug DNA.

Many constituents present in herbal drugs are inhibitory to enzyme driven reactions such as PCR. Care must be taken to ensure that DNA used for testing is pure and sufficiently free from inhibitors. Inhibitors extracted with DNA can be removed by the use of purification procedures, an example is given under: DNA-BASED IDENTIFICATION OF *OCIMUM TENUIFLORUM* LINN (Holy Basil); 2. DNA PURIFICATION.

6. AMPLIFICATION

PCR amplification is conducted using defined cycling parameters, including denaturation and binding temperatures. Many different commercial enzymes and kits are available which can be used once validated for the intended purpose. When commercial kits are used the manufacturers' guidelines for optimisation should be followed.

The concentrations shown below may be used as a generic starting point for method optimisation: Polymerase buffer (1x), $MgCl_2$ (2.5 mM), DNA polymerase (1 Unit), Primers (0.1 µM each), dNTPs (0.1 µM each), DNA (10 ng may be suitable) and *water MB*.

To conduct a PCR the number of reactions required must be known. From each DNA extraction, 2 PCRs are required together with a positive and a negative control reaction. All common components should be measured and combined together to create a master mix, usually this will contain all solutions except the DNA. The master mix is divided equally between each reaction tube and the DNA is added individually.

All PCR tests should include relevant positive and negative controls. To the positive control reaction validated DNA should be added. A solution of *trnH-psbA* BPNARM is recommended. To the negative control, *water MB* should be added in place of the DNA.

7. DETECTION

Specific amplicons may be detected based on their size or composition.

Detection by size can be achieved by agarose gel electrophoresis or capillary electrophoresis.

Detection by sequence can be achieved by probe hybridisation or enzyme cleavage. Amplicon size and sequence both contribute to melt-curve analyses.

A valid test is only achieved if the positive control reaction(s) gives an unambiguously positive result and the negative control reaction gives an unambiguously negative result. A positive result can be shown by the presence of a band of the expected size using agarose gel electrophoresis. A negative result will be the absence of a band.

8. SEQUENCING

Sanger sequencing can be conducted using various commercially available kits and/or service providers. Any validated protocol may be followed and manufacturers' guidelines may be used with commercial kits. A minimum of two reads must be produced for each DNA amplification, with at least one in either direction, and assembled into a contig. The contig should have an overall Phred score of at least 20; a value of 30 and over is preferable.

The text below is provided for information and describes the application of DNA-Based Identification techniques to a herbal drug.

DNA-BASED IDENTIFICATION OF *OCIMUM TENUIFLORUM* LINN. (HOLY BASIL)

1. DNA EXTRACTION

Follow the procedure described in Appendix XI V, 5. DNA EXTRACTION.

2. DNA PURIFICATION

Care should be taken to ensure that inhibitory substances commonly found in *Ocimum tenuiflorum* herbal drug are removed from DNA subsequent to extraction. Many methods are available for this, as for instance the method using propan-2-ol MB described below:
— to 50 µL of DNA extraction solution add 35 µL of *propan-2-ol MB* at 4°, mix by pipetting;
— centrifuge at 14,000 revolutions per minute for 30 minutes at 4°;
— remove the supernatant, taking care not to disturb the pellet which may not be visible;

— re-suspend the pellet in 200 µL of *ethanol (70%)* at ambient temperature;
— centrifuge at 14,000 revolutions per minute for 10 minutes at ambient temperature;
— remove the supernatant liquid taking care not to disturb the pellet, allow any excess ethanol to evaporate at ambient temperature for at least 25 minutes or until dry.
— dissolve the pellet in 50 µL of *tris-EDTA buffer pH 8.0*;
— dilute 1 volume of the DNA solution with 9 volumes of *tris-EDTA buffer pH 8.0* immediately prior to testing.

3. AMPLIFICATION

Prepare as described in Appendix XI V, 6. AMPLIFICATION.

Amplification of the *trnH-psbA* region of the plastid genome is achieved using the following primers and cycling programme.

Primer name	Sequence (5′ to 3′)
tmH	CGCGCATGGTGGATTCACAATCC
psbA	GTTATGCATGAACGTAATGCTC

Cycling programme:

initial denaturation step at 95° for 5 minutes;

35 cycles consisting of
— 95° for 1 minute;
— 30 seconds at touchdown temperature;
— 72° for 1 minute;

final extension period at 72° for 7 minutes;

The touchdown temperature begins at 58° and is reduced by 1° per cycle until 48°, then continued at 48° for the remainder of the program.

If amplification is unsuccessful after purification and dilution of the DNA sample, secondary PCR using 1 µL of the initial amplification as the template for a second is acceptable using the same parameters.

4. AMPLICON DETECTION

Any validated method may be used for this purpose; agarose gel electrophoresis incorporating a DNA specific dye is suitable. Recommended parameters are a 1.0% w/v gel made with 0.5x *TBE buffer MB*, run at 60V for 1 hour and subsequently visualised in an appropriate system such as a UV transilluminator.

A positive result is shown by the presence of a band of the expected size (approximately 400 bp); a DNA ladder should be run adjacent to the samples to show size separation.
A negative result is shown by the absence of a band at this position on the gel.

5. SEQUENCING

The amplicon is sequenced using the amplification primers and is conducted as described under Appendix XI V, 8. SEQUENCING

6. *OCIMUM TENUIFLORUM TRNH-PSBA* REGION SEQUENCE

The sequence of the *trnH-psbA* spacer region for *Ocimum tenuiflorum* is given below, with the bases shown in lower case text being the key bases for identification. These must be checked against all contigs produced using multiple alignment software (Clustal Omega may be suitable).

```
>Ocimum_trnH-psbA_reference_sequence
GTTATGCATGAACGTAATGCTCATAACTTCCCTCTAGACCTAGCTGCTAT
TGAAGCTCCAACAAATGGCTAAGacttgttCTTGGTGTGTAGAAGTTTTT
GAAATATAGATAAATATAAGGAGCAATAAACCCTTTCTTGTTCTATCAAA
AGAGGGTTTATTGCTCCTTTCTTTTCTTTTCAATTATGAGTcctttTCTA
TTTTTCTagtAGTATTGGACTTACCTAGACTTTTCTTTCCATAAAAGAAA
GAAGATAAAAAAAATGATTGAAATTTTATCTTTTGTTTTACAATTTTGCA
AAAAAAATTCAAATTGAAAAAGTGAATTCGTaaataAATAGAAAATTTTC
TTTTTAGATTTTTAGAGTAGAGGGGCGGATGTAGCCAAGTGGATCAAGGC
AGTGGattgtGAATCCACCCATGCGCG
```

7. SEQUENCE MATCHING

trnH-psbA sequences for the identification of *Ocimum tenuiflorum* samples must be a minimum of 300 bp in length, and must cover the key bases for identification. Overall matching of the sequences should be above 95%, and the key bases must match 100% with no gaps.

DNA-BASED IDENTIFICATION OF *ANETHUM GRAVEOLENS* SOWA

1. DNA EXTRACTION

Follow the procedure described in Appendix XI V, 5. DNA EXTRACTION.

2. DNA PURIFICATION

DNA extracted from *Anethum graveolens* Sowa does not require further purification; however, a 1:10 dilution in *Tris-EDTA buffer pH 8.0* can enhance results.

3. AMPLIFICATION

Prepare as described in Appendix XI V, 6. AMPLIFICATION.

Amplification of the ITS2 region of the nuclear genome is achieved using the following primers and cycling programme.

Primer name	Sequence (5′ to 3′)
ITS2 Forward	ATGCGATACTTGGTGTGAAT
ITS2 Reverse	GACGCTTCTCCAGACTACAAT

Cycling programme:
— initial denaturation step at 95° for 5 minutes;
— 40 cycles consisting of
— 95° for 30 seconds;
— 56° for 30 seconds;
— 72° for 45 seconds;
— final extension period at 72° for 10 minutes.

4. AMPLICON DETECTION

Any validated method, which incorporates a DNA ladder, may be used for this purpose; agarose gel electrophoresis incorporating a DNA specific dye is suitable. Recommended parameters are a 2.0% w/v gel made with 0.5x *TBE buffer MB*, run at 60 V for 1 hour and subsequently visualised in an appropriate system such as a UV transilluminator.

A positive result is shown by the presence of a band of the expected size (approximately 480 bp); a DNA ladder should be run adjacent to the samples to show size separation.
A negative result is shown by the absence of a band at this position on the gel.

5. SEQUENCING

The amplicon is sequenced using the amplification primers and is conducted as described under Appendix XI V, 8. SEQUENCING

6. *ANETHUM GRAVEOLENS* SOWA ITS2 REGION SEQUENCE

The sequence of the ITS2 region for *Anethum graveolens* Sowa is given below, with the bases shown in lower case text being the key bases for identification. These must be checked

against all contigs produced using multiple alignment software (Clustal Omega may be suitable).

```
>Anethum_graveolens_Sowa_ITS2_reference_sequence
TTTGCTTGCCCCAACCACTCACTCCTTGATGAGATGTGCTGGTTTTTGGG
CGGAAATTGGCCTCCCGTGCCTTGTTGTGCGGTTGGTGCAAAAGCGAGTC
TCCGGCGTTGGACGTCGTGACATCGGTGGTTGTAAAAGACCCTCTTGACT
TGTCGCACGAATCCTCGTCATCTAAGTGAGCTCTAGGACCCTTgggcacc
acACAATCTGTTTGCCCTAACTGTGACCCCAGGTCAGGCG
```

7. SEQUENCE MATCHING

ITS2 sequences for the identification of *Anethum graveolens* Sowa samples must be a minimum of 240 bp in length, and must cover the key bases for identification. Overall matching of the sequences should be above 95%, and the key bases must match 100% with no gaps.

DNA-BASED IDENTIFICATION OF *TRIBULUS TERRESTRIS* FRUIT

1. DNA EXTRACTION

Follow the procedure described in Appendix XI V, 5. DNA EXTRACTION.

2. DNA PURIFICATION

DNA extracted from *Tribulus terrestis* Fruit does not require further purification.

3. AMPLIFICATION

Prepare as described in Appendix XI V, 6. AMPLIFICATION.

Amplification of the ITS2 region of the nuclear genome is achieved using the following primers and cycling programme.

Primer name	Sequence (5′ to 3′)
ITS1 Forward	TCCGTAGGTGAACCTGCGG
ITS4 Reverse	TCCTCCGCTTATTGATATGC

Cycling programme:
— initial denaturation step at 95° for 5 minutes;
— 35 cycles consisting of
— 95° for 60 seconds;
— 60° for 30 seconds;
— 72° for 60 seconds;
— final extension period at 72° for 7 minutes.

4. AMPLICON DETECTION

Any validated method, which incorporates a DNA ladder, may be used for this purpose; agarose gel electrophoresis incorporating a DNA specific dye is suitable. Recommended parameters are a 2.0% w/v gel made with 0.5x *TBE buffer MB*, run at 60 V for 1 hour and subsequently visualised in an appropriate system such as a UV transilluminator.

A positive result is shown by the presence of a band of the expected size (approximately 700 bp); a DNA ladder should be run adjacent to the samples to show size separation.
A negative result is shown by the absence of a band at this position on the gel.

5. SEQUENCING

The amplicon is sequenced using the amplification primers and is conducted as described under Appendix XI V, 8. SEQUENCING

6. *TRIBULUS TERRESTRIS* FRUIT ITS REGION SEQUENCE

The sequence of the ITS region for *Tribulus terrestris* Fruit is given below, with the bases shown in lower case text being the key bases for identification. These must be checked against all contigs produced using multiple alignment software (Clustal Omega may be suitable).

```
>Tribulus_terrestris_ITS_reference_sequence
CACtcgygcgATGCGTTCCACGctctccaCGGGGACTTGgccaccgcgcg
tTGCTTTATCGGATCATAACAAACCCCGGCGCGGAATGCGTCAAGGAATC
TtwaaATGCGTCGGCACGGCCTTGTGACCCTATCGCAGGGCGTCAGTGCC
AGTGCACTATTactACACGAACGACTCTCGGCAACGGATATCTCGGCTCT
CGCATCGATGAAGAACGTAGCGAAATGCGATACTT
```

7. SEQUENCE MATCHING

Amplification of the ITS region for the identification of *Tribulus terrestris* samples yields a product of approximately 700 bp, within the amplification product lies the reference sequence which must be a minimum of 235 bp in length, and must cover the key bases for identification. Overall matching of the sequences should be above 95%, and the key bases must match 100% with no gaps.

GLOSSARY

This glossary of terms relating to Appendix XI V is published for information only

Table XI V-1

Term	Definition
Amplicon	The DNA product of a PCR.
Amplification	The copying of DNA during a PCR.
Base call	The identification of a DNA base by sequencing software.
Base pair (bp)	The complementary pairing of two nucleotides, A&T or G&C, which forms the unit of measurement for the length of a DNA molecule.
BPNARM	British Pharmacopoeia Nucleic Acid Reference Material.
Consensus sequence	The product of the combining of several individual DNA sequencing reads, providing a consensus of the correct sequence.
Contig	A set of overlapping DNA sequencing reads from one sample which can be used to produce a consensus sequence.
Deoxynucleotide (dNTP)	The monomer or individual unit of DNA; Adenine (A), Cytosine (C), Guanine (G) and Thymine (T).
di-deoxynucleotide (ddNTP)	A modified form of the DNA monomer without an -OH group present on the 3′ carbon of the deoxyribose sugar which is required to bind a subsequent nucleotide.
DNA	Deoxyribonucleic Acid, a double stranded, helical molecule.
DNA ladder	Mixture of DNA molecules of known base pair length. These provide a measure of how far a DNA molecule travels during gel electrophoresis.
Internal transcribed spacer (ITS)	The spacer DNA situated between the small-subunit ribosomal RNA (rRNA) and large-subunit rRNA genes in the chromosome or the corresponding transcribed region in the polycistronic rRNA precursor transcript
Mix by pipetting	Drawing up and expelling a substance up to ten times using an automatic pipette, with the aim of mixing the solutions.
Master mix	A mixture containing the common components for several PCRs, this is made in a large batch or master mix which is then divided between individual reactions. Master mixes contain enough reagents for the required number of tests, typically plus one to allow for pipetting errors.
Mix by pipetting	Drawing up and expelling a substance up to ten times using an automatic pipette, with the aim of mixing the solutions.
PCR	Polymerase Chain Reaction - an enzyme driven reaction where DNA molecules are replicated.
Phred score	The likelihood that a base call in a DNA sequence is incorrect, a score of 20 has a 1 in 100 probability of being an incorrect call, 30 is 1 in 1000 etc.
Positive control	A reaction comprising all common PCR components and a known DNA sample, thereby proving the suitability of all reagents.
Primer (oligonucleotide)	A short single stranded DNA molecule which binds to the DNA to be amplified in a PCR. This enables the enzyme to commence replication, and therefore the binding positions define the start and finish point of the PCR.
Probe hybridisation	The complementary binding of an oligonucleotide to a target DNA molecule causing a measurable response.
Sanger sequencing	The method by which a DNA sequence is resolved, developed by Frederick Sanger and colleagues.
Sequencing	Identifying the order of the nucleotide sequence of DNA.
TBE buffer MB	Tris-borate-EDTA Buffer Solution pH 8.4 MB
Thermal cycler	The machine that performs the cycling of temperatures required for a PCR.
Water MB	Deionised, filtered and autoclaved water.

W. High-Performance Thin-Layer Chromatography of Herbal Drugs and Herbal Drug Preparations

(Ph. Eur. method 2.8.25)

High-performance thin-layer chromatography (HPTLC) is used for qualitative analysis of herbal drugs and herbal drug preparations. It is a thin-layer chromatographic technique (*2.2.27*) that usually uses a glass plate coated with a uniform, porous layer (average pore size 6 nm), typically 200 µm thick, of irregular particles of silica gel between 2 µm and 10 µm in size and with an average size of 5 µm, a polymeric binder and a fluorescence indicator (F_{254}). The results are qualified using a system suitability test.

EQUIPMENT

The equipment used for qualitative HPTLC typically consists of:

- glass plates, as described above, usually 20 × 10 cm in size;
- devices suitable for the application of specified volumes of solutions as bands and allowing control of the dimensions and position of application;
- a device suitable for conditioning the stationary phase at the prescribed relative humidity;
- a suitable chromatographic tank (for example, a twin trough chamber);
- a device suitable for the reproducible drying of the developed plate;
- devices suitable for the application of reagents to, and heating of, the plate as part of the derivatisation procedure;
- a system suitable for the electronic documentation of chromatograms under 254 nm UV, 366 nm UV and white light.

NOTE Normal thin-layer chromatographic methods using glass plates or sheets coated with particles of 5-40 µm or HPTLC aluminium-backed sheets may be used, provided that the results obtained fulfil the general system suitability criteria that the bands develop perpendicular to the lower edge of the plate and the solvent front is parallel to the upper edge of the plate, and satisfy the system suitability test stated in the individual monograph.

METHOD

Preparation of test solution

The test solution is prescribed in the individual monograph and is usually prepared as follows.

For dry herbal drugs or dry herbal extracts, mix 0.5 g of the powdered herbal drug or 0.1 g of the dry herbal extract with 5.0 mL of *methanol R* and sonicate for 15 min; filter or centrifuge and use the filtrate or supernatant as the test solution.

For essential oils, dissolve 50 µL of the essential oil in 1.0 mL of *toluene R* and use this solution as the test solution.

Preparation of reference solutions

Reference solutions are prescribed in the individual monograph and are usually prepared as follows. Prepare a 1 mg/mL solution (identified as 'R' in the chromatograms associated with the monograph) of suitable reagent(s) or reference standard(s) in *methanol R* or, for essential oils, in *toluene R*. Prepare a second reference solution (diluted reference solution, identified as 'R1/4' in the chromatograms associated with the monograph) by mixing 1 volume of this solution and 3 volumes of the same solvent (in some cases a different diluted reference solution, e.g. 'R1/20', may be prescribed instead). Both solutions are used as intensity references.

Intensity marker

Use one or more of the substances in the reference solution and in the diluted reference solution as intensity marker(s) for the evaluation of the chromatogram.

Preparation of system suitability test solution

Prepare the system suitability test (SST) solution as stated in the individual monograph.

Sample application and plate layout

Samples are usually applied as narrow bands 8 mm in length at a distance of 8 mm from the lower edge of the plate. The centre of the first track, which is used for the system suitability test solution, is positioned 20 mm from the left edge of the plate. The minimum distance between tracks (centre to centre) is 11 mm. A maximum of 15 tracks are applied onto a standard plate. If no electronic solvent front detection device is used, the development distance is marked with a pencil close to the right or left edge of the plate.

Conditioning of the plate

Following sample application and unless otherwise stated in the individual monograph, expose the plate to air with a suitable relative humidity obtained using a saturated solution of *magnesium chloride R* (for example, by allowing the plate to stand in a closed chamber containing such a solution for 1 h or by using preconditioned air).

Preparation of the tank and development of the plate

Unless otherwise stated in the individual monograph, the chromatographic separation is performed in a saturated tank. Where a twin trough chamber is used, place a piece of filter paper in the rear trough. Load the tank with a sufficient quantity of mobile phase to wet the filter paper completely and achieve a level of 5 mm in both troughs. With the lid closed, leave the tank for 20 min for saturation. Introduce the plate in a vertical position into the front trough of the tank so that the coating layer faces the filter paper. When the mobile phase has reached the prescribed distance (usually 70 mm from the lower edge of the plate), remove the plate from the tank and dry in a vertical position in a stream of air at room temperature. Other tank configurations and development distances may be specified in an individual monograph.

NOTE Other tanks may be employed if the results obtained fulfil all of the system suitability criteria.

Visualisation

Chromatograms on the plate are visualised as stated in the individual monograph under Detection. Where derivatisation reagents are used, typically 3.5 mL of reagent solution is homogenously sprayed onto a plate of size 20 × 10 cm, or the plate is immersed into the reagent solution, typically at a speed of 5 mm/s for a dwell time of 1 s. Observation may be performed under 254 nm UV, 366 nm UV or white light prior to and/or after derivatisation. When pictures are digitally recorded, exposure time should be adjusted based on the track used for the system suitability test solution.

System suitability test

This test is based on the separation of 2 substances that have similar retardation factors (R_F values) but that are barely separable under the specified chromatographic conditions (for example, chlorogenic acid and hyperoside in chromatographic systems used for flavonoids). The results for the test and reference solutions are only valid when the

system suitability test solution satisfies the separation requirement stated in the individual monograph.

Visual evaluation

The chromatograms obtained with the test and reference solutions are compared with the descriptions in the results section of the individual monograph, with respect to zone position and colour, as well as intensity for the test solution. Zones of the test solution described as 'equivalent' or without an indication of intensity have intensities similar to the zone of the intensity marker in the reference solution (R). Zones described as 'intense' are visually more intense than the zone of the intensity marker in the reference solution; zones described as 'faint' are visually less intense than the zone of the intensity marker in the reference solution, but equal to or more intense than the zone of the intensity marker in the diluted reference solution (R1/4, R1/20, etc.); zones described as 'very faint' are visually less intense than the zone of the intensity marker in the diluted reference solution.

Appendix XII

A. Disintegration

1. Disintegration of Tablets and Capsules[1]

(Ph. Eur. method 2.9.1)

This test is provided to determine whether tablets or capsules disintegrate within the prescribed time when placed in a liquid medium under the experimental conditions presented below.

For the purposes of this test, disintegration does not imply complete dissolution of the unit or even of its active constituent. Complete disintegration is defined as that state in which any residue of the unit, except fragments of insoluble coating or capsule shell, remaining on the screen of the test apparatus or adhering to the lower surface of the discs, if used, is a soft mass having no palpably firm core.

♦Use apparatus A for tablets and capsules that are not greater than 18 mm long. For larger tablets or capsules use apparatus B.♦

TEST A - TABLETS AND CAPSULES OF NORMAL SIZE

Apparatus

The apparatus consists of a basket-rack assembly, a 1 L, low-form beaker, 149 ± 11 mm in height and having an inside diameter of 106 ± 9 mm for the immersion fluid, a thermostatic arrangement for heating the fluid between 35 °C and 39 °C, and a device for raising and lowering the basket in the immersion fluid at a constant frequency rate between 29 and 32 cycles per minute, through a distance of 55 ± 2 mm. The volume of the fluid in the vessel is such that at the highest point of the upward stroke the wire mesh remains at least 15 mm below the surface of the fluid, and descends to not less than 25 mm from the bottom of the vessel on the downward stroke. At no time should the top of the basket-rack assembly become submerged. The time required for the upward stroke is equal to the time required for the downward stroke, and the change in stroke direction is a smooth transition, rather than an abrupt reversal of motion. The basket-rack assembly moves vertically along its axis. There is no appreciable horizontal motion or movement of the axis from the vertical.

Basket-rack assembly The basket-rack assembly consists of 6 open-ended transparent tubes, each 77.5 ± 2.5 mm long and having an inside diameter of 21.85 ± 1.15 mm and a wall 1.9 ± 0.9 mm thick; the tubes are held in a vertical position by 2 plates, each 90 ± 2 mm in diameter and 6.75 ± 1.75 mm in thickness, with 6 holes, each 24 ± 2 mm in diameter, equidistant from the centre of the plate and equally spaced from one another. Attached to the under surface of the lower plate is a woven stainless steel wire cloth, which has a plain square weave with 2.0 ± 0.2 mm mesh apertures and with a wire diameter of 0.615 ± 0.045 mm. The parts of the apparatus are assembled and rigidly held by means of 3 bolts passing through the 2 plates. A suitable means is provided to suspend the basket-rack assembly from the raising and lowering device using a point on its axis.

The design of the basket-rack assembly may be varied somewhat provided the specifications for the glass tubes and the screen mesh size are maintained. The basket-rack

[1] *This chapter has undergone pharmacopoeial harmonisation. See chapter 5.8 Pharmacopoeial harmonisation.*

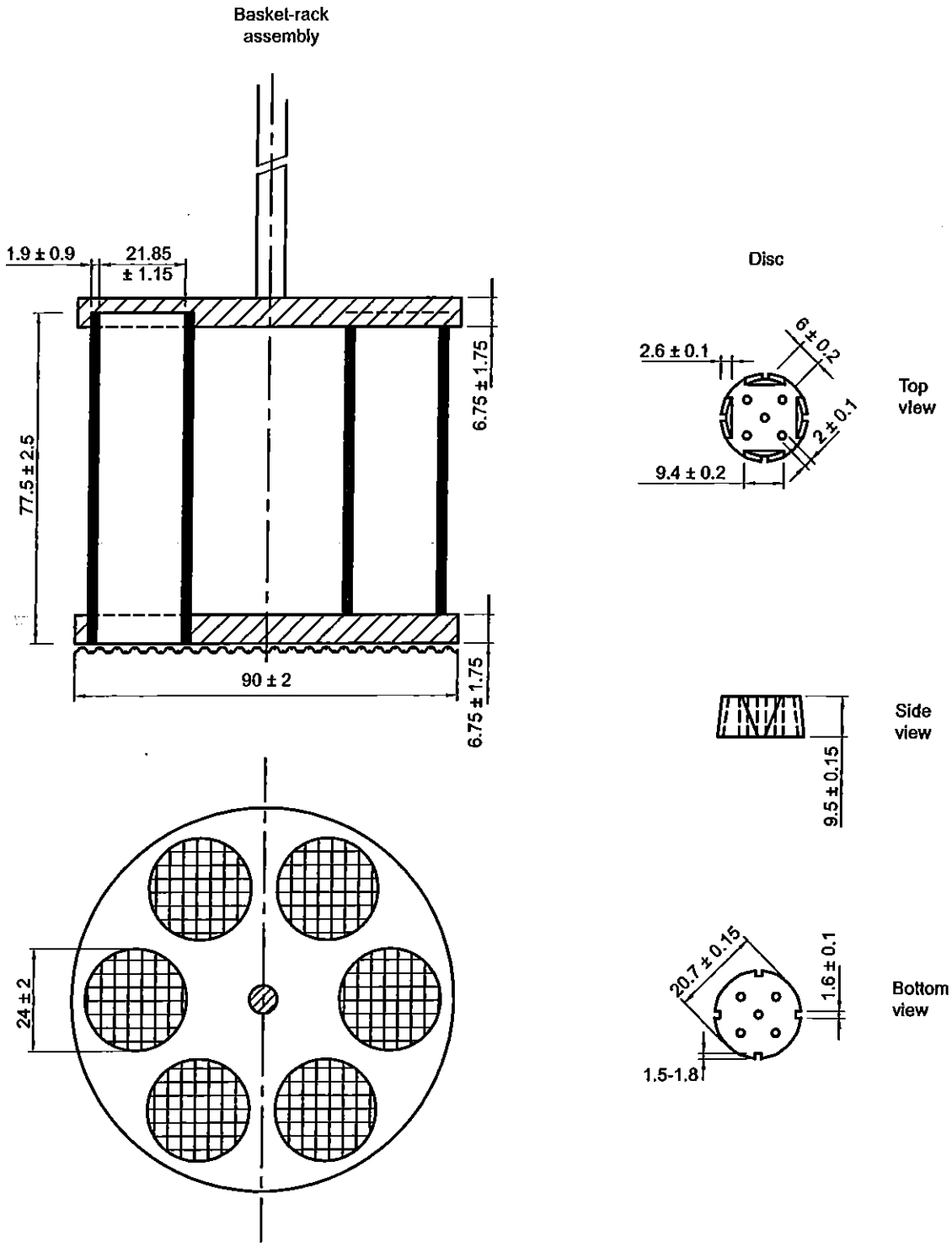


Figure 2.9.1.-1. – *Disintegration apparatus A*
Dimensions in millimetres

assembly conforms to the dimensions shown in Figure 2.9.1.-1.

Discs The use of discs is permitted only where specified or allowed. Each tube is provided with a cylindrical disc 9.5 ± 0.15 mm thick and 20.7 ± 0.15 mm in diameter. The disc is made of a suitable, transparent plastic material having a specific gravity of 1.18-1.20. 5 parallel 2 ± 0.1 mm holes extend between the ends of the cylinder. One of the holes is centered on the cylindrical axis. The other holes are parallel to the cylindrical axis and centered 6 ± 0.2 mm from the axis on imaginary lines perpendicular to the axis and to each other, as defined in Figure 2.9.1.-1. 4 identical trapezoidal-shaped planes are cut into the wall of the cylinder, nearly perpendicular to the ends of the cylinder. The trapezoidal shape is symmetrical; its parallel sides coincide with the ends of the cylinder and are parallel to an imaginary line connecting the centres of 2 adjacent holes 6 mm from the cylindrical axis. The parallel side of the trapezoid on the bottom of the cylinder has a length of 1.6 ± 0.1 mm and its bottom edges lie at a depth of 1.5 mm to 1.8 mm from the cylinder's circumference. The parallel

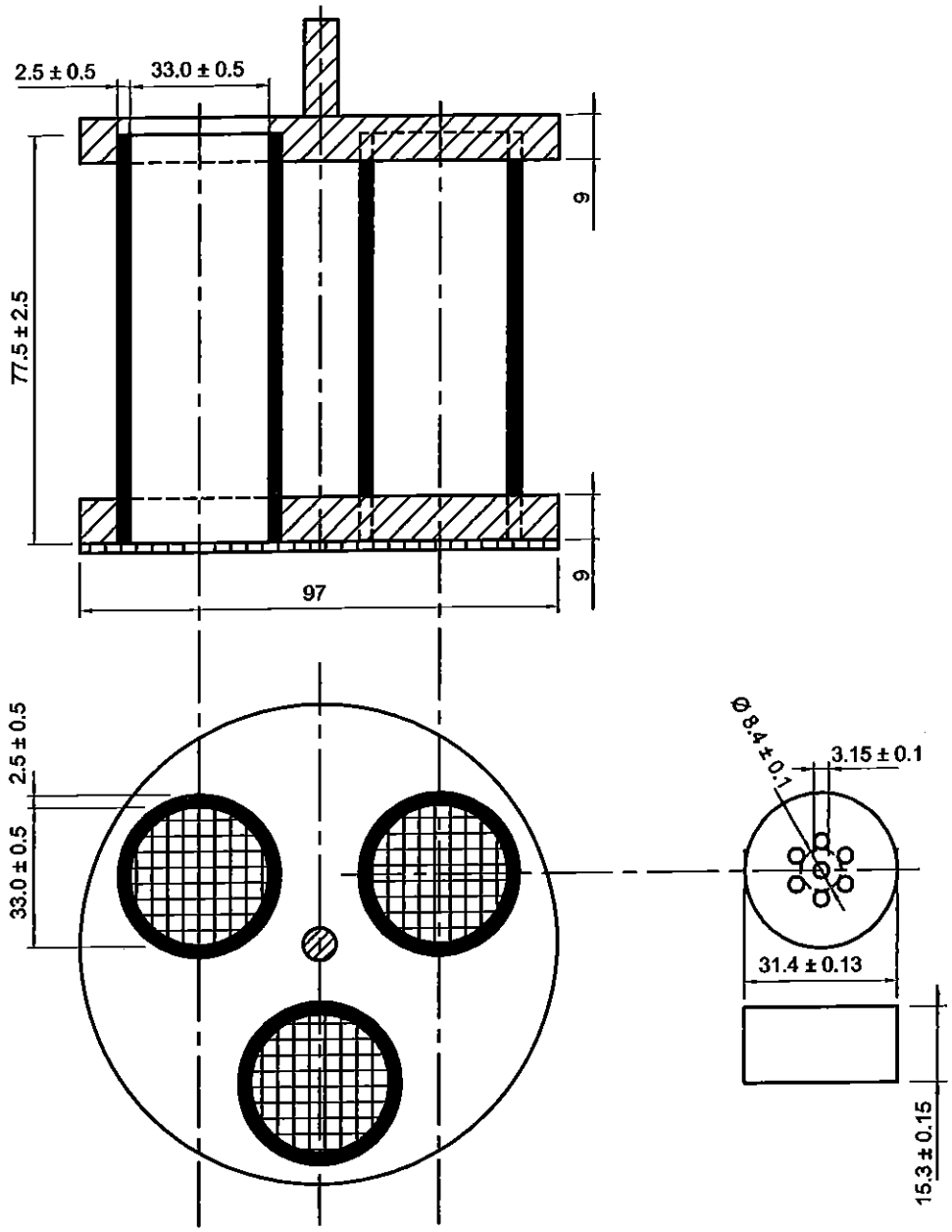


Figure 2.9.1.-2. – *Disintegration apparatus B*
Dimensions in millimetres

side of the trapezoid on the top of the cylinder has a length of 9.4 ± 0.2 mm and its centre lies at a depth of 2.6 ± 0.1 mm from the cylinder's circumference. All surfaces of the disc are smooth.

If the use of discs is specified, add a disc to each tube and operate the apparatus as directed under Procedure. The discs conform to the dimensions shown in Figure 2.9.1.-1.

The use of automatic detection employing modified discs is permitted where the use of discs is specified or allowed. Such discs must comply with the requirements of density and dimension given in this chapter.

Procedure

Place 1 dosage unit in each of the 6 tubes of the basket and, if prescribed, add a disc. Operate the apparatus using the specified medium, maintained at 37 ± 2 °C, as the immersion fluid. At the end of the specified time, lift the basket from the fluid and observe the dosage units: all of the dosage units have disintegrated completely. If 1 or 2 dosage units fail to disintegrate, repeat the test on 12 additional dosage units. The requirements of the test are met if not less than 16 of the 18 dosage units tested have disintegrated.

♦TEST B – LARGE TABLETS AND LARGE CAPSULES

Apparatus

The main part of the apparatus (Figure 2.9.1.-2.) is a rigid basket-rack assembly supporting 3 cylindrical transparent tubes 77.5 ± 2.5 mm long, 33.0 mm ± 0.5 mm in internal diameter, and with a wall thickness of 2.5 ± 0.5 mm. Each tube is provided with a cylindrical disc 31.4 ± 0.13 mm in diameter and 15.3 ± 0.15 mm thick, made of transparent plastic with a relative density of 1.18-1.20. Each disc is pierced by 7 holes, each 3.15 ± 0.1 mm in diameter, 1 in the centre and the other 6 spaced equally on a circle of radius 4.2 mm from the centre of the disc. The tubes are held vertically by 2 separate and superimposed rigid plastic

plates 97 mm in diameter and 9 mm thick, with 3 holes. The holes are equidistant from the centre of the plate and equally spaced. Attached to the under side of the lower plate is a piece of woven gauze made from stainless steel wire 0.63 ± 0.03 mm in diameter and having mesh apertures of 2.0 ± 0.2 mm. The plates are held rigidly in position and 77.5 mm apart by vertical metal rods at the periphery.
A metal rod is also fixed to the centre of the upper plate to enable the assembly to be attached to a mechanical device capable of raising and lowering it smoothly at a constant frequency of between 29 and 32 cycles per minute, through a distance of 55 ± 2 mm.

The assembly is suspended in the specified liquid medium in a suitable vessel, preferably a 1 L beaker. The volume of the liquid is such that when the assembly is in the highest position the wire mesh is at least 15 mm below the surface of the liquid, and when the assembly is in the lowest position the wire mesh is at least 25 mm above the bottom of the beaker and the upper open ends of the tubes remain above the surface of the liquid. A suitable device maintains the temperature of the liquid at 35-39 °C.

The design of the basket-rack assembly may be varied provided the specifications for the tubes and wire mesh are maintained.

Method

Test 6 tablets or capsules either by using 2 basket-rack assemblies in parallel or by repeating the procedure. In each of the 3 tubes, place 1 tablet or capsule and, if prescribed, add a disc; suspend the assembly in the beaker containing the specified liquid. Operate the apparatus for the prescribed period, withdraw the assembly and examine the state of the tablets or capsules. To pass the test, all 6 of the tablets or capsules must have disintegrated.♦

2. Disintegration Test for Suppositories and Pessaries

(Ph. Eur. method 2.9.2)

The disintegration test determines whether the suppositories or pessaries soften or disintegrate within the prescribed time when placed in a liquid medium in the experimental conditions described below.

Disintegration is considered to be achieved when:

a) dissolution is complete,

b) the components of the suppository or pessary have separated: melted fatty substances collect on the surface of the liquid, insoluble powders fall to the bottom and soluble components dissolve, depending on the type of preparation, the components may be distributed in one or more of these ways,

c) there is softening of the sample that may be accompanied by appreciable change of shape without complete separation of the components, the softening is such that the suppository or pessary no longer has a solid core offering resistance to pressure of a glass rod,

d) rupture of the gelatin shell of rectal or vaginal capsules occurs allowing release of the contents,

e) no residue remains on the perforated disc or if a residue remains, it consists only of a soft or frothy mass having no solid core offering resistance to pressure of a glass rod (vaginal tablets).

Apparatus The apparatus (Figure 2.9.2.-1) consists of a sleeve of glass or suitable transparent plastic, of appropriate thickness, to the interior of which is attached by means of three hooks a metal device consisting of two perforated stainless metal discs each containing 39 holes 4 mm in diameter; the diameter of the discs is similar to that of the interior of the sleeve; the discs are about 30 mm apart. The test is carried out using three such apparatuses each containing a single sample. Each apparatus is placed in a beaker with a capacity of at least 4 L filled with water maintained at 36-37 °C, unless otherwise prescribed. The apparatuses may also be placed together in a vessel with a capacity of at least 12 L. The beaker is fitted with a slow stirrer and a device that will hold the cylinders vertically not less than 90 mm below the surface of the water and allow them to be inverted without emerging from the water.

Method Use three suppositories or pessaries. Place each one on the lower disc of a device, place the latter in the sleeve and secure. Invert the apparatuses every 10 min. Examine the samples after the period prescribed in the monograph. To pass the test all the samples must have disintegrated.

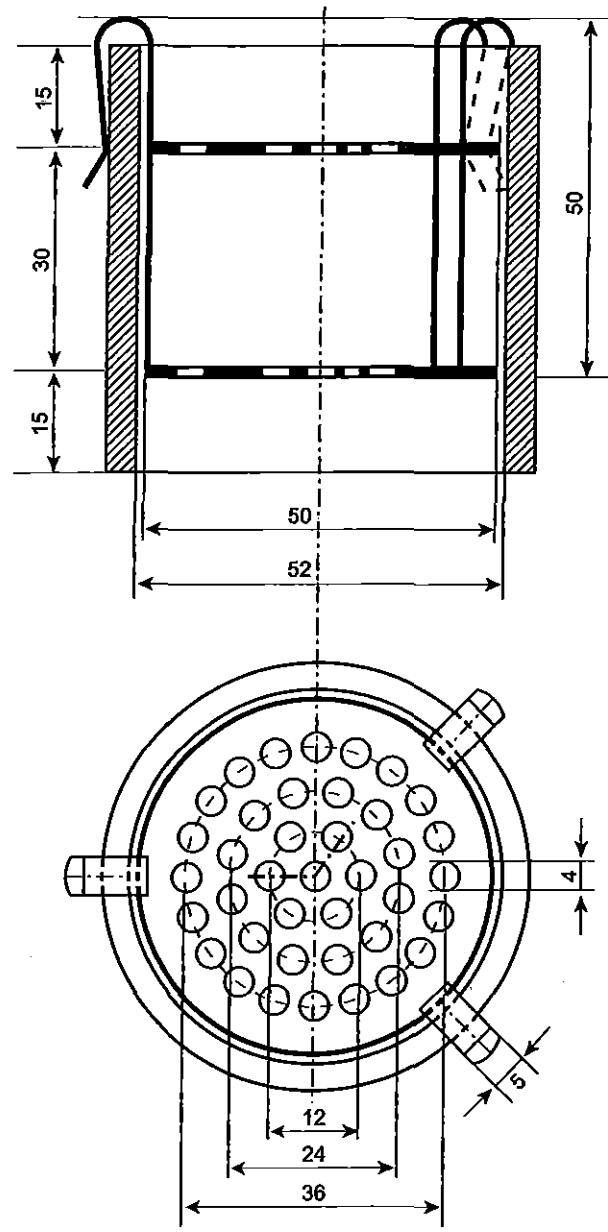
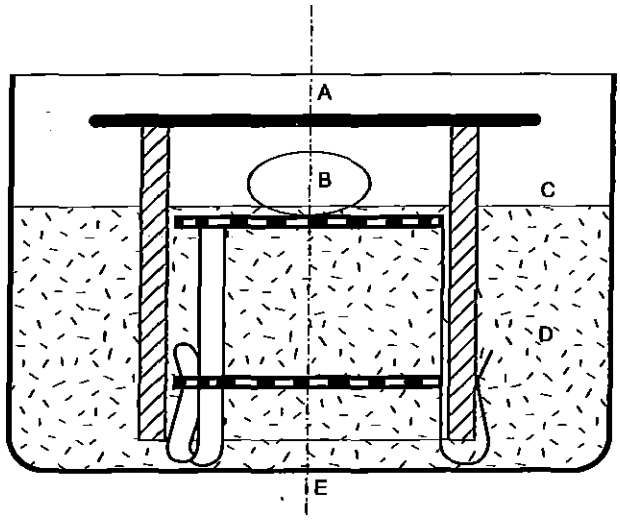


Figure 2.9.2.-1. – *Apparatus for disintegration of suppositories and pessaries*

Dimensions in millimetres

METHOD OF OPERATION FOR VAGINAL TABLETS

Use the apparatus described above, arranged so as to rest on the hooks (see Figure 2.9.2.-2). Place it in a beaker of suitable diameter containing water maintained at 36-37 °C with the level just below the upper perforated disc. Using a pipette, adjust the level with water at 36-37 °C until a uniform film covers the perforations of the disc. Use three vaginal tablets. Place each one on the upper plate of an apparatus and cover the latter with a glass plate to maintain appropriate conditions of humidity. Examine the state of the samples after the period prescribed in the monograph.
To pass the test all the samples must have disintegrated.



A. glass plate
B. vaginal tablet
C. water surface
D. water
E. dish, beaker

Figure 2.9.2.-2.

Monographs of the British Pharmacopoeia

The following additional points apply to monographs of the British Pharmacopoeia.

ACCEPTANCE CRITERIA

For moulded suppositories, disintegration occurs in not more than 30 minutes for fat-based suppositories and in not more than 60 minutes for water-soluble suppositories, unless otherwise justified and authorised.

For moulded pessaries, disintegration occurs in not more than 60 minutes unless otherwise justified and authorised.

For rectal capsules and vaginal tablets and capsules, disintegration occurs in not more than 30 minutes.

Recommendations on Dissolution Testing

(Ph. Eur. general texts 5.17.1)

This general chapter is non-mandatory; it provides information on dissolution testing, on recommended dissolution media and on the expression of dissolution specifications for oral dosage forms (see general chapter 2.9.3. Dissolution test for solid dosage forms). This information represents generally accepted parameters used in the field of dissolution.

In the determination of the dissolution rate of the active substance(s) of a solid dosage form, the following are to be specified:

- the apparatus to be used, and in cases where the flow-through apparatus is specified, which flow-through cell is to be used;
- the composition, the volume and the temperature of the dissolution medium;
- the rotation speed or the flow rate of the dissolution medium;
- the time, the method and the amount for sampling of the test solution or the conditions for continuous monitoring;
- the method of analysis;
- the acceptance criteria.

The choice of apparatus to be used depends on the physico-chemical characteristics of the dosage form. When a large quantity of dissolution medium is required to ensure sink conditions, or when a change of pH is necessary, the flow-through apparatus may be preferred.

EXPERIMENTAL TESTING CONDITIONS

The use of the basket and the paddle apparatus and the reciprocating cylinder apparatus is generally based on the principle of operating under sink conditions, i.e. in such a manner that the material already in solution does not exert a significant modifying effect on the rate of dissolution of the remainder. Sink conditions normally occur in a volume of dissolution medium that is at least 3-10 times the saturation volume.

In general, an aqueous medium is used. The composition of the medium is chosen on the basis of the physico-chemical characteristics of the active substance(s) and excipient(s) within the range of conditions to which the dosage form is likely to be exposed after its administration. This applies in particular to the pH and the ionic strength of the dissolution medium.

The pH of the dissolution medium is usually set between pH 1 and pH 8. In justified cases, a higher pH may be needed. For the lower pH values in the acidic range, *0.1 M hydrochloric acid* is normally used. Recommended dissolution media are described hereafter.

Water is recommended as a dissolution medium only when it is proven that the pH variations do not have an influence on the dissolution characteristics.

In specific cases, and subject to approval by the competent authority, dissolution media may contain enzymes, surfactants, further inorganic substances and organic substances. For the testing of preparations containing poorly aqueous-soluble active substances, modification of the medium may be necessary. In such circumstances, a low concentration of surfactant is recommended; it is recommended to avoid the use of organic solvents.

Gases dissolved in the dissolution medium can affect the results of the dissolution test. This is true in particular for the flow-through apparatus, where de-aeration of the medium is necessary to avoid the formation of gas bubbles in the flow-through cell. A suitable method of de-aeration is as follows: heat the medium while stirring gently to about 41 °C, immediately filter under vacuum using a filter with a porosity of 0.45 µm or less, with vigorous stirring, and continue stirring under vacuum for about 5 min. Other de-aeration techniques for removal of dissolved gases may be used.

Using the paddle or basket apparatus, the volume of dissolution medium is normally 500-1000 mL. A stirring speed of between 50 r/min and 100 r/min is normally chosen; it must not exceed 150 r/min.

For the flow-through apparatus, the liquid flow rate is normally set between 4 mL/min and 50 mL/min.

RECOMMENDED DISSOLUTION MEDIA

The following dissolution media may be used.

Table 5.17.1.-1. – *Examples of dissolution media*

pH	Dissolution media
pH 1.0	HCl
pH 1.2	NaCl, HCl
pH 1.5	NaCl, HCl
pH 4.5	Phosphate or acetate buffer
pH 5.5 and pH 5.8	Phosphate or acetate buffer
pH 6.8	Phosphate buffer
pH 7.2 and pH 7.5	Phosphate buffer

The composition and preparation of these various media are indicated below.

Hydrochloric acid media

— *0.2 M hydrochloric acid.*

— *0.2 M sodium chloride.* Dissolve 11.69 g of *sodium chloride R* in *water R* and dilute to 1000.0 mL with the same solvent.

For preparing media with the pH values indicated in Table 5.17.1.-2, mix 250.0 mL of 0.2 M sodium chloride and the specified volume of *0.2 M hydrochloric acid*, and dilute to 1000.0 mL with *water R*.

Table 5.17.1.-2. – *Hydrochloric acid media*

pH	HCl (mL)
1.2	425.0
1.3	336.0
1.4	266.0
1.5	207.0
1.6	162.0
1.7	130.0
1.8	102.0
1.9	81.0
2.0	65.0
2.1	51.0
2.2	39.0

The hydrochloric acid media may also be prepared by replacing sodium chloride with potassium chloride.

Acetate buffer solutions

— *2 M acetic acid.* Dilute 120.0 g of *glacial acetic acid R* to 1000.0 mL with *water R*.

— *Acetate buffer solution pH 4.5.* Dissolve 2.99 g of *sodium acetate R* in *water R*. Add 14.0 mL of 2 M acetic acid and dilute to 1000.0 mL with *water R*.

— *Acetate buffer solution pH 5.5.* Dissolve 5.98 g of *sodium acetate R* in *water R*. Add 3.0 mL of 2 M acetic acid and dilute to 1000.0 mL with *water R*.

— *Acetate buffer solution pH 5.8.* Dissolve 6.23 g of *sodium acetate R* in *water R*. Add 2.1 mL of 2 M acetic acid and dilute to 1000.0 mL with *water R*.

Phosphate buffer solutions

For preparing buffers with the pH values indicated in Table 5.17.1.-3, mix 250.0 mL of *0.2 M potassium dihydrogen phosphate R* and the specified volume of *0.2 M sodium hydroxide*, and dilute to 1000.0 mL with *water R*.

Table 5.17.1.-3. – *Phosphate buffer solutions*

pH	5.8	6.0	6.2	6.4	6.6	6.8
NaOH (mL)	18.0	28.0	40.5	58.0	82.0	112.0
pH	7.0	7.2	7.4	7.6	7.8	8.0
NaOH (mL)	145.5	173.5	195.5	212.0	222.5	230.5

Other phosphate buffer solutions

— *Phosphate buffer solution pH 4.5.* Dissolve 13.61 g of *potassium dihydrogen phosphate R* in 750 mL of *water R*. Adjust the pH if necessary with *0.1 M sodium hydroxide* or with *0.1 M hydrochloric acid*. Dilute to 1000.0 mL with *water R*.

— *Phosphate buffer solution pH 5.5 R.*

— Phosphate buffer solution pH 6.8 R1.

— *Buffer solution pH 7.2 R.*

— *0.33 M phosphate buffer solution pH 7.5 R.*

Simulated intestinal fluid pH 6.8

Mix 77.0 mL of *0.2 M sodium hydroxide*, 250.0 mL of a solution containing 6.8 g of *potassium dihydrogen phosphate R*, and 500 mL of *water R*. Add 10.0 g of *pancreas powder R*, mix and adjust the pH if necessary. Dilute to 1000.0 mL with *water R*.

Simulated gastric fluid

Dissolve 2.0 g of *sodium chloride R* and 3.2 g of *pepsin powder R* in *water R*, add 80 mL of *1 M hydrochloric acid* and dilute to 1000.0 mL with *water R*. If required, pepsin powder may be omitted.

Increasing pH

For a test involving increasing pH, one of the following sequences may be used:

Time (h)	0 - 1	1 - 2	2 - 3	3 - 4	4 - 5	5 - 6	6 - 7	7
pH	1.0							
pH	1.2	6.8						
pH	1.2	2.5	4.5	7.0		7.5		
pH	1.5	4.5			7.2			

To achieve this pH variation, it is possible either:

— to substitute one buffer solution for another (whole substitution);

— to remove only half of the medium each time (half change method) and replace it with a buffer solution of higher pH: the initial pH is 1.2 and the second solution is phosphate buffer solution pH 7.5; or,

— to an initial solution at pH 1.5, to add a dose of a powder mixture containing *tris(hydroxymethyl)aminomethane R* and *anhydrous sodium acetate R* to obtain pH 4.5 and a second dose to obtain pH 7.2, as described below:

— *hydrochloric acid pH 1.5*: dissolve 2 g of *sodium chloride R* in *water R*, add 31.6 mL of *1 M hydrochloric acid* and dilute to 1000.0 mL with *water R*;
— *buffer solution pH 4.5*: mix 2.28 g of *tris(hydroxymethyl) aminomethane R* with 1.77 g of *anhydrous sodium acetate R*; dissolve this mixture in the hydrochloric acid pH 1.5 described above;
— *buffer solution pH 7.2*: mix 2.28 g of *tris(hydroxymethyl) aminomethane R* with 1.77 g of *anhydrous sodium acetate R*; dissolve this mixture in the buffer solution pH 4.5 described above.

The flow-through cell may be used for the continuous change of pH.

QUALIFICATION AND VALIDATION

Due to the nature of the test method, quality by design is an important qualification aspect for *in vitro* dissolution test equipment. Any irregularities such as vibration or undesired agitation by mechanical imperfections are to be avoided.

Qualification of the dissolution test equipment has to consider the dimensions and tolerances of the apparatus. Critical test parameters, such as temperature and volume of dissolution medium, rotation speed or liquid flow rate, sampling probes and procedures, have to be monitored periodically during the periods of use.

The performance of the dissolution test equipment may be monitored by testing a reference product that is sensitive to hydrodynamic conditions. Such tests may be performed periodically or continuously for comparative reasons with other laboratories.

During testing, critical inspection and observation are required. This approach is especially important to explain any outlying results.

Validation of automated systems, whether concerning the sampling and analytical part or the dissolution media preparation and test performance, has to consider accuracy, precision, and the avoidance of contamination by any dilutions, transfers, cleaning and sample or solvent preparation procedures.

EXPRESSION OF DISSOLUTION SPECIFICATIONS FOR ORAL DOSAGE FORMS

The dissolution specification is expressed in terms of the quantity (Q) of active substance dissolved in a specified time, expressed as a percentage of the content stated on the product label.

Conventional-release dosage forms

In most cases, when tested under reasonable and justified test conditions, the acceptance criteria at level S_1 are that at least 80 per cent of the active substance is released within a specified time, typically 45 min or less. This corresponds to a Q value of 75 per cent, since, as referred to in Table 2.9.3.-1, for level S_1 the individual value of each of the 6 units tested is not less than $Q + 5$ per cent, i.e. not less than 80 per cent.

Typically, a single-point acceptance criterion is sufficient to demonstrate that the majority of the active substance has been released, although in certain circumstances it may be necessary to test at additional time point(s), in order to demonstrate adequate dissolution.

Prolonged-release dosage forms

The dissolution test acceptance criteria for prolonged-release dosage forms is normally expected to consist of 3 or more points. The 1st specification point is intended to prevent unintended rapid release of the active substance ('dose dumping'). It is therefore set after a testing period corresponding to a dissolved amount typically of 20 per cent to 30 per cent. The 2nd specification point defines the dissolution pattern and so is set at around 50 per cent release. The final specification point is intended to ensure almost complete release, which is generally understood as more than 80 per cent release.

Delayed-release dosage forms

A delayed-release dosage form may release the active substance(s) fractionally or totally according to the formulation design when tested in different dissolution media, e.g. in increasing pH conditions. Dissolution specifications therefore have to be decided on a case-by-case basis.

Gastro-resistant dosage forms require at least 2 specification points in a sequential test and 2 different specifications in a parallel test. In a sequential test, the 1st specification point represents an upper limit and is set after 1 h or 2 h in acidic medium, and the 2nd after a pre-set time period of testing in an adequate buffer solution (preferably pH 6.8).

In most cases the acceptance criteria at level B_1 are that at least 80 per cent of the active substance is released. This corresponds to a Q value of 75 per cent, since, as referred to in Table 2.9.3.-4, for level B_1 the individual value of each of the 6 units tested is not less than $Q + 5$ per cent, i.e. not less than 80 per cent.

B. Dissolution

1. Dissolution Test for Tablets and Capsules (Dissolution Test for Solid Dosage Forms)[1]

(Ph. Eur. method 2.9.3)

This test is provided to determine compliance with the dissolution requirements for solid dosage forms administered orally. In this chapter, a dosage unit is defined as 1 tablet or 1 capsule or the amount specified.

APPARATUS

Apparatus 1 (Basket apparatus)

The assembly consists of the following: a vessel, which may be covered, made of glass or other inert, transparent material[2]; a motor; a drive shaft; and a cylindrical basket (stirring element). The vessel is partially immersed in a suitable water-bath of any convenient size or heated by a suitable device such as a heating jacket. The water-bath or heating device permits maintaining the temperature inside the vessel at 37 ± 0.5 °C during the test and keeping the dissolution medium in constant, smooth motion. No part of the assembly, including the environment in which the assembly is placed, contributes significant motion, agitation, or vibration beyond that due to the smoothly rotating stirring element. Apparatus that permits observation of the preparation and stirring element during the test is preferable. The vessel is cylindrical, with a hemispherical bottom and a capacity of 1 L. Its height is 160-210 mm and its inside diameter is 98-106 mm. Its sides are flanged at the top. A fitted cover may be used to retard evaporation[3]. The shaft is positioned so that its axis is not more than 2 mm at any point from the vertical axis of the vessel and rotates smoothly

[1] *This chapter has undergone pharmacopoeial harmonisation. See chapter 5.8 Pharmacopoeial harmonisation.*
[2] *The materials must not sorb, react, or interfere with the preparation to be tested.*
[3] *If a cover is used, it provides sufficient openings to allow ready insertion of the thermometer and withdrawal of samples.*

and without significant wobble that could affect the results. A speed-regulating device is used that allows the shaft rotation speed to be selected and maintained at a specified rate, within ± 4 per cent.

Shaft and basket components of the stirring element are fabricated of stainless steel, type 316 or equivalent, to the specifications shown in Figure 2.9.3.-1.

A basket having a gold coating of about 2.5 µm (0.0001 inch) thick may be used. The dosage unit is placed in a dry basket at the beginning of each test. The distance between the inside bottom of the vessel and the bottom of the basket is maintained at 25 ± 2 mm during the test.

Apparatus 2 (Paddle apparatus)

Use the assembly from Apparatus 1, except that a paddle formed from a blade and a shaft is used as the stirring element. The shaft is positioned so that its axis is not more than 2 mm from the vertical axis of the vessel, at any point, and rotates smoothly without significant wobble that could affect the results. The vertical center line of the blade passes through the axis of the shaft so that the bottom of the blade is flush with the bottom of the shaft. The paddle conforms to the specifications shown in Figure 2.9.3.-2. The distance of 25 ± 2 mm between the bottom of the blade and the inside bottom of the vessel is maintained during the test.

The metallic or suitably inert, rigid blade and shaft comprise a single entity. A suitable two-part detachable design may be used provided the assembly remains firmly engaged during the test. The paddle blade and shaft may be coated with a suitable coating so as to make them inert. The dosage unit is allowed to sink to the bottom of the vessel before rotation of the blade is started. A small, loose piece of non-reactive material, such as not more than a few turns of wire helix, may be attached to dosage units that would otherwise float. An alternative sinker device is shown in Figure 2.9.3.-3. Other validated sinker devices may be used.

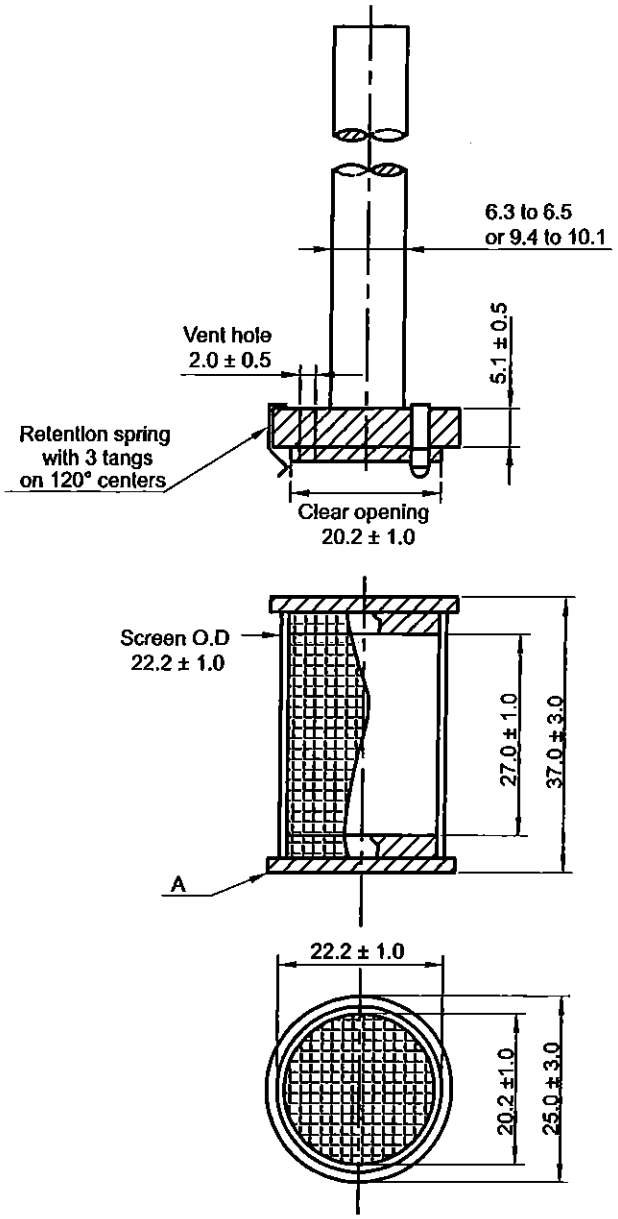
Apparatus 3 (Reciprocating cylinder)

The assembly consists of a set of cylindrical, flat-bottomed glass vessels; a set of glass reciprocating cylinders; inert fittings (stainless steel type 316 or other suitable material) and screens that are made of suitable nonsorbing and nonreactive material, and that are designed to fit the tops and bottoms of the reciprocating cylinders; a motor and drive assembly to reciprocate the cylinders vertically inside the vessels, and if desired, index the reciprocating cylinders horizontally to a different row of vessels. The vessels are partially immersed in a suitable water-bath of any convenient size that permits holding the temperature at 37 ± 0.5 °C during the test. No part of the assembly, including the environment in which the assembly is placed, contributes significant motion, agitation, or vibration beyond that due to the smooth, vertically reciprocating cylinder. A device is used that allows the reciprocation rate to be selected and maintained at the specified dip rate, within ± 5 per cent. An apparatus that permits observation of the preparations and reciprocating cylinders is preferable. The vessels are provided with an evaporation cap that remains in place for the duration of the test. The components conform to the dimensions shown in Figure 2.9.3.-4 unless otherwise specified.

Apparatus 4 (Flow-through cell)

The assembly consists of a reservoir and a pump for the dissolution medium; a flow-through cell; a water-bath that maintains the dissolution medium at 37 ± 0.5 °C. Use the specified cell size.

The pump forces the dissolution medium upwards through the flow-through cell. The pump has a delivery range

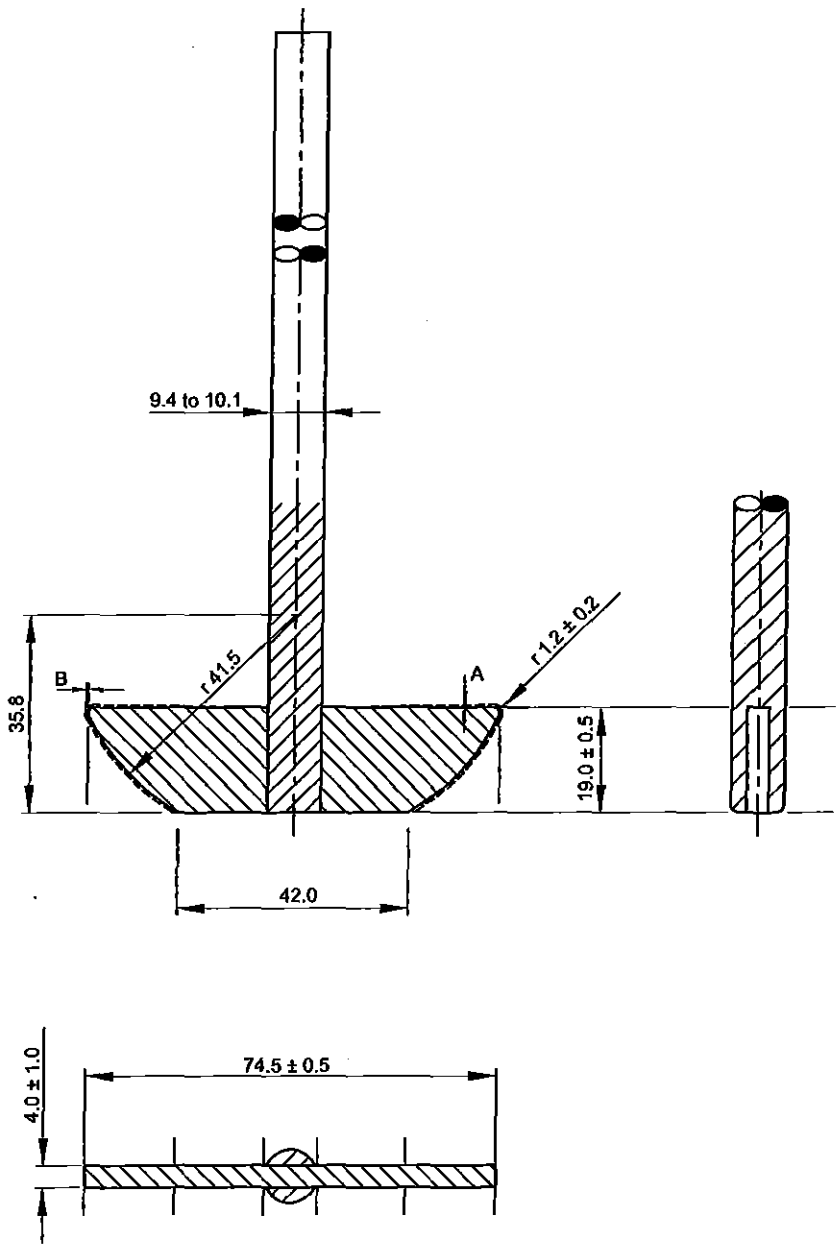


1) Screen with welded seam: 0.22-0.31 mm wire diameter with wire opening of 0.36-0.44 mm. After welding the screen may be slighty altered.
2) Maximum allowable runout at "A" is 1.0 mm when the part is rotated on center line axis with basket mounted.

Figure 2.9.3.-1. – *Apparatus 1, Basket stirring element Dimensions in millimetres*

between 240 mL/h and 960 mL/h, with standard flow rates of 4 mL/min, 8 mL/min, and 16 mL/min. It must deliver a constant flow (± 5 per cent of the nominal flow rate); the flow profile is sinusoidal with a pulsation of 120 ± 10 pulses/min. A pump without pulsation may also be used. Dissolution test procedures using the flow-through cell must be characterised with respect to rate and any pulsation.

The flow-through cell (see Figures 2.9.3.-5 and 2.9.3.-6) of transparent and inert material is mounted vertically, with a filter system that prevents escape of undissolved particles from the top of the cell; standard cell diameters are 12 mm and 22.6 mm; the bottom cone is usually filled with small glass beads of about 1 mm diameter, with 1 bead of about



A and B dimensions do not vary more than 0.5 mm when part is rotated on center line axis. Tolerances are ± 1.0 mm unless otherwise stated.

Figure 2.9.3.-2. – *Apparatus 2, Paddle stirring element*
Dimensions in millimetres

5 mm positioned at the apex to protect the fluid entry tube; a tablet holder (see Figures 2.9.3.-5 and 2.9.3.-6) is available for positioning of special dosage forms. The cell is immersed in a water-bath, and the temperature is maintained at 37 ± 0.5 °C.

The apparatus uses a clamp mechanism and 2 O-rings for the fixation of the cell assembly. The pump is separated from the dissolution unit in order to shield the latter against any vibrations originating from the pump. The position of the pump must not be on a level higher than the reservoir flasks. Tube connections are as short as possible. Use suitably inert tubing, such as polytetrafluoroethylene, with a 1.6 mm inner diameter and inert flanged-end connections.

Apparatus suitability The determination of suitability of the apparatus to perform dissolution testing must include conformance to the dimensions and tolerances of the apparatus as given above. In addition, critical test parameters that have to be monitored periodically during use include volume and temperature of the dissolution medium, rotation speed (Apparatus 1 and 2), dip rate (Apparatus 3), and flow rate of medium (Apparatus 4).

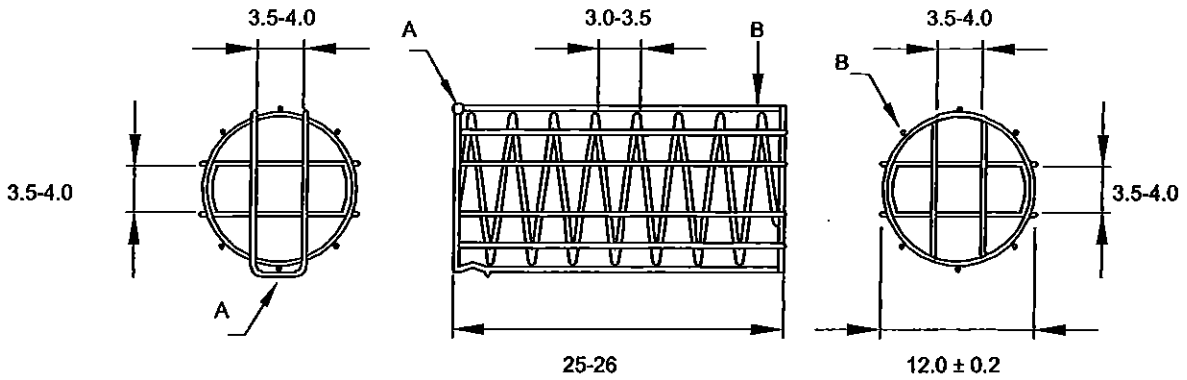
Determine the acceptable performance of the dissolution test assembly periodically.

PROCEDURE

APPARATUS 1 AND 2

Conventional-release solid dosage forms

Procedure Place the stated volume of the dissolution medium (± 1 per cent) in the vessel of the specified apparatus. Assemble the apparatus, equilibrate the dissolution



A: acid-resistant wire clasp B: acid-resistant wire support

Figure 2.9.3.-3. – *Alternative sinker*
Dimensions in millimetres

medium to 37 ± 0.5 °C, and remove the thermometer. ◊The test may also be carried out with the thermometer in place, provided it is shown that results equivalent to those obtained without the thermometer are obtained.◊

Place 1 dosage unit in the apparatus, taking care to exclude air bubbles from the surface of the dosage unit. Operate the apparatus at the specified rate. Within the time interval specified, or at each of the times stated, withdraw a specimen from a zone midway between the surface of the dissolution medium and the top of the rotating basket or blade, not less than 1 cm from the vessel wall. Where multiple sampling times are specified, replace the aliquots withdrawn for analysis with equal volumes of fresh dissolution medium at 37 °C or, where it can be shown that replacement of the medium is not necessary, correct for the volume change in the calculation. Keep the vessel covered for the duration of the test and verify the temperature of the medium at suitable times. Perform the analysis using a suitable assay method[4]. Repeat the test with additional dosage units.

If automated equipment is used for sampling or the apparatus is otherwise modified, verification that the modified apparatus will produce results equivalent to those obtained with the apparatus described in this chapter, is necessary.

Dissolution medium A suitable dissolution medium is used. The volume specified refers to measurements made between 20 °C and 25 °C. If the dissolution medium is a buffered solution, adjust the solution so that its pH is within 0.05 units of the specified pH. Dissolved gases can cause bubbles to form, which may change the results of the test. In such cases, dissolved gases must be removed prior to testing[5].

Time Where a single time specification is given, the test may be concluded in a shorter period if the requirement for minimum amount dissolved is met. Samples are to be withdrawn only at the stated times, within a tolerance of ± 2 per cent.

Prolonged-release solid dosage forms

Procedure Proceed as described for conventional-release dosage forms.

Dissolution medium Proceed as described for conventional-release dosage forms.

Time The test-time points, generally 3, are expressed in hours.

Delayed-release solid dosage forms

Procedure Use Method A or Method B.

Method A

— *Acid stage.* Place 750 mL of *0.1 M hydrochloric acid* in the vessel, and assemble the apparatus. Allow the medium to equilibrate to a temperature of 37 ± 0.5 °C. Place 1 dosage unit in the apparatus, cover the vessel and operate the apparatus at the specified rate. After 2 h of operation in *0.1 M hydrochloric acid*, withdraw an aliquot of the fluid and proceed immediately as directed under Buffer stage. Perform an analysis of the aliquot using a suitable assay method.

— *Buffer stage.* Complete the operations of adding the buffer and adjusting the pH within 5 min. With the apparatus operating at the rate specified, add to the fluid in the vessel 250 mL of a 0.20 M solution of *trisodium phosphate dodecahydrate R* that has been equilibrated to 37 ± 0.5 °C. Adjust, if necessary, with *2 M hydrochloric acid R* or *2 M sodium hydroxide R* to a pH of 6.8 ± 0.05. Continue to operate the apparatus for 45 min, or for the specified time. At the end of the time period, withdraw an aliquot of the fluid and perform the analysis using a suitable assay method.

Method B

— *Acid Stage.* Place 1000 mL of *0.1 M hydrochloric acid* in the vessel and assemble the apparatus. Allow the medium to equilibrate to a temperature of 37 ± 0.5 °C. Place 1 dosage unit in the apparatus, cover the vessel, and operate the apparatus at the specified rate. After 2 h of operation in *0.1 M hydrochloric acid*, withdraw an aliquot of the fluid, and proceed immediately as directed under Buffer stage. Perform an analysis of the aliquot using a suitable assay method.

— *Buffer stage.* For this stage of the procedure use buffer that has previously been equilibrated to a temperature of

[4] *Test specimens are filtered immediately upon sampling unless filtration is demonstrated to be unnecessary. Use an inert filter that does not cause adsorption of the active substance or contain extractable substances that would interfere with the analysis.*

[5] *A method of deaeration is as follows: heat the medium, while stirring gently, to about 41 °C, immediately filter under vacuum using a filter having a porosity of 0.45 µm or less, with vigorous stirring, and continue stirring under vacuum for about 5 min. Other validated deaeration techniques for removal of dissolved gases may be used.*

37 ± 0.5 °C. Drain the acid from the vessel and add 1000 mL of pH 6.8 phosphate buffer, prepared by mixing 3 volumes of *0.1 M hydrochloric acid* with 1 volume of a 0.20 M solution of *trisodium phosphate dodecahydrate R* and adjusting, if necessary, with *2 M hydrochloric acid R* or *2 M sodium hydroxide R* to a pH of 6.8 ± 0.05. This may also be accomplished by removing from the apparatus the vessel containing the acid and replacing it with another vessel, containing the buffer and transferring the dosage unit to the vessel containing the buffer. Continue to operate the apparatus for 45 min, or for the specified time. At the end of the time period, withdraw an aliquot of the fluid and perform the analysis using a suitable assay method.

Time All test times stated are to be observed within a tolerance of ± 2 per cent, unless otherwise specified.

APPARATUS 3

Conventional-release solid dosage forms

Procedure Place the stated volume of the dissolution medium (± 1 per cent) in each vessel of the apparatus. Assemble the apparatus, equilibrate the dissolution medium to 37 ± 0.5 °C, and remove the thermometer. Place 1 dosage unit in each of the reciprocating cylinders, taking care to exclude air bubbles from the surface of each dosage unit, and immediately operate the apparatus as specified. During the upward and downward stroke, the reciprocating cylinder moves through a total distance of 9.9-10.1 cm. Within the time interval specified, or at each of the times stated, raise the reciprocating cylinders and withdraw a portion of the medium from a zone midway between the surface of the dissolution medium and the bottom of each vessel. Perform the analysis as directed. If necessary, repeat the test with additional dosage units.

Replace the aliquot withdrawn for analysis with equal volumes of fresh dissolution medium at 37 °C or, where it can be shown that replacement of the medium is not necessary, correct for the volume change in the calculation. Keep the vessel covered with the evaporation cap for the duration of the test and verify the temperature of the medium at suitable times.

Dissolution medium Proceed as described for conventional-release dosage forms under Apparatus 1 and 2.

Time Proceed as described for conventional-release dosage forms under Apparatus 1 and 2.

Prolonged-release dosage forms

Procedure Proceed as described for conventional-release dosage forms under Apparatus 3.

Dissolution medium Proceed as described for prolonged-release dosage forms under Apparatus 1 and 2.

Time Proceed as described for prolonged-release dosage forms under Apparatus 1 and 2.

Delayed-release dosage forms

Procedure Proceed as described for delayed-release dosage forms, Method B, under Apparatus 1 and 2, using one row of vessels for the acid stage media and the following row of vessels for the buffer stage media, and using the volume of medium specified (usually 300 mL).

Time Proceed as directed for delayed-release dosage forms under Apparatus 1 and 2.

APPARATUS 4

Conventional-release dosage forms

Procedure Place the glass beads into the cell specified. Place 1 dosage unit on top of the beads or, if specified, on a

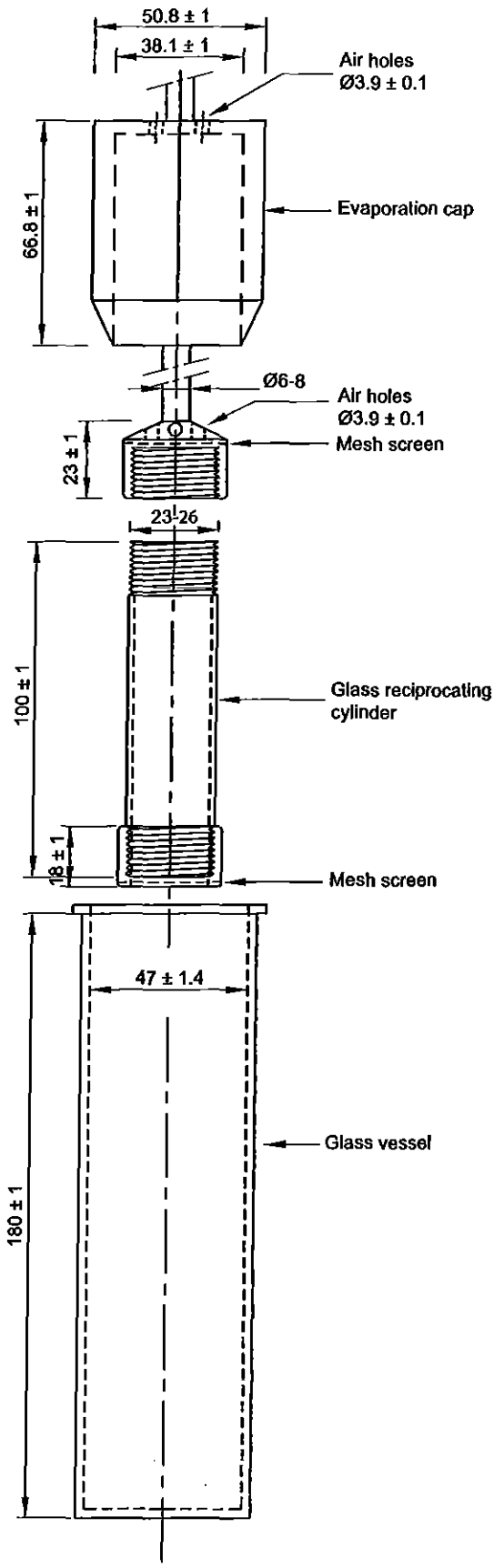


Figure 2.9.3.-4. – *Apparatus 3, glass vessel and reciprocating cylinder*

Dimensions in millimetres

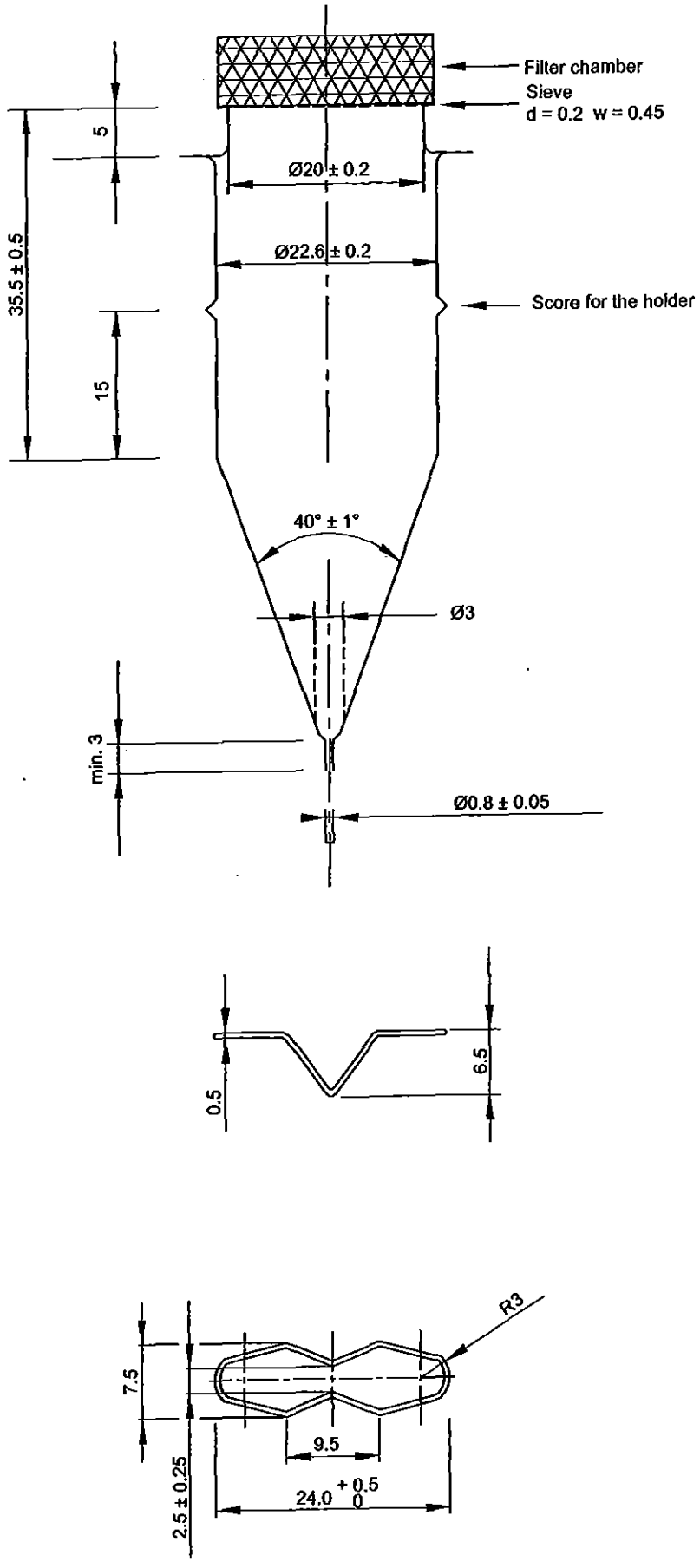


Figure 2.9.3.-5. – *Apparatus 4, large cell for tablets and capsules (top), tablet holder for the large cell (bottom)*
Dimensions in millimetres unless otherwise specified

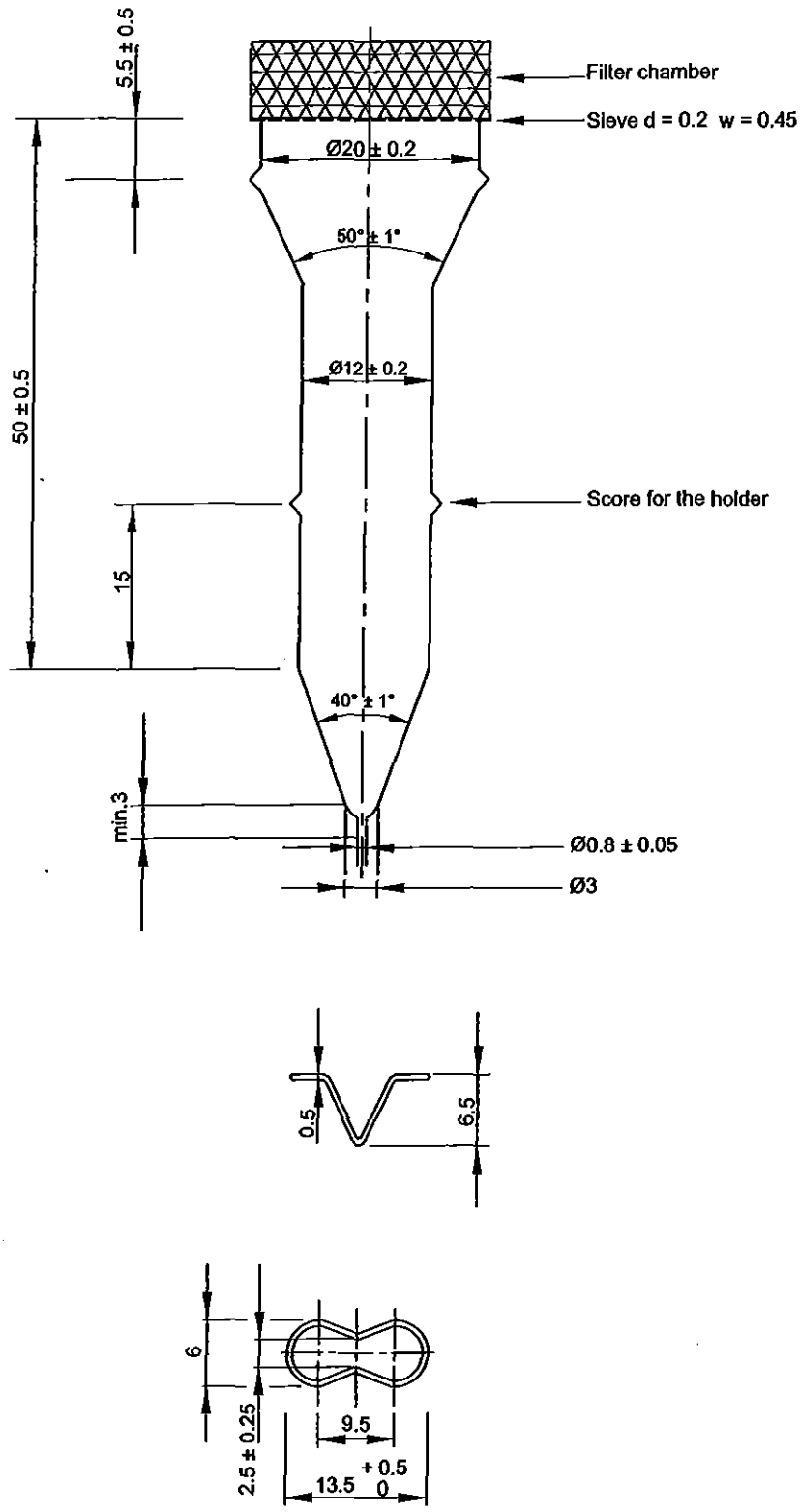


Figure 2.9.3.-6. – *Apparatus 4, small cell for tablets and capsules (top), tablet holder for the small cell (bottom) Dimensions in millimetres unless otherwise specified*

wire carrier. Assemble the filter head and fix the parts together by means of a suitable clamping device. Introduce by the pump the dissolution medium warmed to 37 ± 0.5 °C through the bottom of the cell to obtain the flow rate specified and measured with an accuracy of 5 per cent. Collect the eluate by fractions at each of the times stated. Perform the analysis as directed. Repeat the test with additional dosage units.

Dissolution medium Proceed as described for conventional-release dosage forms under Apparatus 1 and 2.

Time Proceed as described for conventional-release dosage forms under Apparatus 1 and 2.

Prolonged-release dosage forms

Procedure Proceed as described for conventional-release dosage forms under Apparatus 4.

Dissolution medium Proceed as described for conventional-release dosage forms under Apparatus 4.

Time Proceed as described for conventional-release dosage forms under Apparatus 4.

Delayed-release dosage forms

Procedure Proceed as described for delayed-release dosage forms under Apparatus 1 and 2, using the specified media.

Time Proceed as described for delayed-release dosage forms under Apparatus 1 and 2.

INTERPRETATION

Conventional-release solid dosage forms

Unless otherwise specified, the requirements are met if the quantities of active substance dissolved from the dosage units tested conform to Table 2.9.3.-1. Continue testing through the 3 levels unless the results conform at either S_1 or S_2. The quantity Q, is the specified amount of dissolved active substance, expressed as a percentage of the labelled content; the 5 per cent, 15 per cent, and 25 per cent values in the Table are percentages of the labelled content so that these values and Q are in the same terms.

Table 2.9.3.-1

Level	Number tested	Acceptance criteria
S_1	6	Each unit is not less than $Q + 5$ per cent.
S_2	6	Average of 12 units ($S_1 + S_2$) is equal to or greater than Q, and no unit is less than $Q - 15$ per cent.
S_3	12	Average of 24 units ($S_1 + S_2 + S_3$) is equal to or greater than Q, not more than 2 units are less than $Q - 15$ per cent, and no is less than $Q - 25$ per cent.

Prolonged-release dosage forms

Unless otherwise specified, the requirements are met if the quantities of active substance dissolved from the dosage units tested conform to Table 2.9.3.-2. Continue testing through the 3 levels unless the results conform at either L_1 or L_2. Limits on the amounts of active substance dissolved are expressed in terms of the percentage of labelled content. The limits embrace each value of Q_i, the amount dissolved at each specified fractional dosing interval. Where more than one range is specified, the acceptance criteria apply individually to each range.

Table 2.9.3.-2

Level	Number tested	Acceptance criteria
L_1	6	No individual value lies outside each of the stated ranges and no individual value is less than the stated amount at the final test time.
L_2	6	The average value of the 12 units ($L_1 + L_2$) lies within each of the stated ranges and is not less than the stated amount at the final test time; none is more than 10 per cent of labelled content outside each of the stated ranges; and none is more than 10 per cent of labelled content below the stated amount at the final test time.
L_3	12	The average value of the 24 units ($L_1 + L_2 + L_3$) lies within each of the stated ranges, and is not less than the stated amount at the final test time; not more than 2 of the 24 units are more than 10 per cent of labelled content outside each of the stated ranges; not more than 2 of the 24 units are more than 10 per cent of labelled content below the stated amount at the final test time; and none of the units is more than 20 per cent of labelled content outside each of the stated ranges or more than 20 per cent of labelled content below the stated amount at the final test time.

Delayed-release dosage forms

Acid stage Unless otherwise specified, the requirements of this portion of the test are met if the quantities, based on the percentage of the labelled content of active substance dissolved from the units tested conform to Table 2.9.3.-3. Continue testing through the 3 levels unless the results of both acid and buffer stages conform at an earlier level.

Table 2.9.3.-3

Level	Number tested	Acceptance criteria
A_1	6	No individual value exceeds 10 per cent dissolved.
A_2	6	The average value of the 12 units ($A_1 + A_2$) is not more than 10 per cent dissolved, and no individual unit is greater than 25 per cent dissolved.
A_3	12	The average value of the 24 units ($A_1 + A_2 + A_3$) is not more than 10 per cent dissolved, and no individual unit is greater than 25 per cent dissolved.

Buffer stage Unless otherwise specified, the requirements are met if the quantities of active substance dissolved from the units tested conform to Table 2.9.3.-4. Continue testing through the 3 levels unless the results of both stages conform at an earlier level. The value of Q in Table 2.9.3.-4 is 75 per cent dissolved unless otherwise specified. The quantity, Q, is the specified total amount of active substance dissolved in both the acid and buffer stages, expressed as a percentage of the labelled content. The 5 per cent, 15 per cent and 25 per cent values in the Table are percentages of the labelled content so that these values and Q are in the same terms.

Table 2.9.3.-4

Level	Number tested	Acceptance criteria
B_1	6	No unit is less than $Q + 5$ per cent.
B_2	6	The average value of the 12 units ($B_1 + B_2$) is equal to or greater than Q, and no unit is less than $Q - 15$ per cent.
B_3	12	The average value of the 24 units ($B_1 + B_2 + B_3$) is equal to or greater than Q, not more than 2 units are less than $Q - 15$ per cent, and no unit is less than $Q - 25$ per cent.

Recommendations on dissolution testing are given in general chapter 5.17.1.

2. Dissolution Test for Patches

(Ph. Eur. method 2.9.4)

This test is used to determine the dissolution rate of the active substance(s) of patches.

The disk assembly method, the cell method or the rotating cylinder method may be used, as suitable, according to the composition, dimensions and shape of the patch.

A membrane may be used for the purpose of the test. The membrane material (inert porous cellulose, silicone, etc.) must not affect the release kinetics of the active substance(s) from the patch. It must also be free of substances that may interfere with the performance of the patch (e.g. grease). The membrane may be suitably treated before the tests, for example by maintaining it for 24 h in the medium to be used in the test. The membrane is applied over the release surface of the patch in such a way as to avoid the formation of air bubbles.

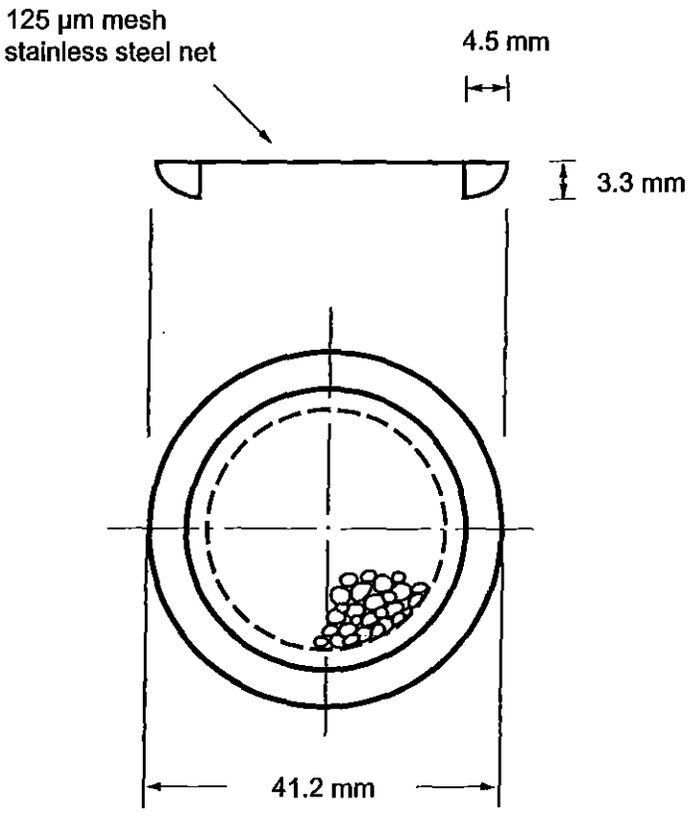


Figure 2.9.4.-1. – *Disk assembly*

1. DISK ASSEMBLY METHOD

Equipment

Use the paddle and vessel assembly from the paddle apparatus described in the dissolution test for solid oral dosage forms (*2.9.3*) with the addition of a stainless steel disk assembly (SSDA) in the form of a net with an aperture of 125 µm (see Figure 2.9.4.-1).

The SSDA holds the system at the bottom of the vessel and is designed to minimise any dead volume between the SSDA and the bottom of the vessel. The SSDA holds the patch flat, with the release surface facing upwards and parallel to the bottom of the paddle blade. A distance of 25 ± 2 mm between the bottom of the paddle blade and the surface of the SSDA is maintained during the test (see Figure 2.9.4.-2). The temperature is maintained at 32 ± 0.5 °C. The vessel may be covered during the test to minimise evaporation.

Procedure

Place the prescribed volume of the dissolution medium in the vessel and equilibrate the medium to the prescribed temperature. Apply the patch to the SSDA, ensuring that the release surface is as flat as possible. The patch may be attached to the SSDA by a prescribed adhesive or by a strip of a double-sided adhesive tape. The adhesive or tape are previously tested for the absence of interference with the assay and of adsorption of the active substance(s). Press the patch, release surface facing upwards, onto the side of the SSDA made adhesive. The applied patch must not overlap the borders of the SSDA. For this purpose and provided that the preparation is homogeneous and uniformly spread on the backing layer, an appropriate and exactly measured piece of the patch may be cut and used to test the dissolution rate. This procedure may also be necessary to achieve appropriate sink conditions. This procedure must not be applied to membrane-type patches. Place the patch mounted on the

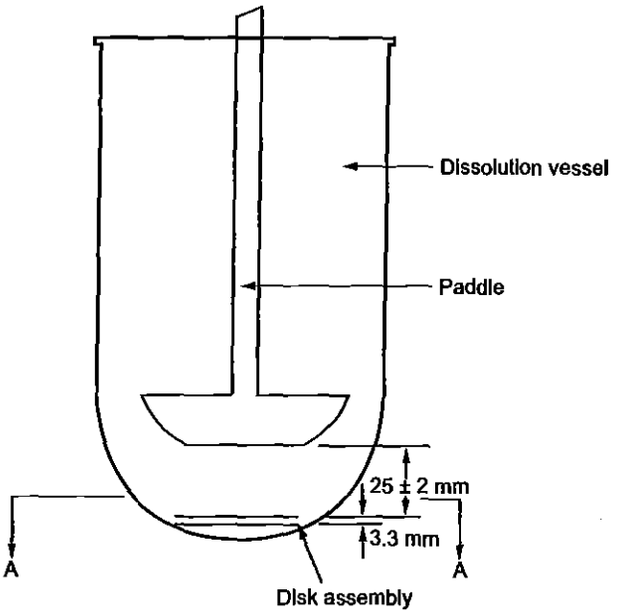


Figure 2.9.4.-2. – *Paddle and disk*

SSDA flat at the bottom of the vessel with the release surface facing upwards. Immediately rotate the paddle, at 100 r/min for example. At predetermined intervals, withdraw a sample from the zone midway between the surface of the dissolution medium and the top of the paddle blade, not less than 1 cm from the vessel wall.

Perform the assay on each sample, correcting for any volume losses, as necessary. Repeat the test with additional patches.

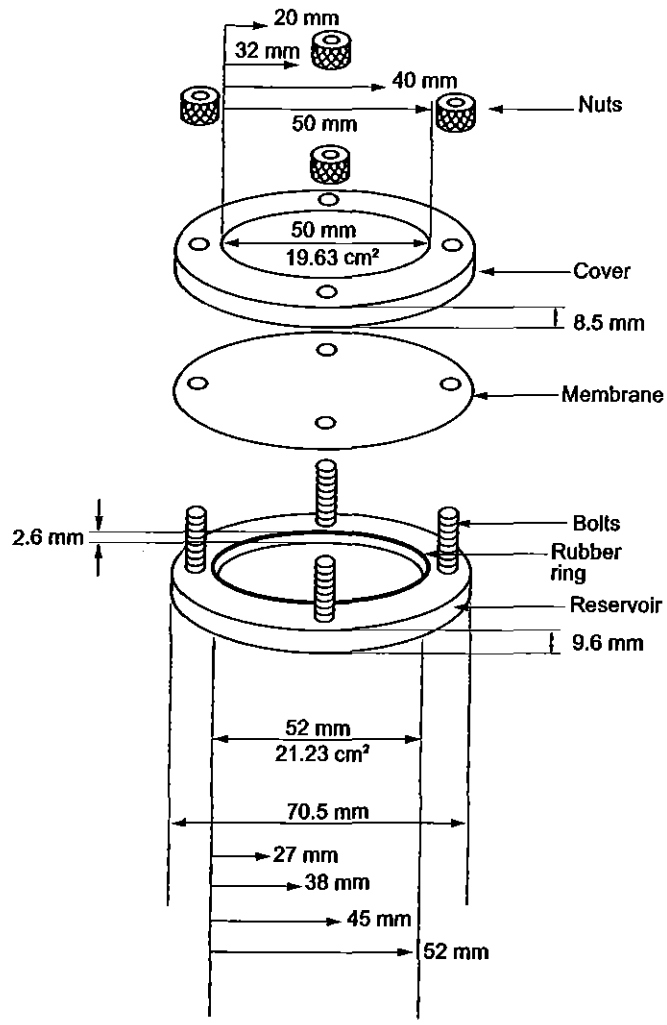


Figure 2.9.4.-3. – *Extraction cell*

2. CELL METHOD

Equipment

Use the paddle and vessel assembly from the paddle apparatus described in the dissolution test for solid oral dosage forms (*2.9.3*) with the addition of the extraction cell (*cell*).

The *cell* is made of chemically inert materials and consists of a *support*, a *cover* and, if necessary, a membrane placed on the patch to isolate it from the medium that may modify or adversely affect the physico-chemical properties of the patch (see Figure 2.9.4.-3).

Support The central part of the support forms a cavity intended to hold the patch. The cavity has a depth of 2.6 mm and a diameter that is appropriate for the size of the patch to be examined. The following diameters can be used: 27 mm, 38 mm, 45 mm, 52 mm, corresponding to volumes of 1.49 mL, 2.95 mL, 4.14 mL, 5.52 mL, respectively.

Cover The cover has a central opening with a diameter selected according to the size of the patch to be examined. The patch can thus be precisely centred, and its release surface limited. The following diameters may be used: 20 mm, 32 mm, 40 mm, 50 mm corresponding to areas of 3.14 cm^2, 8.04 cm^2, 12.57 cm^2, 19.63 cm^2, respectively. The cover is held in place by nuts screwed onto bolts projecting from the support. The cover is sealed to the support by a rubber ring set on the reservoir.

Extraction cell The *cell* holds the patch flat, with the release surface facing upwards and parallel to the bottom of the paddle blade. A distance of 25 ± 2 mm is maintained between the lower surface of the paddle blade and the upper surface of the cell (see Figure 2.9.4.-4). The temperature is maintained at 32 ± 0.5 °C. The vessel may be covered during the test to minimise evaporation.

Procedure

Place the prescribed volume of the dissolution medium in the vessel and equilibrate the medium to the prescribed temperature. Precisely centre the patch in the *cell* with the release surface facing upwards. Close the *cell*, if necessary applying a hydrophobic substance (for example, petrolatum) to the flat surfaces to ensure the seal, and ensure that the patch stays in place. Introduce the *cell* flat into the bottom of the vessel with the cover facing upwards. Immediately rotate the paddle, at 100 r/min for example. At predetermined intervals, withdraw a sample from the zone midway between the surface of the dissolution medium and the top of the paddle blade, not less than 1 cm from the vessel wall.

Perform the assay on each sample, correcting for any volume losses, as necessary. Repeat the test with additional patches.

3. ROTATING CYLINDER METHOD

Equipment

Use the assembly of the paddle apparatus described in the dissolution test for solid oral dosage forms (*2.9.3*). Replace the paddle and shaft with a stainless steel cylinder stirring

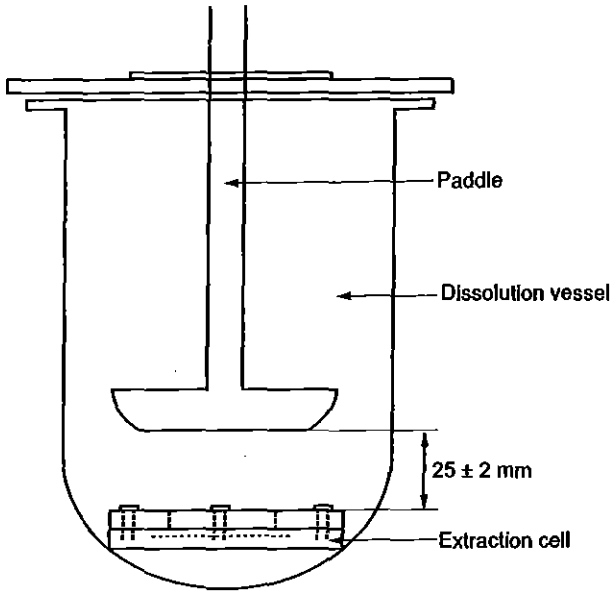


Figure 2.9.4.-4. – *Paddle over extraction cell*

element (*cylinder*) (see Figure 2.9.4.-5). The patch is placed on the *cylinder* at the beginning of each test. The distance between the inside bottom of the vessel and the *cylinder* is maintained at 25 ± 2 mm during the test. The temperature is maintained at 32 ± 0.5 °C. The vessel is covered during the test to minimise evaporation.

Procedure

Place the prescribed volume of the dissolution medium in the vessel and equilibrate the medium to the prescribed temperature. Remove the protective liner from the patch and place the adhesive side on a piece of suitable inert porous membrane that is at least 1 cm larger on all sides than the patch. Place the patch on a clean surface with the membrane in contact with this surface. Two systems for adhesion to the *cylinder* may be used:

— apply a suitable adhesive to the exposed membrane borders and, if necessary, to the back of the patch;
— apply a double-sided adhesive tape to the external wall of the *cylinder*.

Using gentle pressure, carefully apply the non-adhesive side of the patch to the *cylinder*, so that the release surface is in contact with the dissolution medium and the long axis of the patch fits around the circumference of the *cylinder*.

The system for adhesion used is previously tested for absence of interference with the assay and of adsorption of the active substance(s).

Place the *cylinder* in the apparatus and immediately rotate the *cylinder*, at 100 r/min for example. At predetermined intervals, withdraw a sample from the zone midway between the surface of the dissolution medium and the top of the rotating *cylinder*, not less than 1 cm from the vessel wall.

Perform the assay on each sample, correcting for any volume withdrawn, as necessary. Repeat the test with additional patches.

4. INTERPRETATION

The requirements are met if the quantity of active substance(s) released from the patch, expressed as the amount per surface area per time unit, is within the prescribed limits at the defined sampling times.

3. Dissolution Test for Lipophilic Solid Doseage Forms

(Ph. Eur. method 2.9.42)

APPARATUS

The apparatus (see Figure 2.9.42.-1) consists of:

— A reservoir for the dissolution medium.
— A pump that forces the dissolution medium upwards through the flow-through cell.
— A flow-through cell shown in Figure 2.9.42.-2 specifically intended for lipophilic solid dosage forms such as suppositories and soft capsules. It consists of 3 transparent parts which fit into each other. The lower part (1) is made up of 2 adjacent chambers connected to an overflow device.

The dissolution medium passes through chamber A and is subjected to an upwards flow. The flow in chamber B is downwards directed to a small-size bore exit which leads upwards to a filter assembly. The middle part (2) of the cell has a cavity designed to collect lipophilic excipients which float on the dissolution medium. A metal grill serves as a rough filter. The upper part (3) holds a filter unit for paper, glass fibre or cellulose filters.

— A water-bath that will maintain the dissolution medium at 37 ± 0.5 °C.

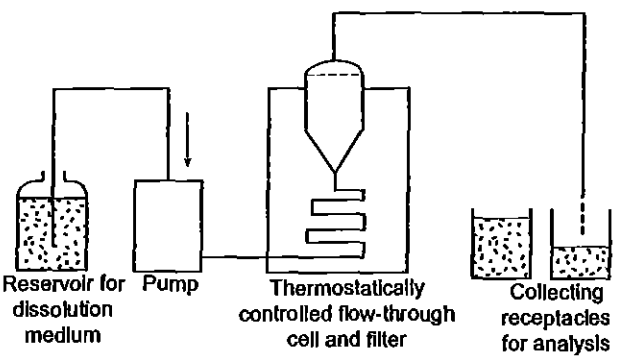


Figure 2.9.42.-1. – *Flow-through apparatus*

Dissolution medium

If the dissolution medium is buffered, adjust its pH to within ± 0.05 units of the prescribed value. Remove any dissolved gases from the dissolution medium before the test since they can cause the formation of bubbles that significantly affect the results.

METHOD

Place 1 unit of the preparation to be examined in chamber A. Close the cell with the prepared filter assembly. At the beginning of the test, chamber A requires air removal via a small orifice connected to the filter assembly. Heat the dissolution medium to an appropriate temperature taking the melting point of the preparation into consideration. Using a suitable pump, introduce the warmed dissolution medium through the bottom of the cell to obtain a suitable continuous flow through an open or closed circuit at the prescribed rate (± 5 per cent). When the dissolution medium reaches the overflow, air starts to escape through the capillary and chamber B fills with the dissolution medium. The preparation spreads through the dissolution medium according to its physico-chemical properties.

In justified and authorised cases, representative fractions of large volume suppositories may be tested.

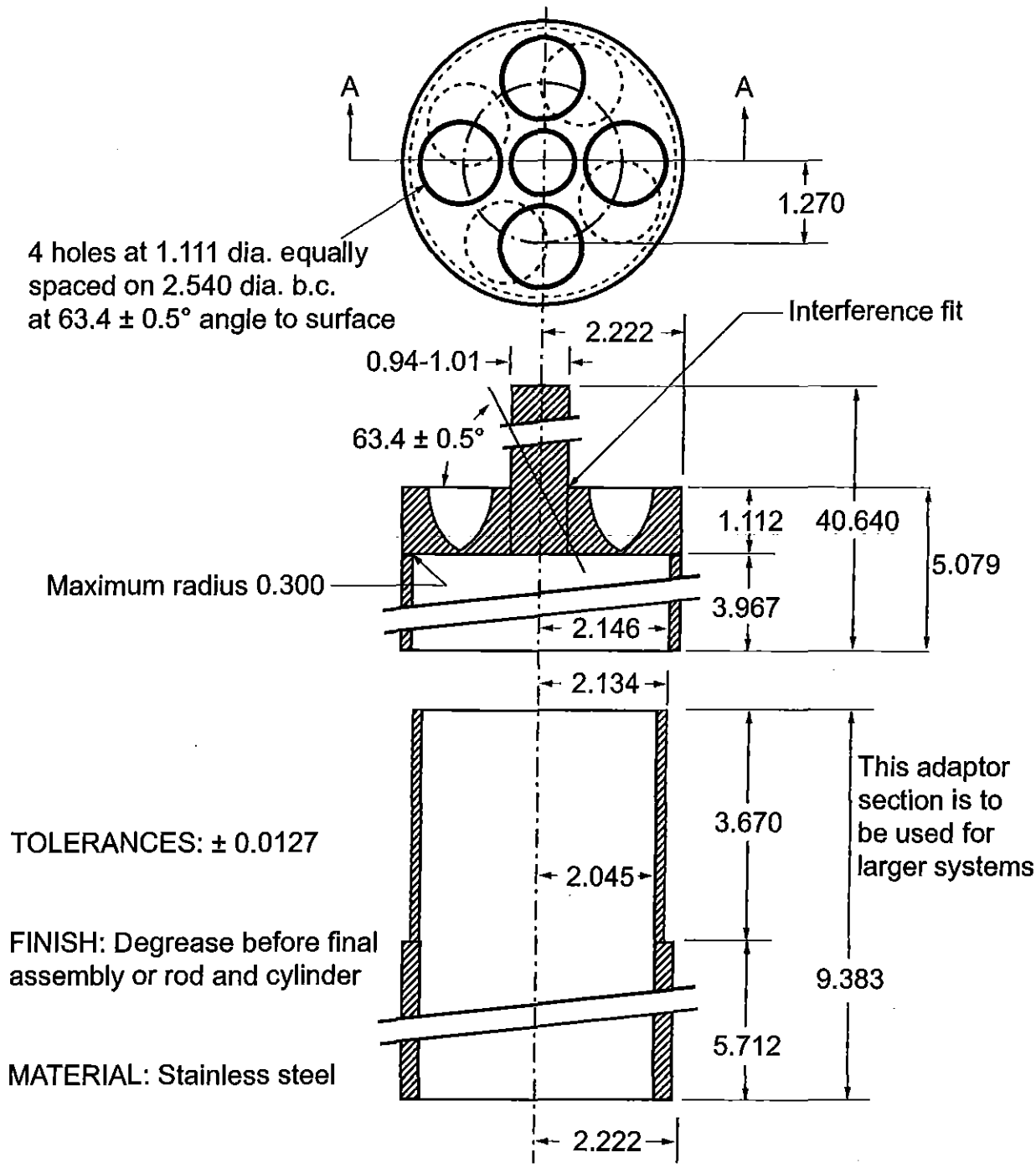


Figure 2.9.4.-5. – *Cylinder stirring element*
Dimensions in centimetres

SAMPLING AND EVALUATION

Samples are always collected at the outlet of the cell, irrespective of whether the circuit is opened or closed.

Filter the liquid removed using an inert filter of appropriate pore size that does not cause significant adsorption of the active substance from the solution and does not contain substances extractable by the dissolution medium that would interfere with the prescribed analytical method. Proceed with analysis of the filtrate as prescribed.

The quantity of the active substance dissolved in a specified time is expressed as a percentage of the content stated on the label.

4. Dissolution Test for Medicated Chewing Gum
(Ph. Eur. method 2.9.25)

PRINCIPLE

The test is used to determine the dissolution rate of active substances in medicated chewing gums. This is done by applying a mechanical kneading procedure to a piece of gum placed in a small chamber designed to simulate the process of chewing.

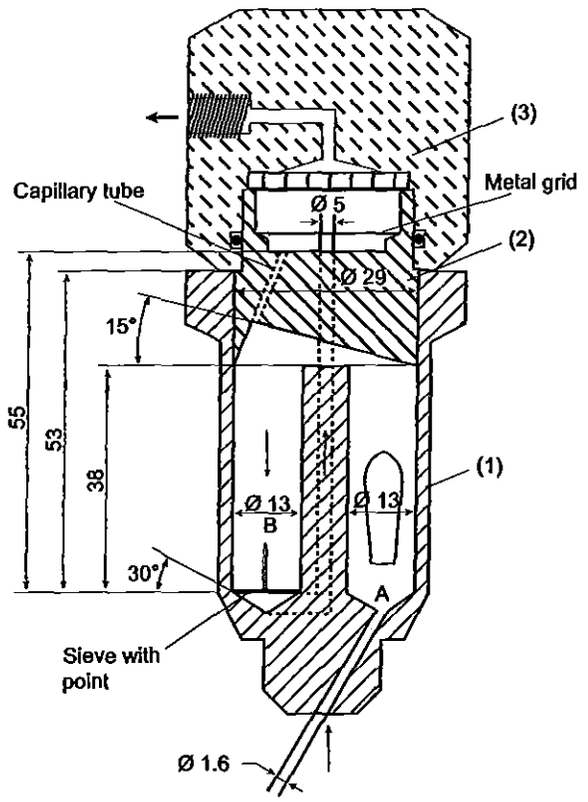


Figure 2.9.42.-2. – *Flow-through cell*
Dimensions in millimetres

APPARATUS A

Chewing apparatus A (Figure 2.9.25.-1) consists of:
— 1 chewing chamber;
— 1 vertical piston;
— 2 horizontal pistons with O-rings and sealing rings.

The chewing chamber consists of 4 individual parts:
— 1 central chamber;
— 1 funnel (Figure 2.9.25.-2);
— 2 guides with bushes (Figure 2.9.25.-3).

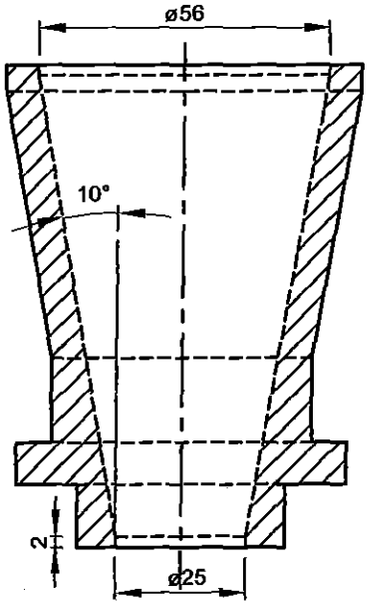


Figure 2.9.25.-2 – *Funnel*
Dimensions in millimetres

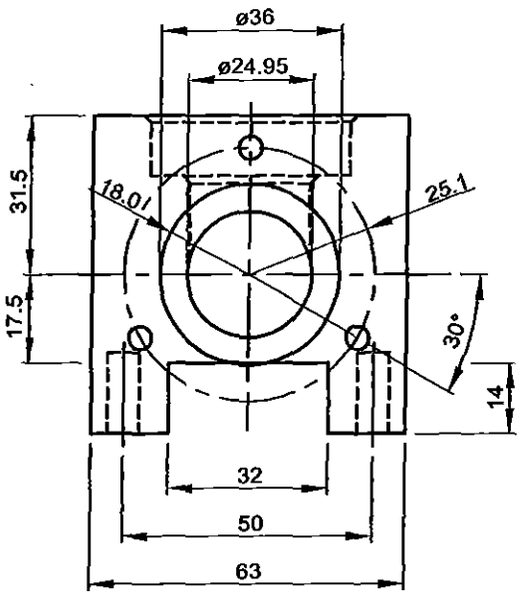


Figure 2.9.25.-3 – *Guide (section G-G)*
Dimensions in millimetres

The funnel and guides are mounted on the central chamber. The O-rings are incorporated in the piston recess with the sealing ring around it; the sealing rings ensure that the chamber is watertight. The horizontal pistons are placed in the chewing chamber through the guides.

The gum is artificially chewed by the horizontal pistons, and the vertical piston ensures that the gum stays in the right place between chews.

Machine speed is controlled to ensure a constant cycle. One cycle (chew) is defined as follows: the horizontal pistons start from their outermost position, move to their innermost position then return to their outermost position. Within one cycle, the vertical piston moves from its lowest position to its uppermost position and back to its lowest position.

Each horizontal piston has a stroke of 25.0 mm. The maximum distance between these 2 pistons is 50 mm. The minimum distance between the 2 horizontal pistons is 0.1 mm to 1.0 mm. The vertical piston has a stroke of 22.0 mm.

Horizontal piston movement is controlled so that the 2 pistons are at their innermost position at the same time. Vertical piston movement is controlled so that it does not conflict with the movement of the horizontal pistons.

If necessary, the machine can be constructed so that the horizontal pistons rotate around their own axes in opposite direction to each other by the end of the chew in order to obtain maximum chewing.

All parts of the apparatus that may come into contact with the preparation or the dissolution medium are chemically inert and do not adsorb, react with or interfere with the sample.

APPARATUS B

Chewing apparatus B (Figure 2.9.25.-4) consists of:
— 1 test cell (Figure 2.9.25.-5 or 2.9.25.-6);
— 1 vertical axle with upper chewing surface (Figures 2.9.25.-7 and 2.9.25.-8);
— 1 base chamber with lower chewing surface (Figures 2.9.25.-9 and 2.9.25.-10);
— 1 device for up-and-down chewing motion;
— 1 revolving device for the vertical axle.

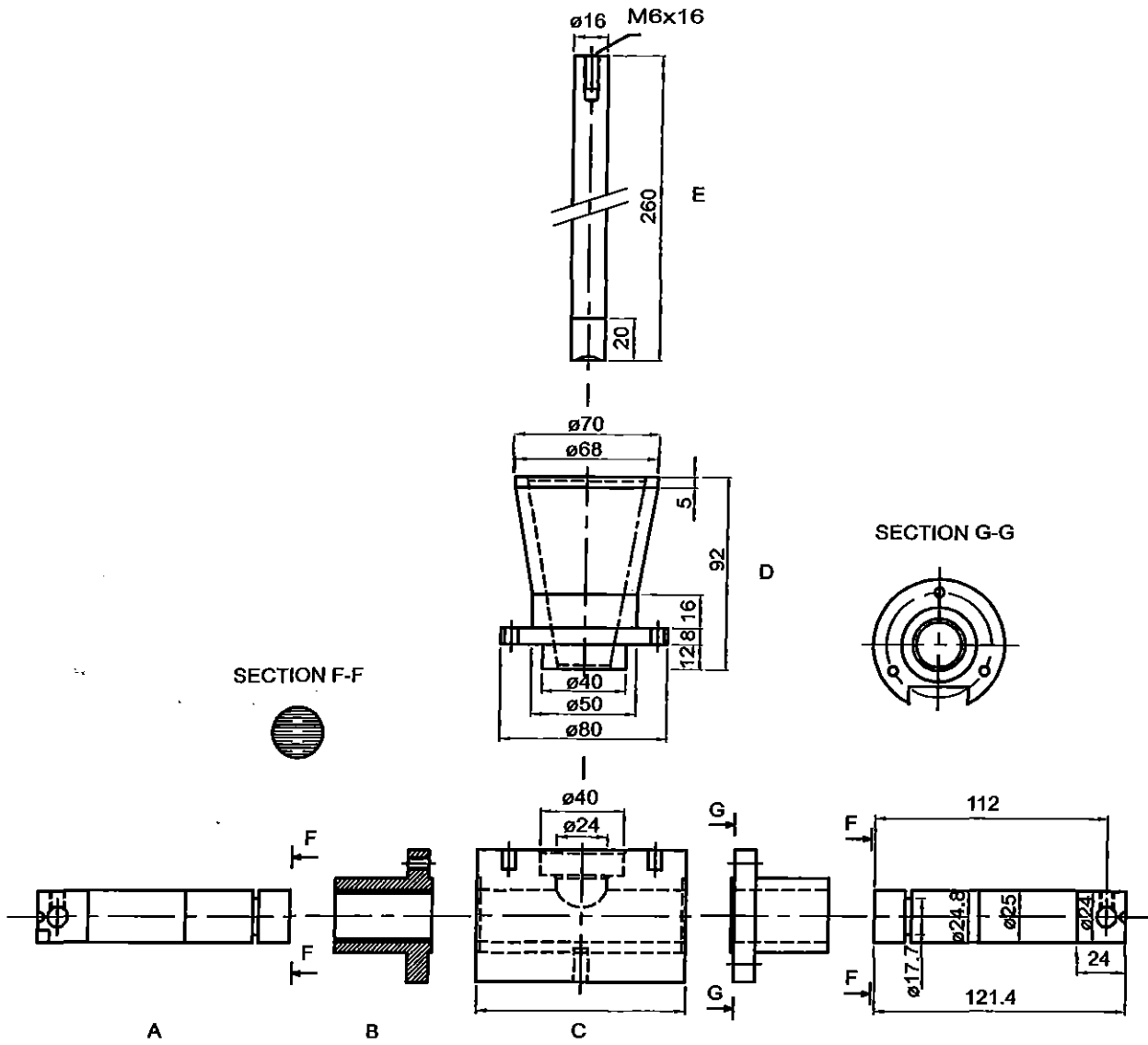


Figure 2.9.25.-1 – *Apparatus A - Chewing chamber and pistons*
Dimensions in millimetres

The gum is artificially chewed by the lower and upper chewing surfaces. Machine speed is controlled to ensure a constant cycle. The distance between the lower and upper chewing surfaces may be set up to 5 mm. The turning angle of the revolving device is about 20°.

The test cells may also be equipped with 1 or 2 glass sampling tubes, coming through the thermostatic double wall. These tubes also make it possible to have an external sink, which may be necessary to achieve sink conditions for sparingly soluble substances.

The gum is usually sandwiched between 2 circular plastic nets to prevent disintegration.

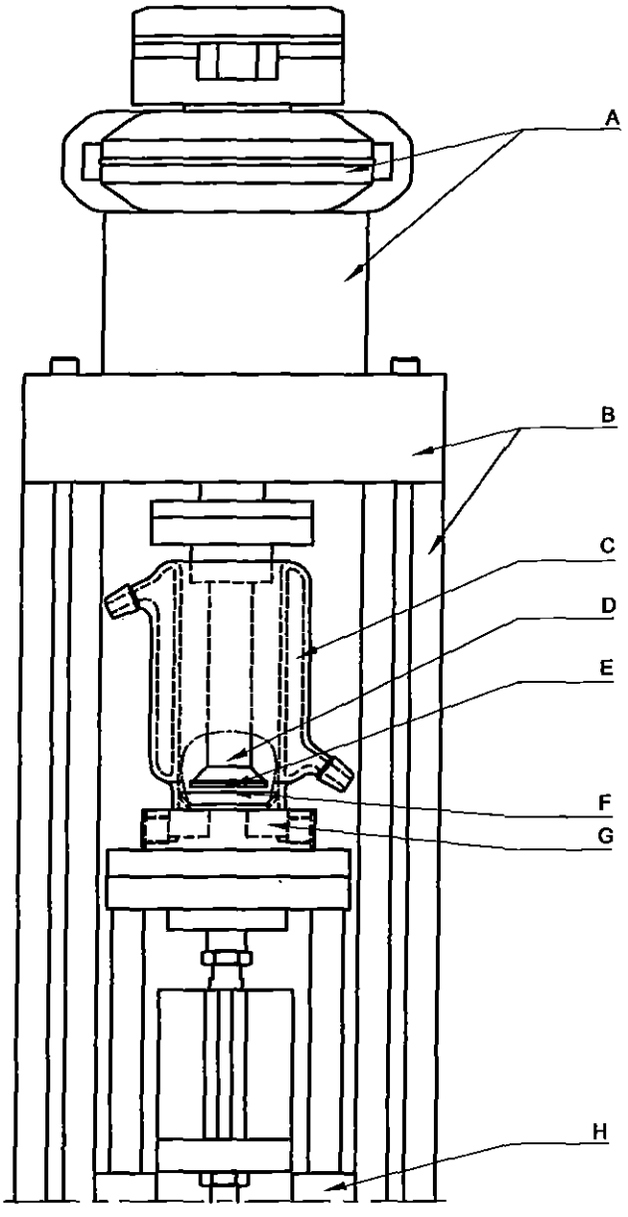
Nets made from nylon (PA6) with an aperture of 1.4 mm and a wire diameter of 0.405 mm may be used.

All parts of the apparatus that may come into contact with the preparation or the dissolution medium are chemically inert and do not adsorb, react with or interfere with the sample.

PROCEDURE

For each determination, the following information is needed:
- — apparatus used (type A or type B);
- — composition, volume and temperature of the dissolution medium;
- — number of chews per minute;
- — time and sampling method;
- — whether the analysis is performed on the gum residue or on the dissolution medium;
- — method of analysis.

Place the prescribed volume of dissolution medium in the chewing chamber, usually 20 mL of *phosphate buffer solution pH 6.0 R2*. Maintain the medium temperature at 37 ± 0.5 °C using an electrical device with external control (apparatus A) or with a thermostat (apparatus B). Set the machine speed at the prescribed number of chews per minute (typically up to 60). Accurately weigh a portion of gum or the whole gum, put it into the chewing chamber and start the machine.



A. revolving device for the upper chewing surface
B. stand
C. test cell
D. axle
E. upper chewing surface
F. lower chewing surface
G. base chamber
H. device for up-and-down chewing motion

Figure 2.9.25.-4 – *Apparatus B*

SAMPLING AND EVALUATION

Stop the apparatus at the prescribed time. Remove the gum residue and take a sample of the dissolution medium. Determine the content of active substance(s) by a suitable method. Medium replacement may be made after each sampling procedure; compensation by calculation of medium volume change or sample dilution is needed. Alternatively, determine the content of active substance(s) remaining in the gum residue. Carry out the test successively on 6 medicated chewing gums.

The quantity of active substance(s) dissolved in a specified time is expressed as a percentage of the content stated on the label.

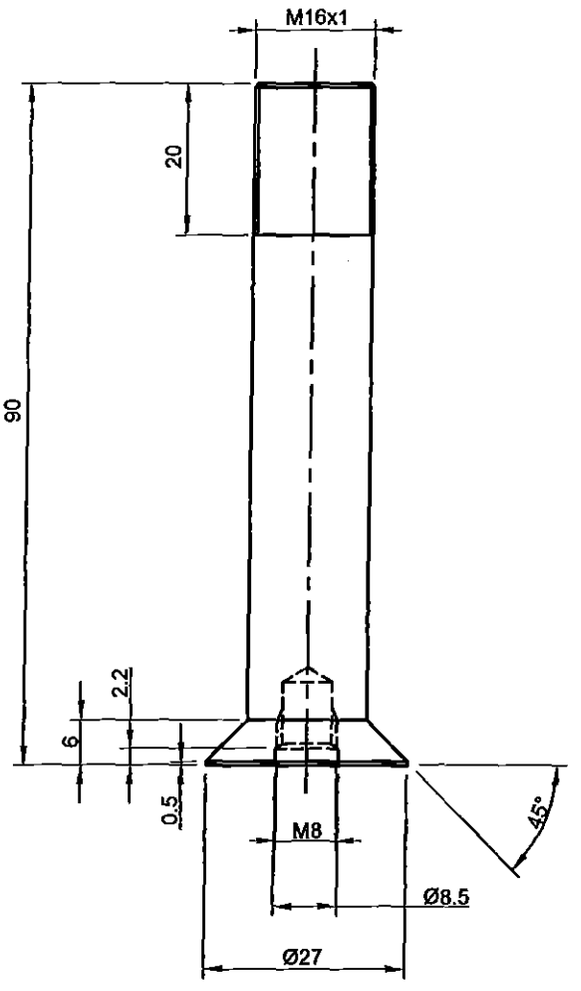


Figure 2.9.25.-7 – *Axle*
Dimensions in millimetres

5. Intrinsic Dissolution

(Ph. Eur. method 2.9.29)

The test is intended to determine the intrinsic dissolution rate of pure solid substances following compaction. It is carried out under specified experimental conditions such that a practical measure of the intrinsic dissolution rate is obtained.

The intrinsic dissolution rate is a theoretical value referring to pure solid substances having null porosity, but, practically, intrinsic dissolution rate is determined on substances having a minimal porosity.

PRINCIPLE

The intrinsic dissolution rate is defined as the dissolution rate of pure substances following compaction under the condition of constant surface area. Its assessment is useful in the characterisation of active substances and excipients.

The dissolution rate of pure substances can be affected by all the solid state properties such as crystal habit, crystallinity, amorphism, polymorphism, pseudo-polymorphism, particle size and specific surface area. In addition, it can also be influenced by extrinsic factors (test conditions), such as hydrodynamics, temperature, viscosity, pH, buffer strength and ionic strength of the dissolution medium.

The assessment of intrinsic dissolution rate of a solid substance involves the preparation of a compact. Assurance

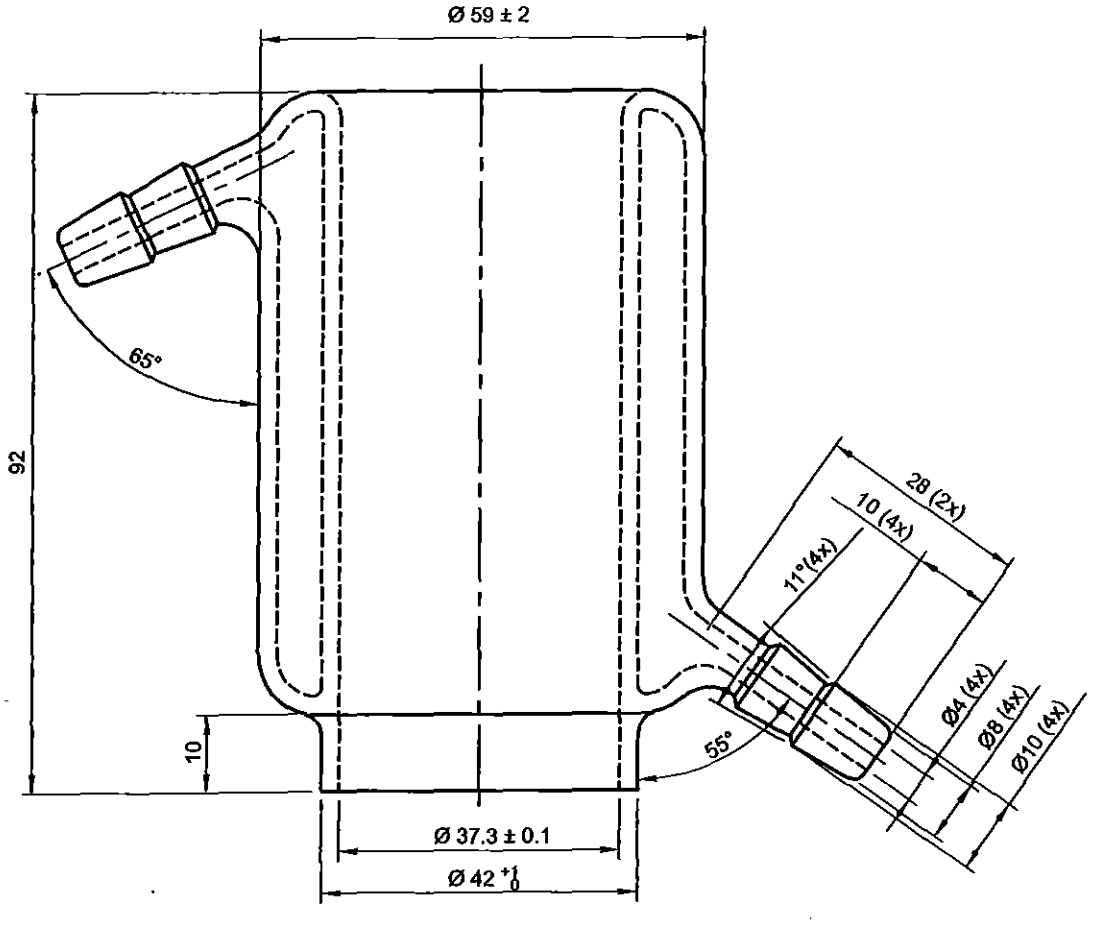


Figure 2.9.25.-5 – *Test cell*
Dimensions in millimetres

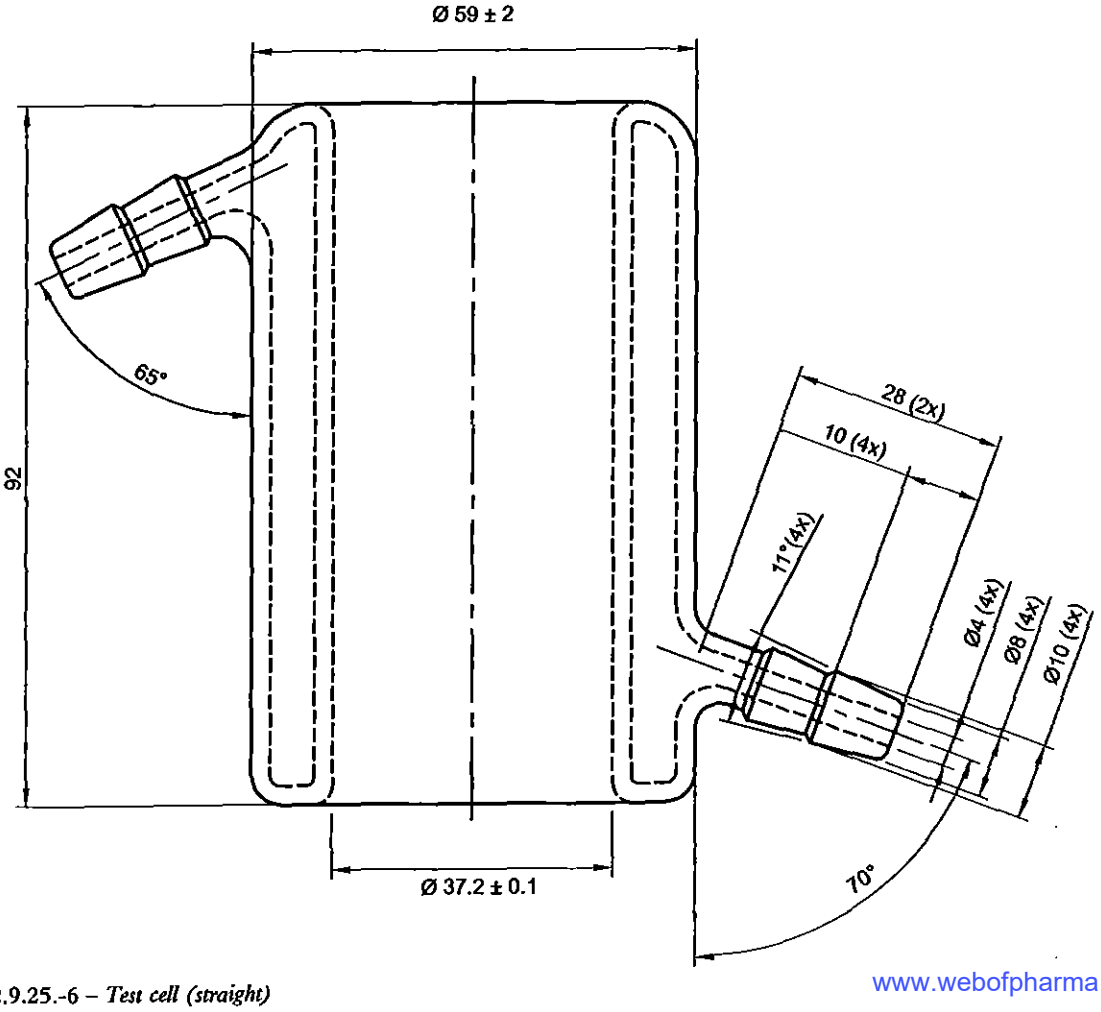


Figure 2.9.25.-6 – *Test cell (straight)*
Dimensions in millimetres

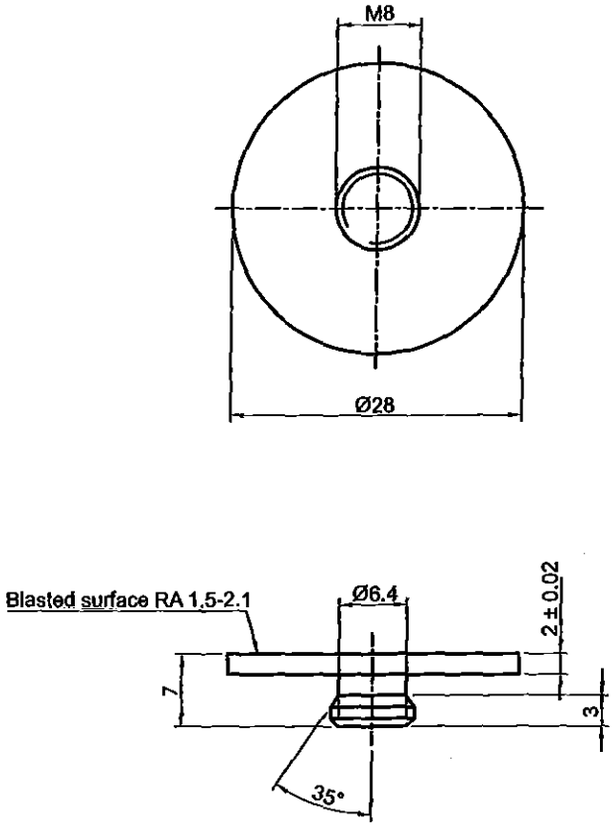


Figure 2.9.25.-8 – *Upper chewing surface*
Dimensions in millimetres

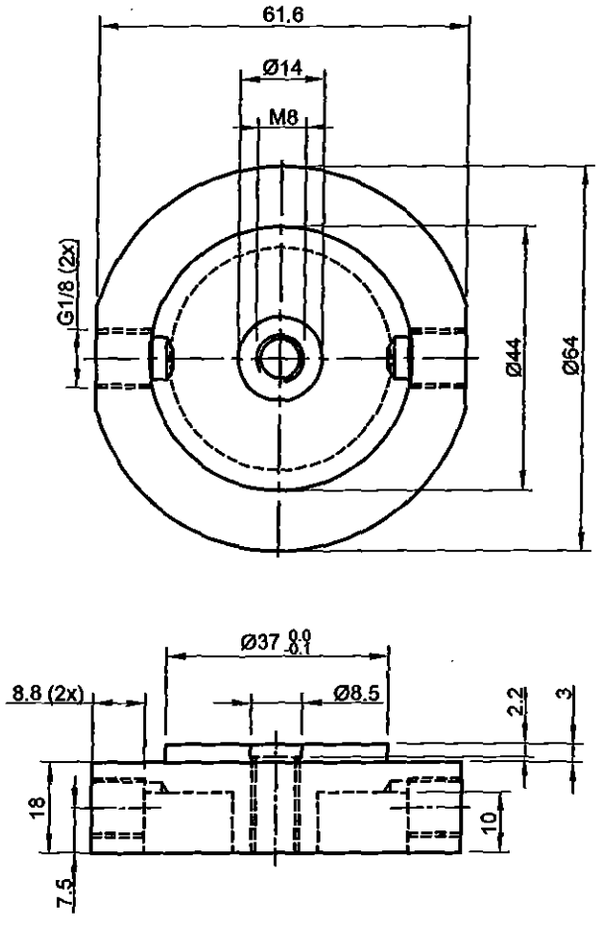


Figure 2.9.25.-9 – *Base chamber*
Dimensions in millimetres

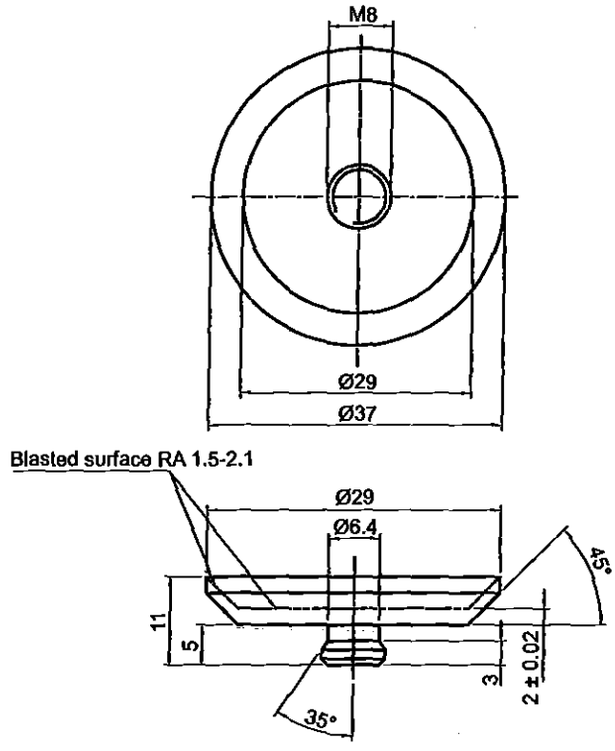


Figure 2.9.25.-10 – *Lower chewing surface*
Dimensions in millimetres

of appropriate compaction properties of the powder to be tested is needed prior to performing the test.

The intrinsic dissolution rate is determined by exposing a constant area of the compacted substance to an appropriate dissolution medium, while maintaining constant stirring rate, temperature, ionic strength and pH.

The intrinsic dissolution rate is expressed in terms of dissolved mass of substance per time per exposed area, typically in milligrams per minute per square centimetre ($mg \cdot min^{-1} \cdot cm^{-2}$).

APPARATUS

A typical apparatus consists of a punch and die fabricated out of hardened steel. The base of the die has 3 threaded holes for the attachment of a surface plate made of polished steel, providing a mirror-smooth base for the compact. The die has a 0.1-1.0 cm diameter cavity into which a measured amount of the powder to be tested is placed. The punch is then inserted in the die cavity and the material is compressed, generally using a benchtop hydraulic press. A hole through the head of the punch allows insertion of a metal rod to facilitate removal from the die after the test. A compact is formed in the cavity with a single face of defined area exposed on the bottom of the die (Figure 2.9.29.-1). The bottom of the die cavity is threaded so that at least 50-75 per cent of the compact can dissolve without falling out of the die. The top of the die has a threaded shoulder that allows it to be attached to a holder. The holder is mounted on a laboratory stirring device, and the entire die, with the compact still in place, is immersed in the dissolution medium and rotated by the stirring device.

PROCEDURE

Weigh the material onto a piece of weighing paper. Attach the surface plate to the underside of the die, and secure it with the 3 provided screws. Transfer the sample of powder tested into the die cavity. Place the punch into the chamber,

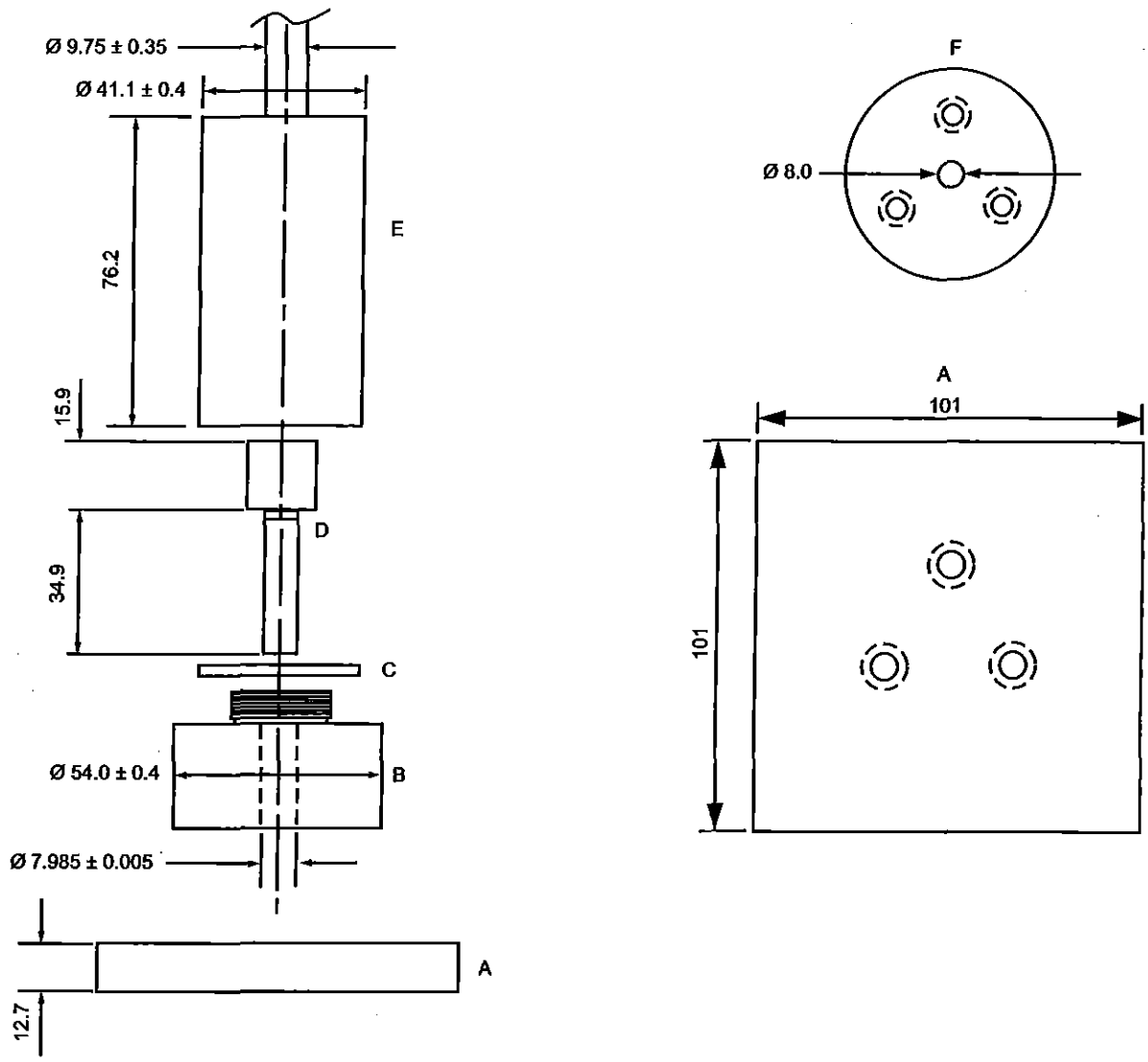


Figure 2.9.29.-1. – *Typical apparatus used to obtain the compact for the determination of the intrinsic dissolution*
Dimensions in millimetres

and secure the metal plate on the top of the assembly. Compress the powder using a hydraulic press by applying a suitable pressure for a sufficient dwell time to ensure a stable compact with minimal porosity; the disintegration of the compact has to be prevented as far as possible, since it would cause an increase in surface area and hence in dissolution rate. Detach the surface plate, and screw the die with punch still in place into the holder. Tighten securely. Remove all loose powder from the surface of the die by blowing compressed air or nitrogen across the surface of the compact.

Slide the die-holder assembly into the dissolution test chuck and tighten. Position the shaft in the spindle so that when the test head is lowered, the exposed surface of the compact will be 3.8 cm from the bottom of the vessel. The disc assembly is aligned to minimise wobble and air bubbles are not allowed to form as this could decrease the compact surface in contact with the dissolution medium. If possible, sink conditions are maintained throughout the test. However, in order to obtain detectable concentrations of solute, the use of a relatively small volume of medium may be necessary as a consequence of the limited surface available for dissolution.

Warm the dissolution medium to the temperature chosen for the test. Lower the test head into position before rotation. Care should be taken to ensure that air bubbles are excluded from the surface of the compact as this could decrease the compact surface in contact with the dissolution medium. Operate the apparatus immediately at the speed of rotation chosen for the test.

Collect samples at fixed time intervals and assay them by means of an analytical method of suitable sensitivity and accuracy.

ASSESSMENT OF THE RESULTS

The data for the cumulative amount dissolved at each time point are corrected for sampling losses. To calculate the intrinsic dissolution rate, plot the cumulative amount of sample dissolved per unit area of the compact against time. The cumulative amount dissolved per unit area is given by the cumulative amount dissolved at each time point divided by the surface area exposed. Linear regression is then performed on the normalised experimental data relevant to an appropriate time interval preceding the possible disintegration of the compact. The intrinsic dissolution rate of the substance tested, expressed in milligrams per minute per square centimetre, is determined from the slope of the regression line. The result for intrinsic dissolution rate must be accompanied by a statement of the precise conditions of compact preparation and test method (dissolution medium, volume of medium used, stirring rate, temperature etc.).

NOTE When necessary and justified, an apparatus with a different configuration may be used, such as a die holder that holds the compact in a fixed vertical position, with agitation provided by a paddle positioned at a defined distance from the surface of the compact.

6. Apparent Dissolution

(Ph. Eur. method 2.9.43)

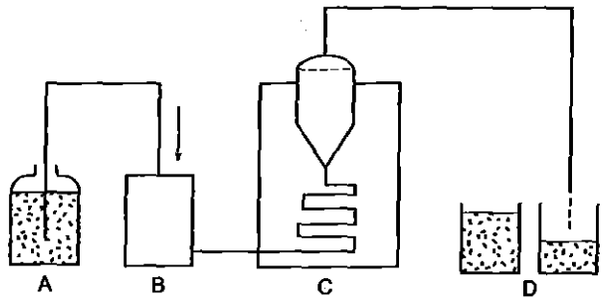
This method is mainly used to determine the apparent dissolution rate of pure solid substances. It may also be used for the determination of the apparent dissolution rate of active substances in preparations presented as powders or granules.

APPARATUS

All parts of the apparatus that may come into contact with the sample or the dissolution medium are chemically inert and do not adsorb, react with, or interfere with the test sample. No part of the assembly or its environment contributes significant motion, agitation or vibration beyond that resulting from the flow-through system.

Apparatus that permits observation of the sample is preferable.

The apparatus (see Figure 2.9.43.-1) consists of:
- a reservoir for the dissolution medium;
- a pump that forces the dissolution medium upwards through the flow-through cell;
- a flow-through cell, preferably of transparent material, mounted vertically with a filter system preventing escape of undissolved particles;
- a water-bath that will maintain the dissolution medium at the chosen temperature (generally 37 ± 0.5 °C).



A. reservoir for dissolution medium | B. pump | C. thermostatically controlled flow-through cell and filter | D. collecting vessels for analysis

Figure 2.9.43.-1. – *Flow-through apparatus*

The flow-through cell shown in Figure 2.9.43.-2 consists of 3 parts that fit into each other. The lower part supports a system of grids and filters on which the powder is placed. The middle part, which fits onto the lower part, contains an insert that sieves the sample when the dissolution medium flows through the cell. This insert is made up of 2 parts: a conical sieve that is placed on the sample and a clip placed midway down the middle part to hold the sieve in place when the dissolution medium passes through. A 2nd filtration assembly (grid and filter) is placed on top of the middle part before fitting the upper part through which the dissolution medium flows out of the cell.

DISSOLUTION MEDIUM

If the dissolution medium is buffered, adjust its pH to within ± 0.05 units. Remove any dissolved gases from the dissolution medium before the test, since they can cause the formation of bubbles, which significantly affect the results.

METHOD

Place a bead of 5 ± 0.5 mm diameter at the bottom of the cone of the lower part followed by glass beads of suitable size, preferably of 1 ± 0.1 mm diameter. Place a sieve (with 0.2 mm apertures), a suitable filter and a 2nd sieve on top of the lower part. Fit the middle part onto the lower part. Weigh the assembly. Place the sample on the filtration assembly and weigh the sample in the cell. Place the sieve of the insert, cone upwards, on the sample, and position the clip midway down the middle part. Place a sieve (with 0.2 mm apertures) and a suitable filter on top of the middle part. Fit the upper part. Heat the dissolution medium to the chosen temperature. Using a suitable pump, introduce the dissolution medium through the bottom of the cell to obtain a suitable continuous flow through an open or closed circuit at the prescribed rate ± 5 per cent.

SAMPLING

Samples of dissolution medium are collected at the outlet of the cell, irrespective of whether the circuit is opened or closed.

Immediately filter the liquid removed using an inert filter of appropriate pore size that does not cause significant adsorption of the substances from the solution and does not contain substances extractable by the dissolution medium that would interfere with the prescribed analytical method. Proceed with the analysis of the filtrate as prescribed.

ASSESSMENT OF THE RESULTS

When the test is performed for batch release purposes, an adequate number of replicates is carried out.

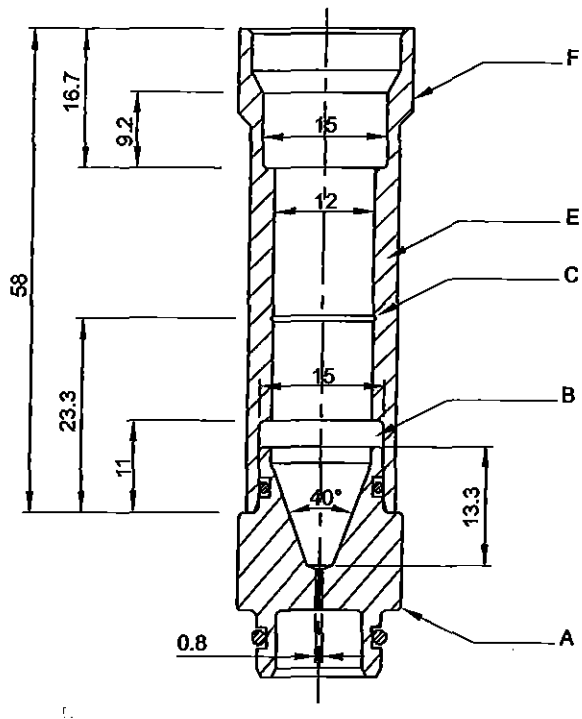
The results are expressed as:
- the amount of dissolved substance by time unit (if the dissolution is linear);
- the dissolution time of the whole sample and at appropriate intermediate stages.

Monographs of the British Pharmacopoeia

The following additional points apply to monographs of the British Pharmacopoeia.

APPARATUS

The choice of the apparatus to be used depends on the physico-chemical characteristics of the dosage form. When this Appendix is invoked in an individual tablet or capsule monograph of the British Pharmacopoeia, use Apparatus I unless otherwise directed.



A. lower part C. clip E. middle part
B. sieve D. insert F. upper part

Figure 2.9.43.-2. – *Flow-through cell*
Dimensions in millimetres

PROCEDURE

The dissolution medium is that specified in the individual monograph. Unless otherwise indicated in the monograph, withdraw samples at 45 minutes.

Where one tablet or capsule is directed to be placed in the apparatus, for each of the six tablets or capsules tested the amount of active ingredient in solution is not less than 70% of the prescribed or stated amount, unless otherwise specified in the monograph, except that if one fails this requirement a further six may be tested individually and all must comply. Where two or more tablets or capsules are directed to be placed together in the apparatus, a total of six replicate tests are carried out. In each test the amount of active ingredient in solution per tablet or capsule is not less than 70% of the prescribed or stated amount, unless otherwise specified in the monograph. No retesting is permitted.

Where capsule shells interfere with the analysis, remove the contents of no fewer than six capsules as completely as possible and dissolve the empty capsule shells in the specified volume of dissolution medium. Carry out the test as directed in the individual monograph and make any necessary correction. Correction factors should not be greater than 25% of the labelled content.

C. Consistency of Formulated Preparations

1. Uniformity of Weight (Mass)

(Ph. Eur. method 2.9.5)

Weigh individually 20 units taken at random or, for single-dose preparations presented in individual containers, the contents of 20 units, and determine the average mass.

Not more than 2 of the individual masses deviate from the average mass by more than the percentage deviation shown in Table 2.9.5.-1 and none deviates by more than twice that percentage.

For capsules and powders for parenteral administration, proceed as described below.

CAPSULES

Weigh an intact capsule. Open the capsule without losing any part of the shell and remove the contents as completely as possible. For soft shell capsules, wash the shell with a suitable solvent and allow to stand until the odour of the solvent is no longer perceptible. Weigh the shell. The mass of the contents is the difference between the weighings. Repeat the procedure with another 19 capsules.

Table 2.9.5.-1

Pharmaceutical Form	Average Mass	Percentage deviation
Tablets (uncoated and film-coated)	80 mg or less	10
	More than 80 mg and less than 250 mg	7.5
	250 mg or more	5
Capsules, granules (uncoated, single-dose) and powders (single-dose)	Less than 300 mg	10
	300 mg or more	7.5
Powders for parenteral administration* (single-dose)	More than 40 mg	10
Suppositories and pessaries	All masses	5
Powders for eye-drops and powders for eye lotions (single-dose)	Less than 300 mg	10
	300 mg or more	7.5

* When the average mass is equal to or below 40 mg, the preparation is not submitted to the test for uniformity of mass but to the test for uniformity of content of single-dose preparations (2.9.6).

POWDERS FOR PARENTERAL ADMINISTRATION

Remove any paper labels from a container and wash and dry the outside. Open the container and without delay weigh the container and its contents. Empty the container as completely as possible by gentle tapping, rinse it if necessary with *water R* and then with *alcohol R* and dry at 100-105 °C for 1 h, or, if the nature of the container precludes heating at this temperature, dry at a lower temperature to constant mass. Allow to cool in a desiccator and weigh. The mass of the contents is the difference between the weighings. Repeat the procedure with another 19 containers.

2. Uniformity of Weight (Mass) of Delivered Doses from Multidose Containers

(Ph. Eur. method 2.9.27)

The following test is intended for oral dosage forms such as granules, powders for oral use and liquids for oral use, which are supplied in multidose containers provided at manufacture with a measuring device.

Weigh individually 20 doses taken at random from one or more containers with the measuring device provided and determine the individual and average masses. Not more than 2 of the individual masses deviate from the average mass by more than 10 per cent and none deviates by more than 20 per cent.

3. Uniformity of Content

(Ph. Eur. method 2.9.6)

The test for uniformity of content of single-dose preparations is based on the assay of the individual contents of active substance(s) of a number of single-dose units to determine

whether the individual contents are within limits set with reference to the average content of the sample.

The test is not required for multivitamin and trace-element preparations and in other justified and authorised circumstances.

Method Using a suitable analytical method, determine the individual contents of active substance(s) of 10 dosage units taken at random.

Apply the criteria of test A, test B or test C as specified in the monograph for the dosage form in question.

TEST A

The preparation complies with the test if each individual content is between 85 per cent and 115 per cent of the average content. The preparation fails to comply with the test if more than one individual content is outside these limits or if one individual content is outside the limits of 75 per cent to 125 per cent of the average content.

If one individual content is outside the limits of 85 per cent to 115 per cent but within the limits of 75 per cent to 125 per cent, determine the individual contents of another 20 dosage units taken at random. The preparation complies with the test if not more than one of the individual contents of the 30 units is outside 85 per cent to 115 per cent of the average content and none is outside the limits of 75 per cent to 125 per cent of the average content.

TEST B

The preparation complies with the test if not more than one individual content is outside the limits of 85 per cent to 115 per cent of the average content and none is outside the limits of 75 per cent to 125 per cent of the average content. The preparation fails to comply with the test if more than 3 individual contents are outside the limits of 85 per cent to 115 per cent of the average content or if one or more individual contents are outside the limits of 75 per cent to 125 per cent of the average content.

If 2 or 3 individual contents are outside the limits of 85 per cent to 115 per cent but within the limits of 75 per cent to 125 per cent, determine the individual contents of another 20 dosage units taken at random. The preparation complies with the test if not more than 3 individual contents of the 30 units are outside the limits of 85 per cent to 115 per cent of the average content and none is outside the limits of 75 per cent to 125 per cent of the average content.

TEST C

The preparation complies with the test if the average content of the 10 dosage units is between 90 per cent and 110 per cent of the content stated on the label and if the individual content of each dosage unit is between 75 per cent and 125 per cent of the average content.

4. Uniformity of Dosage Units[1]

(Ph. Eur. method 2.9.40)

To ensure the consistency of dosage units, each unit in a batch should have an active substance content within a narrow range around the label claim. Dosage units are defined as dosage forms containing a single dose or a part of a dose of an active substance in each dosage unit. ◊Unless otherwise stated,◊ the uniformity of dosage units specification is not intended to apply to solutions, suspensions, emulsions or gels in single-dose containers intended for local action following cutaneous administration. ◊The test for content uniformity is not required for multivitamin, single-vitamin and trace-element preparations.◊

The term 'uniformity of dosage unit' is defined as the degree of uniformity in the amount of the active substance among dosage units. Therefore, the requirements of this chapter apply to each active substance being comprised in dosage units containing 1 or more active substances, unless otherwise specified elsewere in this Pharmacopoeia.

The uniformity of dosage units can be demonstrated by either of 2 methods: content uniformity or mass variation (see Table 2.9.40.-1).

The test for content uniformity of preparations presented in dosage units is based on the assay of the individual contents of active substance(s) of a number of dosage units to determine whether the individual contents are within the limits set. The content uniformity method may be applied in all cases.

The test for mass variation is applicable for the following dosage forms:

(1) solutions enclosed in single-dose containers and in soft capsules;

(2) solids (including powders, granules and sterile solids) that are packaged in single-dose containers and contain no added active or inactive substances;

(3) solids (including sterile solids) that are packaged in single-dose containers, with or without added active or inactive substances, that have been prepared from true solutions and freeze-dried in the final containers and are labelled to indicate this method of preparation;

(4) hard capsules, uncoated tablets, or film-coated tablets, containing 25 mg or more of an active substance comprising 25 per cent or more, by mass, of the dosage unit or, in the case of hard capsules, the capsule contents, except that uniformity of other active substances present in lesser proportions is demonstrated by meeting content uniformity requirements.

The test for content uniformity is required for all dosage forms not meeting the above conditions for the mass variation test. ♦Alternatively, products that do not meet the 25 mg/25 per cent threshold limit may be tested for uniformity of dosage units by mass variation instead of the content uniformity test on the following condition: the concentration Relative Standard Deviation (RSD) of the active substance in the final dosage units is not more than 2 per cent, based on process validation data and development data, and if there has been regulatory approval of such a change. The concentration RSD is the RSD of the concentration per dosage unit (*m/m* or *m/V*), where concentration per dosage unit equals the assay result per dosage unit divided by the individual dosage unit mass. See the RSD formula in Table 2.9.40.-2.♦

CONTENT UNIFORMITY

Select not fewer than 30 units, and proceed as follows for the dosage form designated. Where different procedures are used for assay of the preparation and for the content uniformity test, it may be necessary to establish a correction factor to be applied to the results of the latter.

Solid dosage forms

Assay 10 units individually using an appropriate analytical method. Calculate the acceptance value (see Table 2.9.40.-2).

[1] *This chapter has undergone pharmacopoeial harmonisation. See chapter 5.8 Pharmacopoeial harmonisation.*

Table 2.9.40.-1. – *Application of Content Uniformity (CU) and Mass Variation (MV) test for dosage forms*

Dosage forms	Type	Sub-Type	Dose and ratio of active substance	
			≥ 25 mg and ≥ 25 per cent	< 25 mg or < 25 per cent
Tablets	uncoated		MV	CU
	coated	film-coated	MV	CU
		others	CU	CU
Capsules	hard		MV	CU
	soft	suspensions, emulsions, gels	CU	CU
		solutions	MV	MV
Solids in single-dose containers	single component		MV	MV
	multiple components	solution freeze-dried in final container	MV	MV
		others	CU	CU
Solutions enclosed in single-dose containers			MV	MV
Others: dosage forms not addressed by the other categories in this table including but not limited to suppositories, transdermal patches and semi-solid preparations applied cutaneously and intended for systemic distribution of the active substance			CU	CU

Liquid or semi-solid dosage forms
Assay 10 units individually using an appropriate analytical method. Carry out the assay on the amount of well-mixed material that is removed from an individual container in conditions of normal use. Express the results as delivered dose. Calculate the acceptance value (see Table 2.9.40.-2).

Calculation of Acceptance Value
Calculate the Acceptance Value (*AV*) using the formula:

$$|M - \overline{X}| + ks$$

for which the terms are as defined in Table 2.9.40.-2.

MASS VARIATION
Carry out an assay for the active substance(s) on a representative sample of the batch using an appropriate analytical method. This value is result *A*, expressed as percentage of label claim (see Calculation of Acceptance Value). Assume that the concentration (mass of active substance per mass of dosage unit) is uniform. Select not fewer than 30 dosage units, and proceed as follows for the dosage form designated.

Uncoated or film-coated tablets
Accurately weigh 10 tablets individually. Calculate the active substance content, expressed as percentage of label claim, of each tablet from the mass of the individual tablets and the result of the assay. Calculate the acceptance value.

Hard capsules
Accurately weigh 10 capsules individually, taking care to preserve the identity of each capsule. Remove the contents of each capsule by suitable means. Accurately weigh the emptied shells individually, and calculate for each capsule the net mass of its contents by subtracting the mass of the shell from the respective gross mass. Calculate the active substance content in each capsule from the mass of product removed from the individual capsules and the result of the assay. Calculate the acceptance value.

Soft capsules
Accurately weigh 10 intact capsules individually to obtain their gross masses, taking care to preserve the identity of each capsule. Then cut open the capsules by means of a suitable clean, dry cutting instrument such as scissors or a sharp open blade, and remove the contents by washing with a suitable solvent. Allow the occluded solvent to evaporate from the shells at room temperature over a period of about 30 min, taking precautions to avoid uptake or loss of moisture. Weigh the individual shells, and calculate the net contents. Calculate the active substance content in each capsule from the mass of product removed from the individual capsules and the result of the assay. Calculate the acceptance value.

Solid dosage forms other than tablets and capsules
Proceed as directed for hard capsules, treating each unit as described therein. Calculate the acceptance value.

Liquid ◊or semi-solid◊ dosage forms
Accurately weigh the amount of liquid or semi-solid that is removed from each of 10 individual containers in conditions of normal use. If necessary, compute the equivalent volume after determining the density. Calculate the active substance content in each container from the mass of product removed from the individual containers and the result of the assay. Calculate the acceptance value.

Calculation of Acceptance Value
Calculate the acceptance value (*AV*) as shown in content uniformity, except that the individual contents of the units

Table 2.9.40.-2.

Variable	Definition	Conditions	Value
$\overline{X}$	Mean of individual contents (x_1, x_2,..., x_n), expressed as a percentage of the label claim		
$x_1, x_2,..., x_n$	Individual contents of the dosage units tested, expressed as a percentage of the label claim		
n	Sample size (number of dosage units in a sample)		
k	Acceptability constant	If n = 10, then	2.4
		If n = 30, then	2.0
s	Sample standard deviation		$\left[\frac{\sum_{i=1}^{n}(x_i-\overline{X})^2}{n-1}\right]^{1/2}$
RSD	Relative standard deviation		$\frac{100s}{\overline{X}}$
M (case 1) To be applied when $T \leq$ 101.5	Reference value	If 98.5 per cent $\leq \overline{X} \leq$ 101.5 per cent, then	$M = \overline{X}$ ($AV = ks$)
		If $\overline{X}$ < 98.5 per cent, then	M = 98.5 per cent (AV = 98.5 $-\overline{X} + ks$)
		If $\overline{X}$ > 101.5 per cent, then	M = 101.5 per cent ($AV = \overline{X} - 101.5 + ks$)
M (case 2) To be applied when $T >$ 101.5	Reference value	If 98.5 per cent $\leq \overline{X} \leq T$, then	$M = \overline{X}$ ($AV = ks$)
		If $\overline{X}$ < 98.5 per cent, then	M = 98.5 per cent (AV = 98.5 $-\overline{X} + ks$)
		If $\overline{X} > T$, then	$M = T$ per cent ($AV = \overline{X} - T + ks$)
Acceptance value (AV)			General formula: $\lvert M - \overline{X} \rvert + ks$ Calculations are specified above for the different cases.
$L1$	Maximum allowed acceptance value		$L1$ = 15.0 unless otherwise specified
$L2$	Maximum allowed range for deviation of each dosage unit tested from the calculated value of M	On the low side, no dosage unit result can be less than 0.75 M while on the high side, no dosage unit result can be greater than 1.25 M (This is based on $L2$ value of 25.0)	$L2$ = 25.0 unless otherwise specified
T	Target content per dosage unit at time of manufacture, expressed as a percentage of the label claim. Unless otherwise stated, T is equal to 100 per cent or T is the manufacturer's approved target content per dosage unit		

are replaced with the individual estimated contents defined below.

$x_1, x_2, \ldots, x_n$ = individual estimated contents of the dosage units tested;

where

$$x_i = w_i \times \frac{A}{\overline{W}}$$

$w_1, w_2, \ldots, w_n$ = individual masses of the dosage units tested;
A = content of active substance (percentage of label claim) obtained using an appropriate analytical method (assay);
$\overline{W}$ = mean of individual masses ($w_1, w_2, \ldots, w_n$).

CRITERIA

Apply the following criteria, unless otherwise specified.

Solid, semi-solid and liquid dosage forms

The requirements for dosage uniformity are met if the acceptance value of the first 10 dosage units is less than or equal to $L1$ per cent. If the acceptance value is greater than $L1$ per cent, test the next 20 dosage units and calculate the acceptance value. The requirements are met if the final acceptance value of the 30 dosage units is less than or equal to $L1$ per cent and no individual content of the dosage unit is less than $(1 - L2 \times 0.01)M$ or more than $(1 + L2 \times 0.01)M$ in calculation of acceptance value under content uniformity or under mass variation. Unless otherwise specified, $L1$ is 15.0 and $L2$ is 25.0.

5. Extractable Volume of Parenteral Preparations[2]

(Test for Extractable Volume of Parenteral Preparations, Ph. Eur. method 2.9.17)

Suspensions and emulsions are shaken before withdrawal of the contents and before the determination of the density. Oily and viscous preparations may be warmed according to the instructions on the label, if necessary, and thoroughly shaken immediately before removing the contents. The contents are then cooled to 20-25 °C before measuring the volume.

SINGLE-DOSE CONTAINERS

Select 1 container if the nominal volume is 10 mL or more, 3 containers if the nominal volume is more than 3 mL and less than 10 mL, or 5 containers if the nominal volume is 3 mL or less. Take up individually the total contents of each container selected into a dry syringe of a capacity not exceeding 3 times the volume to be measured, and fitted with a 21-gauge needle not less than 2.5 cm in length. Expel any air bubbles from the syringe and needle, then discharge the contents of the syringe without emptying the needle into a standardised dry cylinder (graduated to contain rather than to deliver the designated volumes) of such size that the volume to be measured occupies at least 40 per cent of its graduated volume. Alternatively, the volume of the contents in millilitres may be calculated as the mass in grams divided by the density.

For containers with a nominal volume of 2 mL or less, the contents of a sufficient number of containers may be pooled to obtain the volume required for the measurement provided that a separate, dry syringe assembly is used for each container. The contents of containers holding 10 mL or more may be determined by opening them and emptying the contents directly into the graduated cylinder or tared beaker.

The volume is not less than the nominal volume in case of containers examined individually, or, in case of containers with a nominal volume of 2 mL or less, is not less than the sum of the nominal volumes of the containers taken collectively.

MULTIDOSE CONTAINERS

For injections in multidose containers labelled to yield a specific number of doses of a stated volume, select one container and proceed as directed for single-dose containers using the same number of separate syringe assemblies as the number of doses specified.

The volume is such that each syringe delivers not less than the stated dose.

CARTRIDGES AND PREFILLED SYRINGES

Select 1 container if the nominal volume is 10 mL or more, 3 containers if the nominal volume is more than 3 mL and less than 10 mL, or 5 containers if the nominal volume is 3 mL or less. If necessary, fit the containers with the accessories required for their use (needle, piston, syringe) and transfer the entire contents of each container without emptying the needle into a dry tared beaker by slowly and constantly depressing the piston. Determine the volume in millilitres calculated as the mass in grams divided by the density.

The volume measured for each of the containers is not less than the nominal volume.

PARENTERAL INFUSIONS

Select one container. Transfer the contents into a dry measuring cylinder of such a capacity that the volume to be determined occupies at least 40 per cent of the nominal volume of the cylinder. Measure the volume transferred.

The volume is not less than the nominal volume.

7. Preparations for Inhalation: Aerodynamic Assessment of Fine Particles

(Ph. Eur. method 2.9.18)

This test is used to determine the fine particle characteristics of the aerosol clouds generated by preparations for inhalation.

Unless otherwise justified and authorised, one of the following apparatus and test procedures is used.

Stage mensuration Is performed periodically together with confirmation of other dimensions critical to the effective operation of the impactor.

Re-entrainment (for apparatus D and E) To ensure efficient particle capture, coat each plate with glycerol, silicone oil or similar high viscosity liquid, typically deposited from a volatile solvent. Plate coating must be part of method validation and may be omitted where justified and authorised.

Mass balance The total mass of the active substance is not less than 75 per cent and not more than 125 per cent of the average delivered dose determined during testing for uniformity of delivered dose. This is not a test of the inhaler but it serves to ensure that the results are valid.

APPARATUS A - GLASS IMPINGER

The apparatus is shown in Figure 2.9.18.-1 (see also Table 2.9.18.-1).

Procedure for nebulisers

Introduce 7 mL and 30 mL of a suitable solvent into the upper and lower impingement chambers, respectively.

[2] *This chapter has undergone pharmacopoeial harmonisation. See chapter 5.8 Pharmacopoeial harmonisation.*

Connect all the component parts. Ensure that the assembly is vertical and adequately supported and that the jet spacer peg of the lower jet assembly just touches the bottom of the lower impingement chamber. Connect a suitable pump fitted with a filter (of suitable pore size) to the outlet of the apparatus. Adjust the air flow through the apparatus, as measured at the inlet to the throat, to 60 ± 5 L/min.

Introduce the liquid preparation for inhalation into the reservoir of the nebuliser. Fit the mouthpiece and connect it by means of an adapter to the device.

Switch on the pump of the apparatus and after 10 s switch on the nebuliser.

After 60 s, unless otherwise justified, switch off the nebuliser, wait for about 5 s and then switch off the pump of the apparatus. Dismantle the apparatus and wash the inner surface of the upper impingement chamber collecting the washings in a volumetric flask. Wash the inner surface of the lower impingement chamber collecting the washings in a second volumetric flask. Finally, wash the filter preceding the pump and its connections to the lower impingement chamber and combine the washings with those obtained from the lower impingement chamber. Determine the amount of active substance collected in each of the 2 flasks. Express the results for each of the 2 parts of the apparatus as a percentage of the total amount of active substance.

Procedure for pressurised inhalers

Place the actuator adapter in position at the end of the throat so that the mouthpiece end of the actuator, when inserted to a depth of about 10 mm, lines up along the horizontal axis of the throat and the open end of the actuator, which accepts the pressurised container, is uppermost and in the same vertical plane as the rest of the apparatus.

Introduce 7 mL and 30 mL of a suitable solvent into the upper and lower impingement chambers, respectively.

Connect all the component parts. Ensure that the assembly is vertical and adequately supported and that the lower jet-spacer peg of the lower jet assembly just touches the bottom of the lower impingement chamber. Connect a suitable pump to the outlet of the apparatus. Adjust the air flow through the apparatus, as measured at the inlet to the throat, to 60 ± 5 L/min.

Prime the metering valve by shaking for 5 s and discharging once to waste; after not less than 5 s, shake and discharge again to waste. Repeat a further 3 times.

Shake for about 5 s, switch on the pump to the apparatus and locate the mouthpiece end of the actuator in the adapter, discharge once immediately. Remove the assembled inhaler from the adapter, shake for not less than 5 s, relocate the mouthpiece end of the actuator in the adapter and discharge again. Repeat the discharge sequence. The number of discharges should be minimised and typically would not be greater than 10. After the final discharge wait for not less than 5 s and then switch off the pump. Dismantle the apparatus.

Wash the inner surface of the inlet tube to the lower impingement chamber and its outer surface that projects into the chamber with a suitable solvent, collecting the washings in the lower impingement chamber. Determine the content of active substance in this solution. Calculate the amount of active substance collected in the lower impingement chamber per discharge and express the results as a percentage of the dose stated on the label.

Procedure for powder inhalers

Introduce 7 mL and 30 mL of a suitable solvent into the upper and lower impingement chambers, respectively.

Connect all the component parts. Ensure that the assembly is vertical and adequately supported and that the jet-spacer peg of the lower jet assembly just touches the bottom of the lower impingement chamber. Without the inhaler in place, connect a suitable pump to the outlet of the apparatus. Adjust the air flow through the apparatus, as measured at the inlet to the throat, to 60 ± 5 L/min.

Prepare the inhaler for use and locate the mouthpiece in the apparatus by means of a suitable adapter. Switch on the pump for 5 s. Switch off the pump and remove the inhaler. Repeat the discharge sequence. The number of discharges should be minimised and typically would not be greater than 10. Dismantle the apparatus.

Wash the inner surface of the inlet tube to the lower impingement chamber and its outer surface that projects into the chamber with a suitable solvent, collecting the washings in the lower impingement chamber. Determine the content of active substance in this solution. Calculate the amount of active substance collected in the lower impingement chamber per discharge and express the results as a percentage of the dose stated on the label.

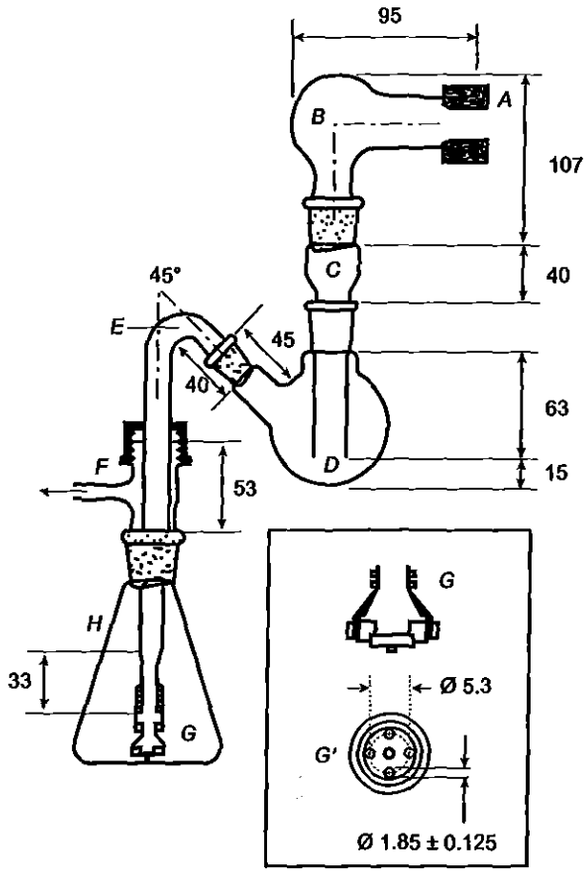


Figure 2.9.18.-1. – *Apparatus A: glass impinger*

Dimensions in millimetres (tolerances ± 1 mm unless otherwise prescribed)

Table 2.9.18.-1. – *Component specification for apparatus A in Figure 2.9.18.-1*

Code	Item	Description	Dimensions*
A	Mouthpiece adaptor	Moulded rubber adapter for actuator mouthpiece.	
B	Throat	Modified round-bottomed flask:	50 mL
		— *ground-glass inlet socket*	29/32
		— *ground-glass outlet cone*	24/29
C	Neck	Modified glass adapter:	
		— *ground-glass inlet socket*	24/29
		— *ground-glass outlet cone*	24/29
		Lower outlet section of precision-bore glass tubing:	
		— *bore diameter*	14
		Selected bore light-wall glass tubing:	
		— *external diameter*	17
D	Upper impingement chamber	Modified round-bottomed flask	100 mL
		— *ground-glass inlet socket*	24/29
		— *ground-glass outlet cone*	24/29
E	Coupling tube	Medium-wall glass tubing:	
		— *ground-glass cone*	14/23
		Bent section and upper vertical section:	
		— *external diameter*	13
		Lower vertical section:	
		— *external diameter*	8
F	Screwthread, side-arm adaptor	Plastic screw cap	28/13
		Silicone rubber ring	28/11
		PTFE washer	28/11
		Glass screwthread:	
		— *thread size*	28
		Side-arm outlet to vacuum pump:	
		— *minimum bore diameter*	5
G	Lower jet assembly	Modified polypropylene filter holder connected to lower vertical section of coupling tube by PTFE tubing.	see Figure 2.9.18.-1
		Acetal circular disc with the centres of four jets arranged on a projected circle of diameter 5.3 mm with an integral jet spacer peg:	10
		— *peg diameter*	2
		— *peg protrusion*	2
H	Lower impingement chamber	Conical flask	250 mL
		— *ground-glass inlet socket*	24/29

* Dimensions in millimetres, unless otherwise stated.

FINE PARTICLE DOSE AND PARTICLE SIZE DISTRIBUTION

APPARATUS C - MULTI-STAGE LIQUID IMPINGER

The multi-stage liquid impinger consists of impaction stages 1 (pre-separator), 2, 3 and 4 and an integral filter stage (stage 5), see Figures 2.9.18.-4/6. An impaction stage comprises an upper horizontal metal partition wall (B)

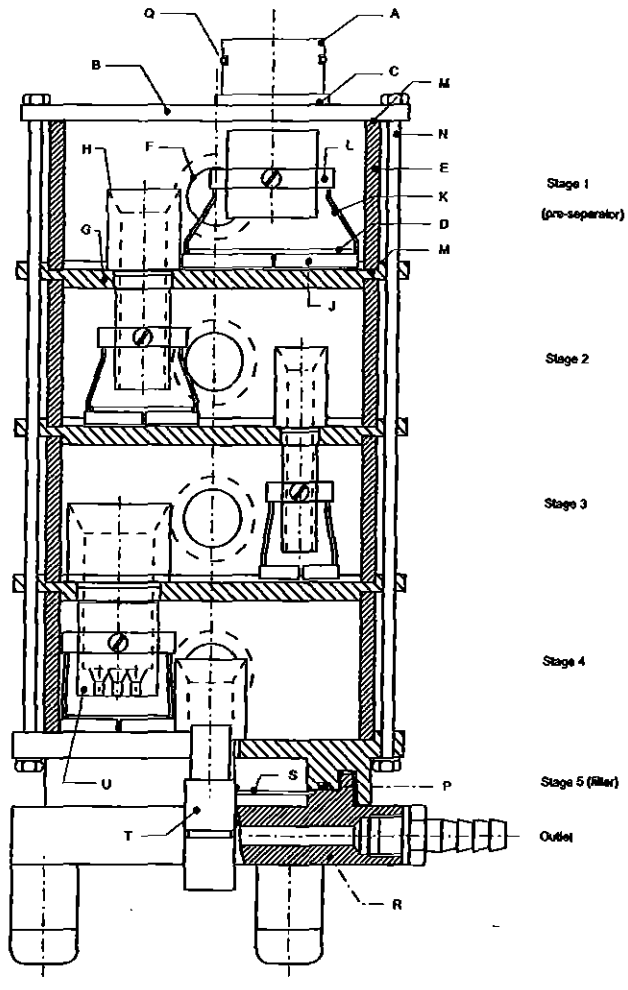


Figure 2.9.18.-4. – *Apparatus C: multi-stage liquid impinger*

through which a metal inlet jet tube (A) with its impaction plate (D) is protruding. A glass cylinder (E) with sampling port (F) forms the vertical wall of the stage, and a lower horizontal metal partition wall (G) through which the tube (H) connects to the next lower stage. The tube into stage 4 (U) ends in a multi-jet arrangement. The impaction plate (D) is secured in a metal frame (J) which is fastened by 2 wires (K) to a sleeve (L) secured on the jet tube.
The horizontal face of the collection plate is perpendicular to the axis of the jet tube and centrally aligned. The upper surface of the impaction plate is slightly raised above the edge of the metal frame. A recess around the perimeter of the horizontal partition wall guides the position of the glass cylinder. The glass cylinders are sealed against the horizontal partition walls with gaskets (M) and clamped together by 6 bolts (N). The sampling ports are sealed by stoppers.
The bottom-side of the lower partition wall of stage 4 has a concentrical protrusion fitted with a rubber O-ring (P) which seals against the edge of a filter placed in the filter holder. The filter holder (R) is constructed as a basin with a concentrical recess in which a perforated filter support (S) is flush-fitted. The filter holder is dimensioned for 76 mm diameter filters. The assembly of impaction stages is clamped onto the filter holder by 2 snap-locks (T). Connect an induction port (see Figure 2.9.18.-7) onto the stage 1 inlet jet tube of the impinger. A rubber O-ring on the jet tube provides an airtight connection to the induction port.
A suitable mouthpiece adapter is used to provide an airtight seal between the inhaler and the induction port. The front

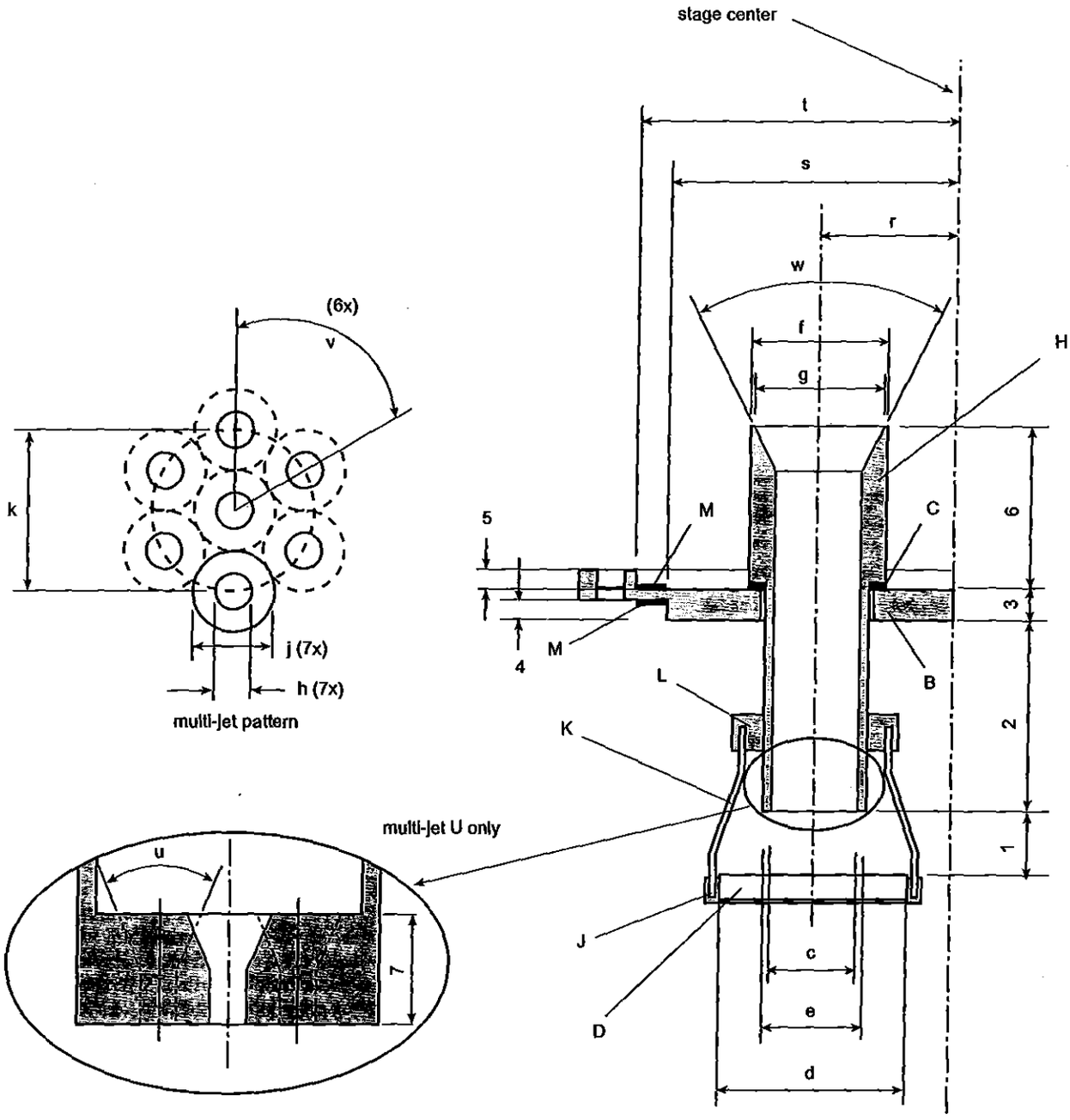


Figure 2.9.18.-5. – *Apparatus C: details of jet tube and impaction plate. Inserts show end of multi-jet tube U leading to stage 4. (Numbers and lowercase letters refer to Table 2.9.18.-3 and uppercase letters refer to Figure 2.9.18.-4).*

face of the inhaler mouthpiece must be flush with the front face of the induction port.

Procedure for pressurised inhalers

Dispense 20 mL of a solvent, capable of dissolving the active substance into each of stages 1 to 4 and replace the stoppers. Tilt the apparatus to wet the stoppers, thereby neutralising electrostatic charge. Place a suitable filter capable of quantitatively collecting the active substance in stage 5 and assemble the apparatus. Place a suitable mouthpiece adapter in position at the end of the induction port so that the mouthpiece end of the actuator, when inserted, lines up along the horizontal axis of the induction port and the inhaler is positioned in the same orientation as intended for use. Connect a suitable vacuum pump to the outlet of the apparatus and adjust the air flow through the apparatus, as measured at the inlet to the induction port, to 30 L/min (± 5 per cent). Switch off the pump.

Unless otherwise prescribed in the patient instructions, shake the inhaler for 5 s and discharge 1 delivery to waste. Switch on the pump to the apparatus, locate the mouthpiece end of the actuator in the adapter and discharge the inhaler into the apparatus, depressing the valve for a sufficient time to ensure complete discharge. Wait for 5 s before removing the assembled inhaler from the adapter. Repeat the procedure. The number of discharges should be minimised and typically would not be greater than 10. The number of discharges is sufficient to ensure an accurate and precise determination of the fine particle dose. After the final discharge, wait for 5 s and then switch off the pump.

Table 2.9.18.-2. – *Component specification for apparatus C in Figures 2.9.18.-4/6*

Code*	Item	Description	Dimensions**
A,H	Jet tube	Metal tube screwed onto partition wall sealed by gasket (C), polished inner surface	see Figure 2.9.18.-5
B,G	Partition wall	Circular metal plate	
		— *diameter*	120
		— *thickness*	see Figure 2.9.18.-5
C	Gasket	e.g. PTFE	to fit jet tube
D	Impaction plate	Porosity 0 sintered-glass disk	
		— *diameter*	see Figure 2.9.18.-5
E	Glass cylinder	Plane polished cut glass tube	
		— *height, including gaskets*	46
		— *outer diameter*	100
		— *wall thickness*	3.5
		— *sampling port (F) diameter*	18
		— *stopper in sampling port*	ISO 24/25
J	Metal frame	L-profiled circular frame with slit	
		— *inner diameter*	to fit impaction plate
		— *height*	4
		— *thickness of horizontal section*	0.5
		— *thickness of vertical section*	2
K	Wire	Steel wire interconnecting metal frame and sleeve (2 for each frame)	
		— *diameter*	1
L	Sleeve	Metal sleeve secured on jet tube by screw	
		— *inner diameter*	to fit jet tube
		— *height*	6
		— *thickness*	5
M	Gasket	e.g. silicone	to fit glass cylinder
N	Bolt	Metal bolt with nut (6 pairs)	
		— *length*	205
		— *diameter*	4
P	O-ring	Rubber O-ring	
		— *diameter × thickness*	66.34 × 2.62
Q	O-ring	Rubber O-ring	
		— *diameter × thickness*	29.1 × 1.6
R	Filter holder	Metal housing with stand and outlet	see Figure 2.9.18.-6
S	Filter support	Perforated sheet metal	
		— *diameter*	65
		— *hole diameter*	3
		— *distance between holes (centre-points)*	4
T	Snap-locks		
U	Multi-jet tube	Jet tube (H) ending in multi-jet arrangement.	see inserts Figure 2.9.18.-5

* Refers to Figure 2.9.18.-4.
** Measures in millimetres with tolerances according to iso 2768-m unless otherwise stated.

Table 2.9.18.-3. – *Dimensions(1) of jet tube with impaction plate of apparatus C*

Type	Code(2)	Stage 1	Stage 2	Stage 3	Stage 4	Filter (stage 5)
Distance	1	9.5 (-.0+.5)	5.5 (-.0+.5)	4.0 (-.0+.5)	6.0 (-.0+.5)	n.a.
Distance	2	26	31	33	30.5	0
Distance	3	8	5	5	5	5
Distance	4	3	3	3	3	n.a.
Distance	5	0	3	3	3	3
Distance	6(3)	20	25	25	25	25
Distance	7	n.a.	n.a.	n.a.	8.5	n.a.
Diameter	c	25	14	8.0 (± .1)	21	14
Diameter	d	50	30	20	30	n.a.
Diameter	e	27.9	16.5	10.5	23.9	n.a.
Diameter	f	31.75 (-.0+.5)	22	14	31	22
Diameter	g	25.4	21	13	30	21
Diameter	h	n.a.	n.a.	n.a.	2.70 (± .5)	n.a.
Diameter	j	n.a.	n.a.	n.a.	6.3	n.a.
Diameter	k	n.a.	n.a.	n.a.	12.6	n.a.
Radius(4)	r	16	22	27	28.5	0
Radius	s	46	46	46	46	n.a.
Radius	t	n.a.	50	50	50	50
Angle	w	10°	53°	53°	53°	53°
Angle	u	n.a.	n.a.	n.a.	45°	n.a.
Angle	v	n.a.	n.a.	n.a.	60°	n.a.

(1) Measures in millimetres with tolerances according to ISO 2768-m unless otherwise stated
(2) Refer to Figure 2.9.18.-5
(3) Including gasket
(4) Relative centreline of stage compartment
n.a. = not applicable

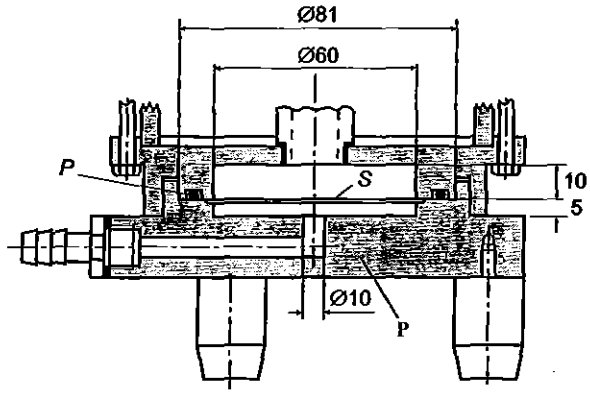
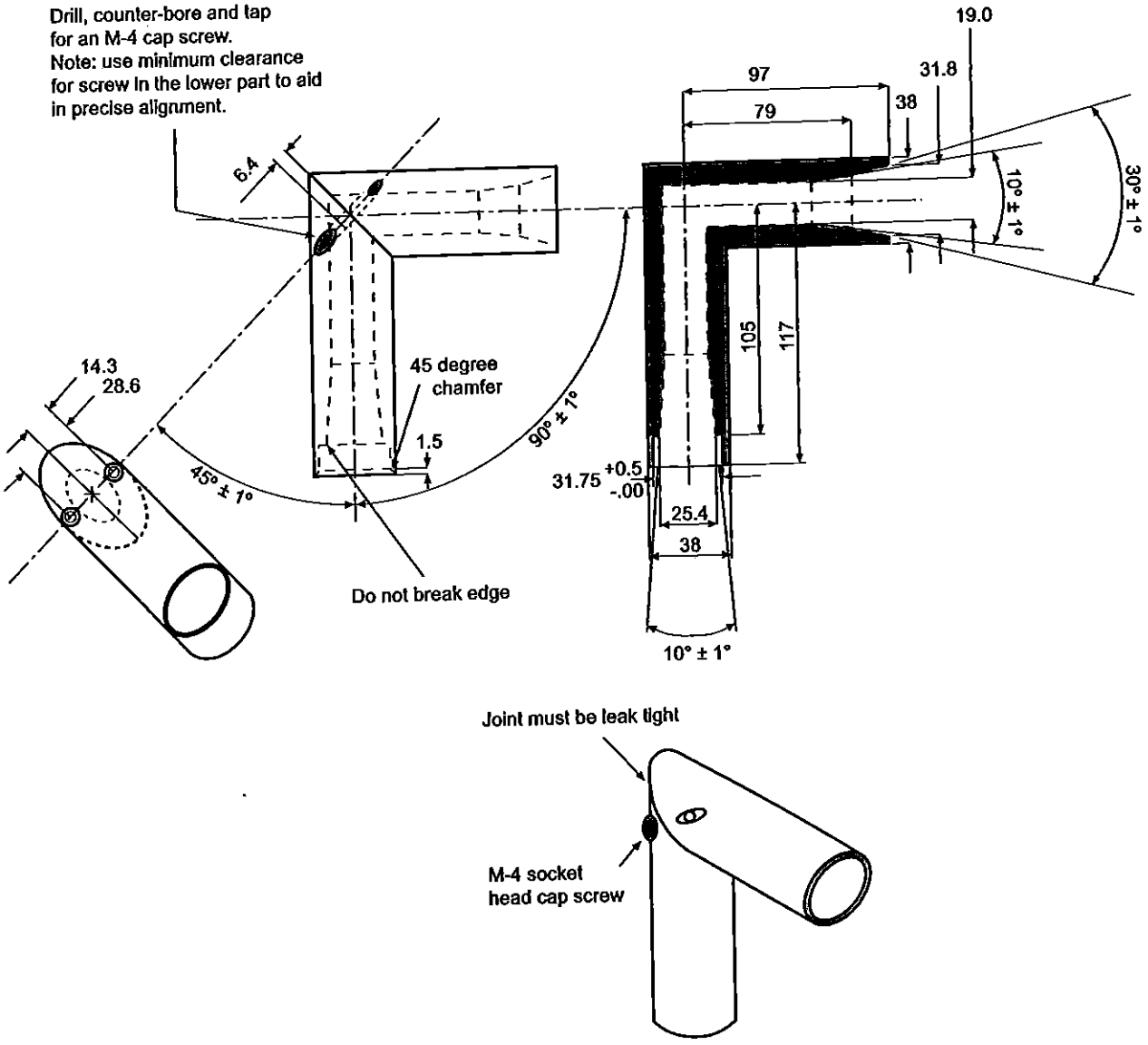


Figure 2.9.18.-6. – *Apparatus C: details of the filter stage (stage 5). Numbers refer to dimensions (Ø = diameter). Uppercase letters refer to Table 2.9.18.-2. Dimensions in millimetres unless otherwise stated*

Dismantle the filter stage of the apparatus. Carefully remove the filter and extract the active substance into an aliquot of the solvent. Remove the induction port and mouthpiece adapter from the apparatus and extract the active substance into an aliquot of the solvent. If necessary, rinse the inside of the inlet jet tube to stage 1 with solvent, allowing the solvent to flow into the stage. Extract the active substance from the inner walls and the collection plate of each of the 4 upper stages of the apparatus into the solution in the respective



Isometric view of induction port

1. Material may be aluminium, stainless steel or other suitable material.
2. Machine from 38 mm bar stock.
3. Bore 19 mm hole through bar.
4. Cut tube to exact 45° as shown.
5. The inner bores and tapers should be smooth – surface roughness Ra approx. 0.4 µm.
6. Mill joining ends of stock to provide a liquid tight leak-free seal.
7. Set up a holding fixture for aligning the inner 19 mm bore and for drilling and tapping M4 × 0.7 threads. There must be virtually no mismatch of the inner bores in the miter joint.

Figure 2.9.18.-7. – *Induction port*
Dimensions in millimetres unless otherwise stated

stage by carefully tilting and rotating the apparatus, observing that no liquid transfer occurs between the stages.

Using a suitable method of analysis, determine the quantity of active substance contained in each of the aliquots of solvent.

Calculate the fine particle dose (see Calculations).

Procedure for powder inhalers

Place a suitable low resistance filter capable of quantitatively collecting the active substance in stage 5 and assemble the apparatus. Connect the apparatus to a flow system according to the scheme specified in Figure 2.9.18.-8 and Table 2.9.18.-4. Unless otherwise defined, conduct the test at the flow rate, Q_{out}, used in the test for uniformity of delivered dose, drawing 4 L of air from the mouthpiece of the inhaler and through the apparatus.

Connect a flowmeter to the induction port. Use a flowmeter calibrated for the volumetric flow leaving the meter, or calculate the volumetric flow leaving the meter (Q_{out}) using the ideal gas law. For a meter calibrated for the entering

volumetric flow (Q_{in}), use the following expression:

$$Q_{out} = \frac{Q_{in} \times P_0}{P_0 - \Delta P}$$

P_0 = atmospheric pressure,
ΔP = pressure drop over the meter.

Adjust the flow control valve to achieve steady flow through the system at the required rate, Q_{out} (± 5 per cent). Switch off the pump. Ensure that critical flow occurs in the flow control valve by the following procedure.

With the inhaler in place and the test flow rate established, measure the absolute pressure on both sides of the control valve (pressure reading points P2 and P3 in Figure 2.9.18.-8). A ratio P3/P2 of less than or equal to 0.5 indicates critical flow. Switch to a more powerful pump and re-measure the test flow rate if critical flow is not indicated.

Dispense 20 mL of a solvent, capable of dissolving the active substance into each of the 4 upper stages of the apparatus and replace the stoppers. Tilt the apparatus to wet the stoppers, thereby neutralising electrostatic charge. Place a suitable mouthpiece adapter in position at the end of the induction port.

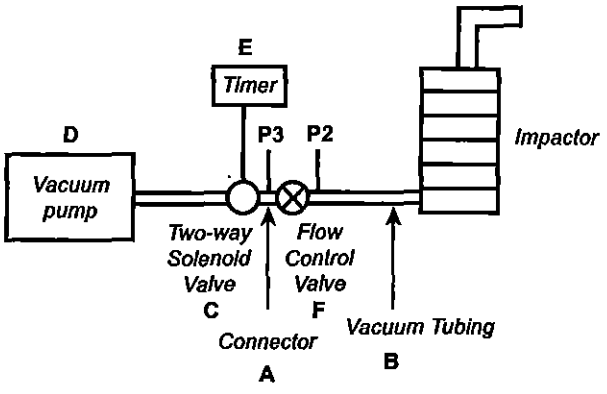


Figure 2.9.18.-8. – *Experimental set-up for testing powder inhalers*

Prepare the powder inhaler for use according to patient instructions. With the pump running and the 2-way solenoid valve closed, locate the mouthpiece of the inhaler in the mouthpiece adapter. Discharge the powder into the apparatus by opening the valve for the required time, T (± 5 per cent). Repeat the procedure. The number of discharges should be minimised and typically would not be greater than 10. The number of discharges is sufficient to ensure an accurate and precise determination of fine particle dose.

Dismantle the filter stage of the apparatus. Carefully remove the filter and extract the active substance into an aliquot of the solvent. Remove the induction port and mouthpiece adapter from the apparatus and extract the active substance into an aliquot of the solvent. If necessary, rinse the inside of the inlet jet tube to stage 1 with solvent, allowing the solvent to flow into the stage. Extract the active substance from the inner walls and the collection plate of each of the 4 upper stages of the apparatus into the solution in the respective stage by carefully tilting and rotating the apparatus, observing that no liquid transfer occurs between the stages.

Using a suitable method of analysis, determine the amount of active substance contained in each of the aliquots of solvent.

Calculate the fine particle dose (see Calculations).

Table 2.9.18.-4. – *Component specification for Figure 2.9.18.-8*

Code	Item	Description
A	Connector	ID ≥ 8 mm, e.g., short metal coupling, with low-diameter branch to P3.
B	Vacuum tubing	A length of suitable tubing having an ID ≥ 8 mm and an internal volume of 25 ± 5 mL.
C	2-way solenoid valve	A 2-way, 2-port solenoid valve having a minimum airflow resistance orifice with ID ≥ 8 mm and an opening time ≤ 100 ms. (e.g. type 256-A08, Bürkert GmbH, D-74653 Ingelfingen), or equivalent.
D	Vacuum pump	Pump must be capable of drawing the required flow rate through the assembled apparatus with the powder inhaler in the mouthpiece adapter (e.g. product type 1023, 1423 or 2565, Gast Manufacturing Inc., Benton Harbor, MI 49022), or equivalent. Connect the pump to the 2-way solenoid valve using short and/or wide (ID ≥ 10 mm) vacuum tubing and connectors to minimise pump capacity requirements.
E	Timer	Timer capable to drive the 2-way solenoid valve for the required duration (e.g. type G814, RS Components International, Corby, NN17 9RS, UK), or equivalent.
P2 P3	Pressure measurements	Determine under steady-state flow condition with an absolute pressure transducer.
F	Flow control valve	Adjustable regulating valve with maximum $C_v \geq 1$, (e.g. type 8FV12LNSS, Parker Hannifin plc., Barnstaple, EX31 1NP, UK), or equivalent.

APPARATUS D - ANDERSEN CASCADE IMPACTOR

The Andersen 1 ACFM non-viable cascade impactor consists of 8 stages together with a final filter. Material of construction may be aluminium, stainless steel or other suitable material. The stages are clamped together and sealed with O-rings. Critical dimensions applied by the manufacturer of apparatus D are provided in Table 2.9.18.-5. In use, some occlusion and wear of holes will occur. In-use mensuration tolerances need to be justified. In the configuration used for pressurised inhalers (Figure 2.9.18.-9) the entry cone of the impactor is connected to an induction port (see Figure 2.9.18.-7). A suitable mouthpiece adapter is used to provide an airtight seal between the inhaler and the induction port. The front face of the inhaler mouthpiece must be flush with the front face of the induction port.

In the configuration for powder inhalers, a pre-separator is placed above the top stage to collect large masses of non-respirable powder. It is connected to the induction port as shown in Figure 2.9.18.-10. To accommodate high flow rates through the impactor, the outlet nipple, used to connect the impactor to the vacuum system is enlarged to have an internal diameter of greater than or equal to 8 mm.

Procedure for pressurised inhalers

Assemble the Andersen impactor with a suitable filter in place. Ensure that the system is airtight. In that respect, follow the manufacturer's instructions. Place a suitable mouthpiece adapter in position at the end of the induction port so that the mouthpiece end of the actuator, when inserted, lines up along the horizontal axis of the induction port and the inhaler unit is positioned in the same orientation as the intended use. Connect a suitable pump to the outlet of

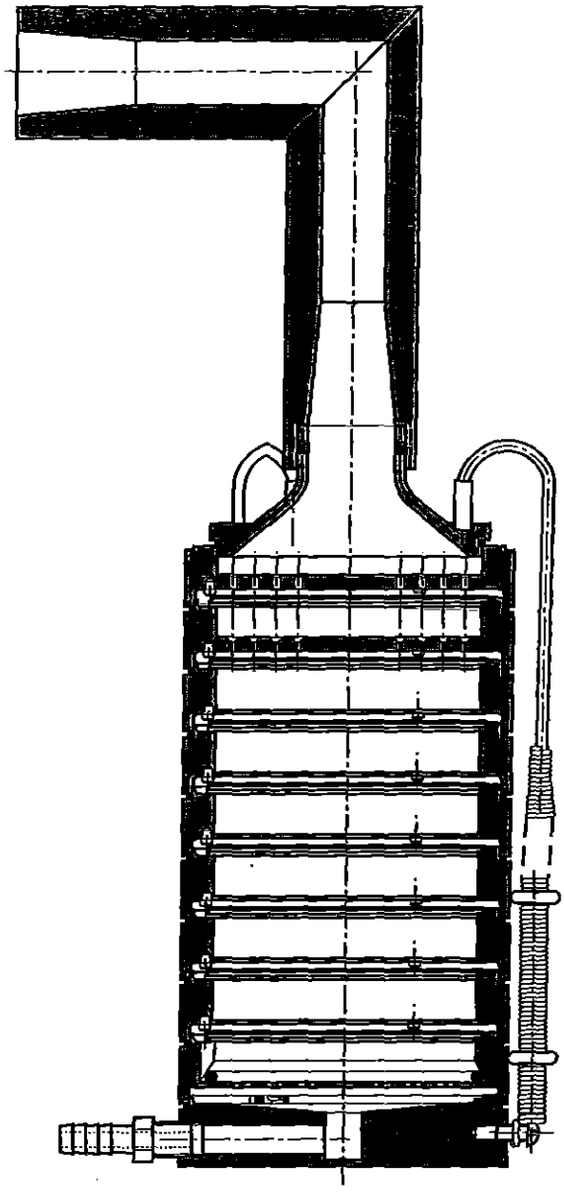


Figure 2.9.18.-9. – *Apparatus D: Andersen cascade impactor used for pressurised inhalers*

Table 2.9.18.-5. – *Critical dimensions for apparatus D*

Description	Number	Dimension (mm)
Stage 0 nozzle diameter	96	2.55 ± 0.025
Stage 1 nozzle diameter	96	1.89 ± 0.025
Stage 2 nozzle diameter	400	0.914 ± 0.0127
Stage 3 nozzle diameter	400	0.711 ± 0.0127
Stage 4 nozzle diameter	400	0.533 ± 0.0127
Stage 5 nozzle diameter	400	0.343 ± 0.0127
Stage 6 nozzle diameter	400	0.254 ± 0.0127
Stage 7 nozzle diameter	201	0.254 ± 0.0127

the apparatus and adjust the air flow through the apparatus, as measured at the inlet to the induction port, to 28.3 L/min (± 5 per cent). Switch off the pump.

Unless otherwise prescribed in the patient instructions, shake the inhaler for 5 s and discharge one delivery to waste. Switch on the pump to the apparatus, locate the mouthpiece end of the actuator in the adapter and discharge the inverted inhaler into the apparatus, depressing the valve for a sufficient time to ensure complete discharge. Wait for 5 s before removing the assembled inhaler from the adapter. Repeat the procedure. The number of discharges should be minimised and typically would not be greater than 10. The number of discharges is sufficient to ensure an accurate and precise determination of the fine particle dose. After the final discharge, wait for 5 s and then switch off the pump.

Dismantle the apparatus. Carefully remove the filter and extract the active substance into an aliquot of the solvent. Remove the induction port and mouthpiece adapter from the apparatus and extract the active substance into an aliquot of the solvent. Extract the active substance from the inner walls and the collection plate of each of the stages of the apparatus into aliquots of solvent.

Using a suitable method of analysis, determine the quantity of active substance contained in each of the aliquots of solvent.

Calculate the fine particle dose (see Calculations).

Procedure for powder inhalers

The aerodynamic cut-off diameters of the individual stages of this apparatus are currently not well-established at flow rates other than 28.3 L/min. Users must justify and validate the use of the impactor in the chosen conditions, when flow rates different from 28.3 L/min are selected.

Assemble the Andersen impactor with the pre-separator and a suitable filter in place and ensure that the system is airtight. Depending on the product characteristics, the pre-separator may be omitted, where justified and authorised. Stages 6 and 7 may also be omitted at high flow rates, if justified. The pre-separator may be coated in the same way as the plates or may contain 10 mL of a suitable solvent. Connect the apparatus to a flow system according to the scheme specified in Figure 2.9.18.-8 and Table 2.9.18.-4.

Unless otherwise defined, conduct the test at the flow rate, Q_{out}, used in the test for uniformity of delivered dose drawing 4 L of air from the mouthpiece of the inhaler and through the apparatus.

Connect a flowmeter to the induction port. Use a flowmeter calibrated for the volumetric flow leaving the meter, or calculate the volumetric flow leaving the meter (Q_{out}) using the ideal gas law. For a meter calibrated for the entering volumetric flow (Q_{in}), use the following expression:

$$Q_{out} = \frac{Q_{in} \times P_0}{P_0 - \Delta P}$$

P_0 = atmospheric pressure,
ΔP = pressure drop over the meter.

Adjust the flow control valve to achieve steady flow through the system at the required rate, Q_{out} (± 5 per cent). Ensure that critical flow occurs in the flow control valve by the procedure described for Apparatus C. Switch off the pump.

Prepare the powder inhaler for use according to the patient instructions. With the pump running and the 2-way solenoid valve closed, locate the mouthpiece of the inhaler in the mouthpiece adapter. Discharge the powder into the apparatus by opening the valve for the required time, T (± 5 per cent). Repeat the discharge sequence. The number of discharges should be minimised and typically would not be greater than 10. The number of discharges is sufficient to ensure an accurate and precise determination of fine particle dose.

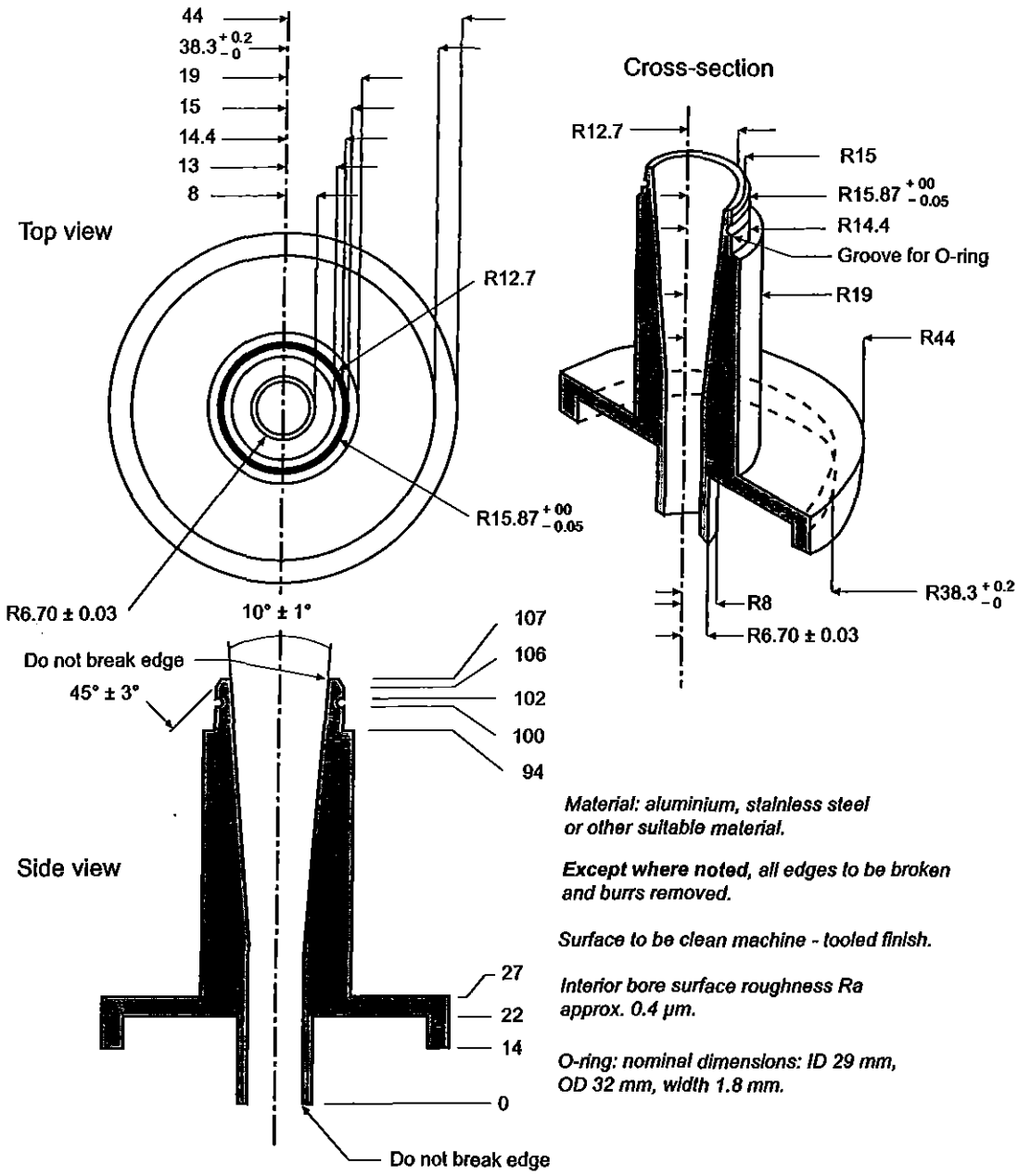


Figure 2.9.18.-10. – *Connection of the induction port to the preseparator of the Andersen cascade impactor Dimensions in millimetres unless otherwise stated*

Dismantle the apparatus. Carefully remove the filter and extract the active substance into an aliquot of the solvent. Remove the pre-separator, induction port and mouthpiece adapter from the apparatus and extract the active substance into an aliquot of the solvent. Extract the active substance from the inner walls and the collection plate of each of the stages of the apparatus into aliquots of solvent.

Using a suitable method of analysis, determine the quantity of active substance contained in each of the aliquots of solvent.

Calculate the fine particle dose (see Calculations).

APPARATUS E

Apparatus E is a cascade impactor with 7 stages and a micro-orifice collector (MOC). Over the flow rate range of 30 L/min to 100 L/min the 50 per cent-efficiency cut-off diameters (D_{50} values) range between 0.24 µm to 11.7 µm, evenly spaced on a logarithmic scale. In this flow range, there are always at least 5 stages with D_{50} values between 0.5 µm and 6.5 µm. The collection efficiency curves for each stage are sharp and minimise overlap between stages.

Material of construction may be aluminium, stainless steel or other suitable material.

The impactor configuration has removable impaction cups with all the cups in one plane (Figures 2.9.18.-11/14). There are 3 main sections to the impactor; the bottom frame that holds the impaction cups, the seal body that holds the jets and the lid that contains the interstage passageways (Figures 2.9.18.-11/12). Multiple nozzles are used at all but the first stage (Figure 2.9.18.-13). The flow passes through the impactor in a saw-tooth pattern.

Critical dimensions are provided in Table 2.9.18.-6.

In routine operation, the seal body and lid are held together as a single assembly. The impaction cups are accessible when this assembly is opened at the end of an inhaler test.

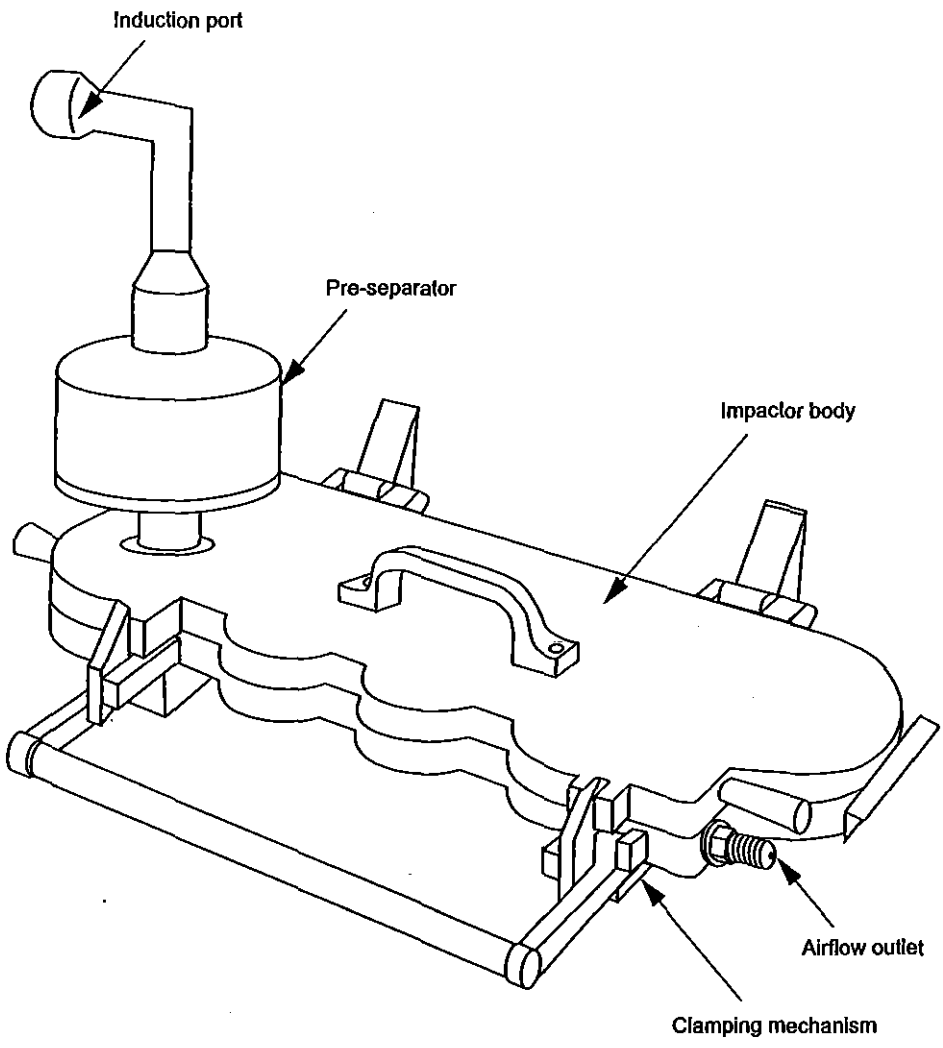


Figure 2.9.18.-11. – *Apparatus E (shown with the pre-separator in place)*

The cups are held in a support tray, so that all cups can be removed from the impactor simultaneously by lifting out the tray.

An induction port with internal dimensions (relevant to the airflow path) defined in Figure 2.9.18.-7 connects to the impactor inlet. A pre-separator can be added when required, typically with powder inhalers, and connects between the induction port and the impactor. A suitable mouthpiece adapter is used to provide an airtight seal between the inhaler and the induction port.

Apparatus E contains a terminal Micro-Orifice Collector (MOC) that for most formulations will eliminate the need for a final filter as determined by method validation. The MOC is an impactor plate with nominally 4032 holes, each approximately 70 µm in diameter. Most particles not captured on stage 7 of the impactor will be captured on the cup surface below the MOC. For impactors operated at 60 L/min, the MOC is capable of collecting 80 per cent of 0.14 µm particles. For formulations with a significant fraction of particles not captured by the MOC, there is an optional filter holder that can replace the MOC or be placed downstream of the MOC (a glass fibre filter is suitable).

Procedure for pressurised inhalers

Place cups into the apertures in the cup tray. Insert the cup tray into the bottom frame, and lower into place. Close the impactor lid with the seal body attached and operate the handle to lock the impactor together so that the system is airtight.

Connect an induction port with internal dimensions defined in Figure 2.9.18.-7 to the impactor inlet. Place a suitable mouthpiece adapter in position at the end of the induction port so that the mouthpiece end of the actuator, when inserted, lines up along the horizontal axis of the induction port. The front face of the inhaler mouthpiece must be flush with the front face of the induction port. When attached to the mouthpiece adapter, the inhaler is positioned in the same orientation as intended for use. Connect a suitable pump to the outlet of the apparatus and adjust the air flow through the apparatus, as measured at the inlet to the induction port, to 30 L/min (± 5 per cent). Switch off the pump.

Unless otherwise prescribed in the patient instructions, shake the inhaler for 5 s and discharge 1 delivery to waste. Switch on the pump to the apparatus. Prepare the inhaler for use according to the patient instructions, locate the mouthpiece end of the actuator in the adapter and discharge the inhaler into the apparatus, depressing the valve for a sufficient time to ensure a complete discharge. Wait for 5 s before removing the assembled inhaler from the adapter. Repeat the procedure. The number of discharges should be minimised, and typically would not be greater than 10. The number of discharges is sufficient to ensure an accurate and precise determination of the fine particle dose. After the final discharge, wait for 5 s and then switch off the pump.

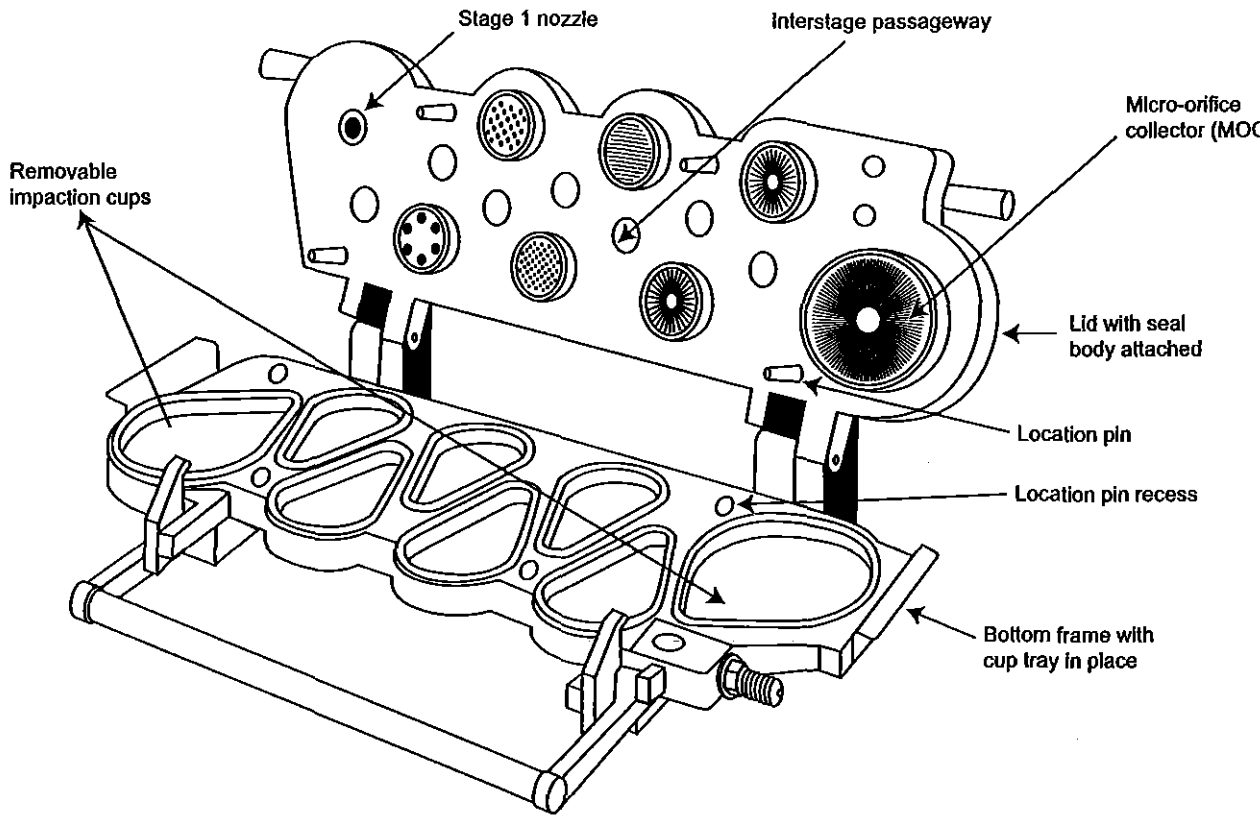


Figure 2.9.18.-12. – *Apparatus E showing component parts*

Table 2.9.18.-6. – *Critical dimensions for apparatus E*

Description	Dimension (mm)
Pre-separator (dimension a - see Figure 2.9.18.-15)	12.8 ± 0.05
Stage 1* Nozzle diameter	14.3 ± 0.05
Stage 2* Nozzle diameter	4.88 ± 0.04
Stage 3* Nozzle diameter	2.185 ± 0.02
Stage 4* Nozzle diameter	1.207 ± 0.01
Stage 5* Nozzle diameter	0.608 ± 0.01
Stage 6* Nozzle diameter	0.323 ± 0.01
Stage 7* Nozzle diameter	0.206 ± 0.01
MOC*	approx. 0.070
Cup depth (dimension b - see Figure 2.9.18.-14)	14.625 ± 0.10
Collection cup surface roughness (Ra)	0.5 - 2 µm
Stage 1 nozzle to seal body distance** - dimension c	0 ± 1.18
Stage 2 nozzle to seal body distance** - dimension c	5.236 ± 0.736
Stage 3 nozzle to seal body distance** - dimension c	8.445 ± 0.410
Stage 4 nozzle to seal body distance** - dimension c	11.379 ± 0.237
Stage 5 nozzle to seal body distance** - dimension c	13.176 ± 0.341
Stage 6 nozzle to seal body distance** - dimension c	13.999 ± 0.071
Stage 7 nozzle to seal body distance** - dimension c	14.000 ± 0.071
MOC nozzle to seal body distance** - dimension c	14.429 to 14.571

* See Figure 2.9.18.-13
** See Figure 2.9.18.-14

Dismantle the apparatus and recover the active substance as follows: remove the induction port and mouthpiece adapter from the apparatus and recover the deposited active substance into an aliquot of solvent. Open the impactor by releasing the handle and lifting the lid. Remove the cup tray, with the collection cups, and recover the active substance in each cup into an aliquot of solvent.

Using a suitable method of analysis, determine the quantity of active substance contained in each of the aliquots of solvent.

Calculate the fine particle dose (see Calculations).

Procedure for powder inhalers

Assemble the apparatus with the pre-separator (Figure 2.9.18.-15). Depending on the product characteristics, the pre-separator may be omitted, where justified.

Place cups into the apertures in the cup tray. Insert the cup tray into the bottom frame, and lower into place. Close the impactor lid with the seal body attached and operate the handle to lock the impactor together so that the system is airtight.

When used, the pre-separator should be assembled as follows: assemble the pre-separator insert into the pre-separator base. Fit the pre-separator base to the impactor inlet. Add 15 mL of the solvent used for sample recovery to the central cup of the pre-separator insert. Place the pre-separator body on top of this assembly and close the 2 catches.

Connect an induction port with internal dimensions defined in Figure 2.9.18.-7 to the impactor inlet or pre-separator inlet. Place a suitable mouthpiece adapter in position at the end of the induction port so that the mouthpiece end of the inhaler, when inserted, lines up along the horizontal axis of the induction port. The front face of the inhaler mouthpiece must be flush with the front face of the induction port. When attached to the mouthpiece adapter, the inhaler is positioned in the same orientation as intended for use. Connect the

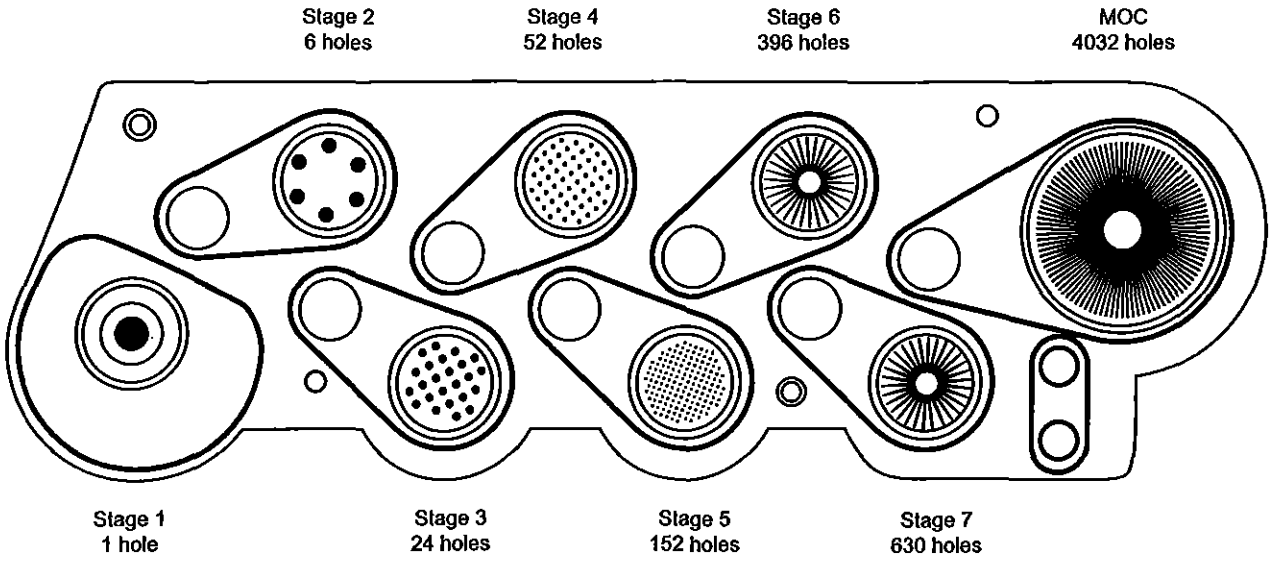


Figure 2.9.18.-13. – *Apparatus E: nozzle configuration*

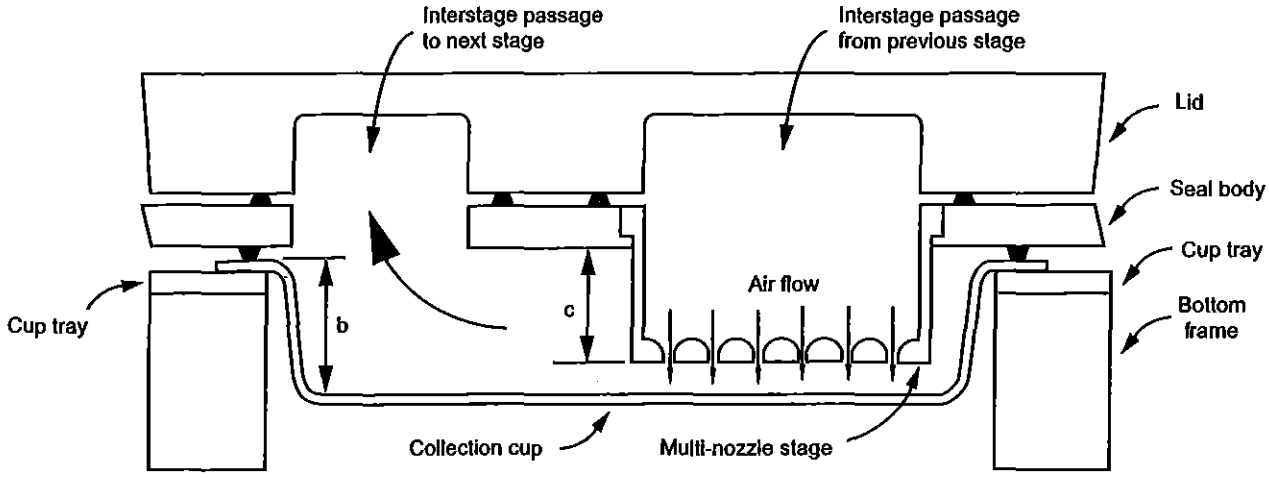


Figure 2.9.18.-14. – *Apparatus E: configuration of interstage passageways*

apparatus to a flow system according to the scheme specified in Figure 2.9.18.-8 and Table 2.9.18.-4.

Unless otherwise prescribed, conduct the test at the flow rate, Q_{out}, used in the test for uniformity of delivered dose drawing 4 L of air from the mouthpiece of the inhaler and through the apparatus. Connect a flowmeter to the induction port. Use a flowmeter calibrated for the volumetric flow leaving the meter, or calculate the volumetric flow leaving the meter (Q_{out}) using the ideal gas law. For a meter calibrated for the entering volumetric flow (Q_{in}), use the following expression:

$$Q_{out} = \frac{Q_{in} \times P_0}{P_0 - \Delta P}$$

P_0 = atmospheric pressure,
ΔP = pressure drop over the meter.

Adjust the flow control valve to achieve steady flow through the system at the required rate, Q_{out} (± 5 per cent). Ensure that critical flow occurs in the flow control valve by the procedure described for Apparatus C. Switch off the pump.

Prepare the powder inhaler for use according to the patient instructions. With the pump running and the 2-way solenoid valve closed, locate the mouthpiece of the inhaler in the mouthpiece adapter. Discharge the powder into the apparatus by opening the valve for the required time, T (± 5 per cent). Repeat the discharge sequence.

The number of discharges should be minimised and typically would not be greater than 10. The number of discharges is sufficient to ensure an accurate and precise determination of fine particle dose.

Dismantle the apparatus and recover the active substance as follows: remove the induction port and mouthpiece adapter from the pre-separator, when used, and recover the deposited active substance into an aliquot of solvent. When used, remove the pre-separator from the impactor, being careful to avoid spilling the cup liquid into the impactor. Recover the active substance from the pre-separator.

Open the impactor by releasing the handle and lifting the lid. Remove the cup tray, with the collection cups, and recover the active substance in each cup into an aliquot of solvent.

Using a suitable method of analysis, determine the quantity of active substance contained in each of the aliquots of solvent.

Calculate the fine particle dose (see Calculations).

CALCULATIONS

From the analysis of the solutions, calculate the mass of active substance deposited on each stage per discharge and the mass of active substance per discharge deposited in the

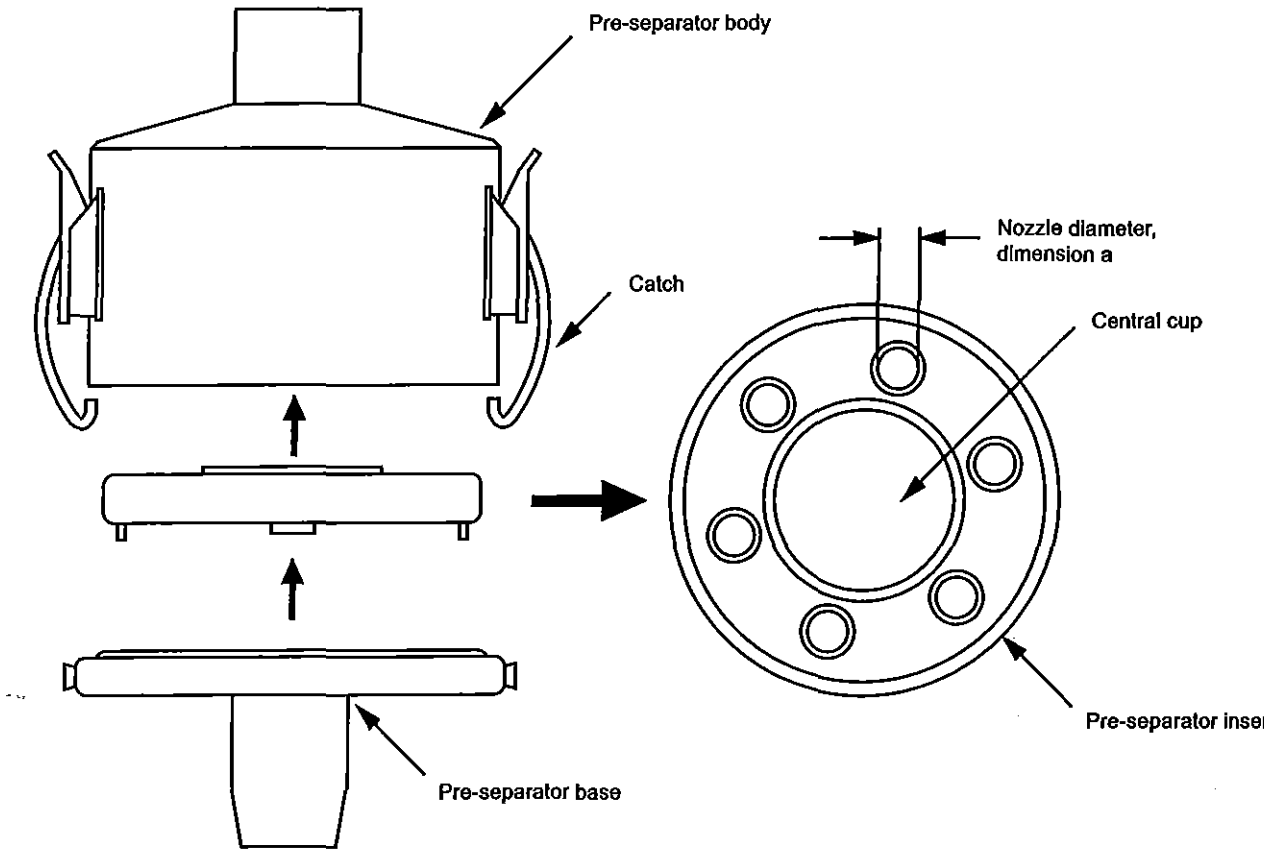


Figure 2.9.18.-15. – *Apparatus E: pre-separator configuration*

induction port, mouthpiece adapter and when used, the pre-separator.

Starting at the final collection site (filter or MOC), derive a table of cumulative mass versus cut-off diameter of the respective stage (see Tables 2.9.18.-7 for Apparatus C, 2.9.18.-8 for Apparatus D, 2.9.18.-9 for Apparatus E). Calculate by interpolation the mass of the active substance less than 5 µm. This is the Fine Particle Dose (FPD).

If necessary, and where appropriate (e.g., where there is a log-normal distribution), plot the cumulative fraction of active substance versus cut-off diameter (see Tables 2.9.18.-7/9) on log probability paper, and use this plot to determine values for the Mass Median Aerodynamic Diameter (MMAD) and Geometric Standard Deviation (GSD) as appropriate. Appropriate computational methods may also be used.

8. Preparations for Nebulisation: Characterisation *(Ph. Eur. method 2.9.44)*

Products used for nebulisation and intended for pulmonary delivery are characterised using the following tests:

— Active substance delivery rate and total active substance delivered;

— Aerodynamic assessment of nebulised aerosols.

These tests standardise the approach given to the assessment of the dose that would be delivered to a patient but are not intended to provide assessment of the nebuliser device itself, which is described in the European standard EN 13544-1:2007+A1:2009, Respiratory therapy equipment - Part 1: Nebulizing systems and their components.

The mass- rather than the number-weighted size distribution is more appropriate to evaluate product performance. Indeed, active substance mass as a function of aerodynamic diameter is more indicative of therapeutic effect within the respiratory tract.

ACTIVE SUBSTANCE DELIVERY RATE AND TOTAL ACTIVE SUBSTANCE DELIVERED

These tests are performed to assess the rate of delivery to the patient and the total active substance delivered to the patient, using standardised conditions of volumetric flow rate. It is essential that breath-enhanced and breath-actuated nebulisers be evaluated by a breathing simulator, as the output of these types of device is highly dependent on inhalation flow rate. The methodology below describes the use of a standard breathing pattern defined for adults. Should a particular product for nebulisation only be indicated for paediatric (i.e. neonate, infant or child) use, then paediatric breathing pattern(s) must be used. Breathing patterns are used, rather than continuous flow rates, to provide a more appropriate measure of the mass of active substance that would be delivered to patients.

Active substance delivery rate and total active substance delivered are appropriate characteristics because they allow the mass delivered to be characterised in a standard way regardless of the nebuliser used. Accordingly, the test methodology described below allows that the mass of active substance delivered in the 1st period (typically 1 min) is measured (consequently giving an assessment of active substance delivery rate) as well as capturing the total mass of active substance delivered.

Table 2.9.18.-7. – *Calculations for Apparatus C. Use* $q = \sqrt{(60/Q)}$, *where Q is the test flow rate in litres per minute* (Q_{ma} *for powder inhalers)*

Cut-off diameter (µm)	Mass of active substance deposited per discharge	Cumulative mass of active substance deposited per discharge	Cumulative fraction of active substance (per cent)
$d_4 = 1.7 \times q$	mass from stage 5, m_5*	$c_4 = m_5$	$f_4 = (c_4/c) \times 100$
$d_3 = 3.1 \times q$	mass from stage 4, m_4	$c_3 = c_4 + m_4$	$f_3 = (c_3/c) \times 100$
$d_2 = 6.8 \times q$	mass from stage 3, m_3	$c_2 = c_3 + m_3$	$f_2 = (c_2/c) \times 100$
	mass from stage 2, m_2	$c = c_2 + m_2$	100

* Stage 5 is the filter stage

Table 2.9.18.-8. – *Calculations for Apparatus D when used at a flow rate of 28.3 L/min*

Cut-off diameter (µm)	Mass of active substance deposited per discharge	Cumulative mass of active substance deposited per discharge	Cumulative fraction of active substance (per cent)
$d_7 = 0.4$	mass from stage 8, m_8	$c_7 = m_8$	$f_7 = (c_7/c) \times 100$
$d_6 = 0.7$	mass from stage 7, m_7	$c_6 = c_7 + m_7$	$f_6 = (c_6/c) \times 100$
$d_5 = 1.1$	mass from stage 6, m_6	$c_5 = c_6 + m_6$	$f_5 = (c_5/c) \times 100$
$d_4 = 2.1$	mass from stage 5, m_5	$c_4 = c_5 + m_5$	$f_4 = (c_4/c) \times 100$
$d_3 = 3.3$	mass from stage 4, m_4	$c_3 = c_4 + m_4$	$f_3 = (c_3/c) \times 100$
$d_2 = 4.7$	mass from stage 3, m_3	$c_2 = c_3 + m_3$	$f_2 = (c_2/c) \times 100$
$d_1 = 5.8$	mass from stage 2, m_2	$c_1 = c_2 + m_2$	$f_1 = (c_1/c) \times 100$
$d_0 = 9.0$	mass from stage 1, m_1	$c_0 = c_1 + m_1$	$f_0 = (c_0/c) \times 100$
	mass from stage 0, m_0	$c = c_0 + m_0$	100

Table 2.9.18.-9. – *Calculations for Apparatus E. Use* $q = (60/Q)^x$, *where Q is the test flow rate in litres per minute, and x is listed in the table*

Cut-off diameter (µm)	x	Mass of active substance deposited per discharge	Cumulative mass of active substance deposited per discharge	Cumulative fraction of active substance (per cent)
$d_7 = 0.34 \times q$	0.67	mass from MOC or terminal filter, m_8	$c_7 = m_8$	$F_7 = (c_7/c) \times 100$
$d_6 = 0.55 \times q$	0.60	mass from stage 7, m_7	$c_6 = c_7 + m_7$	$F_6 = (c_6/c) \times 100$
$d_5 = 0.94 \times q$	0.53	mass from stage 6, m_6	$c_5 = c_6 + m_6$	$F_5 = (c_5/c) \times 100$
$d_4 = 1.66 \times q$	0.47	mass from stage 5, m_5	$c_4 = c_5 + m_5$	$F_4 = (c_4/c) \times 100$
$d_3 = 2.82 \times q$	0.50	mass from stage 4, m_4	$c_3 = c_4 + m_4$	$F_3 = (c_3/c) \times 100$
$d_2 = 4.46 \times q$	0.52	mass from stage 3, m_3	$c_2 = c_3 + m_3$	$F_2 = (c_2/c) \times 100$
$d_1 = 8.06 \times q$	0.54	mass from stage 2, m_2	$c_1 = c_2 + m_2$	$F_1 = (c_1/c) \times 100$
		mass from stage 1, m_1	$c = c_1 + m_1$	100

APPARATUS

Breathing simulator

A commercially available breathing simulator, which is able to generate the breathing profiles specified in Table 2.9.44.-1, is used for the test. The breathing profile indicated for adults is used unless the medicinal product is specifically intended for use in paediatrics, where alternate patterns should be used, as indicated in Table 2.9.44.-1.

Table 2.9.44.-1. – *Breathing simulator specifications*

Item	Specification			
	Adult	Neonate	Infant	Child
Tidal volume	500 mL	25 mL	50 mL	155 mL
Frequency	15 cycles/min	40 cycles/min	30 cycles/min	25 cycles/min
Waveform	sinusoidal	sinusoidal	sinusoidal	sinusoidal
Inhalation/ exhalation ratio	1:1	1:3	1:3	1:2

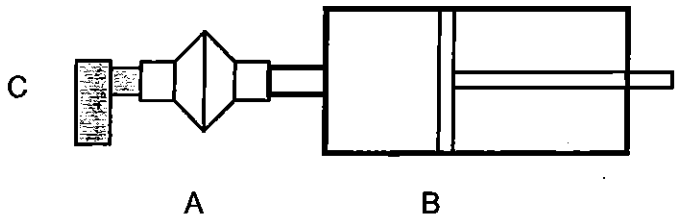
Filter system

A suitably validated low-resistance filter, capable of quantitatively collecting the aerosol and enabling recovery of the active substance with an appropriate solvent, is used for the test. The dead volume of the filter casing does not exceed 10 per cent of the tidal volume used in the breath simulation.

METHOD

Attach the filter (contained in the filter holder) (A) to the breath simulator (B) according to Figure 2.9.44.-1. Fill the nebuliser (C) with the volume of the medicinal product as specified in the patient instructions. Attach the mouthpiece of the nebuliser to the inhalation filter using a mouthpiece adapter if required, ensuring that connections are airtight. Make sure the nebuliser is positioned in the same orientation as intended for use; this may require tilting the breathing simulator and filter holder. Set the breathing simulator to generate the specified breathing pattern.

Start the breathing simulator then, at the beginning of an inhalation cycle, start the nebuliser. Operate the nebuliser for a defined initial time period. The time chosen, usually



A. inhalation filter and filter holder B. breathing simulator C. nebuliser

Figure 2.9.44.-1. – *Experimental set-up for breathing simulator testing*

60 ± 1 s, must allow sufficient active substance deposition on the inhalation filter to allow quantitative analysis. If the quantity of active substance deposited on the inhalation filter in 60 s is insufficient for this analysis, the length of the time interval for aerosol collection can be increased. If the filter is soaked with the preparation, this time can be decreased. At the end of this initial period, stop the nebuliser.

Place a fresh filter and filter holder in position and continue until nebulisation ceases. Interrupt nebulisation and exchange filters if necessary, to avoid filter saturation.

RESULTS

Using a suitable method of analysis, determine the mass of active substance collected on the filters and filter holders during each time interval. Determine the active substance delivery rate by dividing the mass of active substance collected on the first inhalation filter by the time interval used for collection. Determine the total mass of active substance delivered by summing the mass of active substance collected on all inhalation filters and filter holders.

AERODYNAMIC ASSESSMENT OF NEBULISED AEROSOLS

Nebulised products need to be size-characterised at flow rates lower than the range that is normally used for powder inhalers and metered-dose inhalers. A flow rate of 15 L/min is recommended in the European standard because this value represents a good approximation to the mid-inhalation flow rate achievable by a tidally breathing healthy adult (500 mL tidal volume).

Although low-angle laser light scattering instruments (laser diffractometers) can provide rapid size-distribution measurements of nebuliser-generated aerosols, these techniques do not detect the active substance; rather they measure the size distribution of the droplets irrespective of their content. This may not be a problem with homogeneous solutions, but can result in significant error if the product to be nebulised is a suspension, or if droplet evaporation is significant as can be the case with certain nebuliser types. Cascade impactors enable the aerosol to be characterised unambiguously in terms of the mass of active substance as a function of aerodynamic diameter. Laser diffraction may be used if validated against a cascade impaction method.

Apparatus E (see below under Apparatus), a cascade impactor, has been calibrated at 15 L/min specifically to meet the recommendation of the European standard, and is therefore used for this test. Determining mass balance in the same way as for powder inhalers and metered-dose inhalers is not straightforward, in that the dose is being captured as a continuous output, and hence is not included. As part of method development, recovery experiments must be performed to validate the method.

It is also recognised that the control of evaporation of droplets produced by nebulisers may be critical to avoid bias in the droplet size assessment process. Evaporation can be minimised by cooling the impactor to a temperature of about 5 °C, typically achieved by cooling the impactor in a refrigerator for about 90 min. Typically, at least after each day of use, the apparatus must be fully cleaned, including the inter-stage passageways, in view of the greater risk of corrosion caused by the condensation/accumulation of saline-containing droplets on inter-stage metalwork associated with cooling the impactor. It is recommended to dry all surfaces of the apparatus after each test, for example with compressed air. Note: the micro-orifice collector (MOC) should not be dried with compressed air.

APPARATUS

A detailed description of Apparatus E and the induction port is contained in general chapter *2.9.18*, and includes details of critical dimensions and the qualification process for the impactor (stage mensuration).

A back-up filter in addition to the micro-orifice collector (MOC) must be used to ensure quantitative recovery of active substance from the nebulised aerosol at the specified flow rate of 15 L/min. The filter is located below the MOC (internal filter option) or a filter in holder, external to the impactor, is used to capture any fine droplets that pass beyond the last size fractionating stage.

A pre-separator is not used for testing nebuliser-generated aerosols.

METHOD VALIDATION

Impactor stage overloading

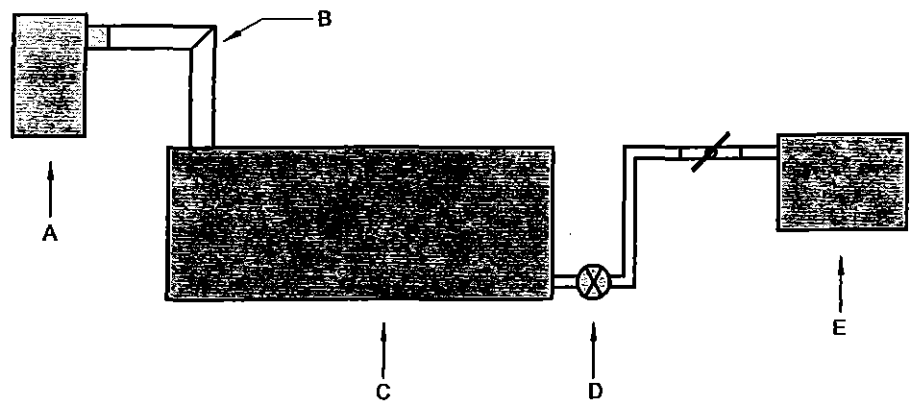
During method development and validation, it is important to confirm that the volume of liquid sampled from the nebuliser does not overload the impactor. Visual inspection of the collection surfaces on stages collecting most of the droplets may reveal streaking if overloading has occurred. This phenomenon is usually also associated with an increase in mass of active substance collected on the final stage and back-up filter. Reducing the sampling period (T_0) is the most effective way to avoid overloading in any given system, balancing overloading with analytical sensitivity.

Re-entrainment

Droplet bounce and re-entrainment are less likely with nebuliser-produced droplets than with solid particles from inhalers and for that reason coating would not normally be required.

METHOD

Pre-cool the assembled impactor and induction port in a refrigerator (set at about 5 °C) for not less than 90 min and start the determination within about 5 min of removal of the impactor from the refrigerator. Other methods that maintain



A. nebuliser B. induction port C. impactor (apparatus B) D. flow control valve E. vacuum source

Figure 2.9.44.-2. – *Apparatus E for measuring the size distribution of preparations for nebulisation*

the impactor at a constant temperature (for example, use of a cooling cabinet) can also be employed when validated.

Set up the nebuliser with a supply of driving gas (usually air or oxygen), or use a compressor, at the pressure and flow rate specified by the manufacturer of the nebuliser. Take precautions to ensure that the gas supply line does not become detached from the nebuliser when under pressure. Fill the nebuliser with the volume of the medicinal product as specified in the patient instructions.

Remove the impactor from the refrigerator. Attach the induction port to the impactor, and connect the outlet of the impactor/external filter to a vacuum source that is capable of drawing air through the system at 15 L/min as specified in Figure 2.9.44.-2. Turn on the flow through the impactor.

Connect a flow meter, calibrated for the volumetric flow leaving the meter, to the induction port. Adjust the flow control valve located between the impactor and the vacuum source to achieve a steady flow through the system at 15 L/min (± 5 per cent). Remove the flow meter.

Make sure the nebuliser is positioned in the same orientation as intended for use then attach the mouthpiece of the nebuliser to the induction port, using a mouthpiece adapter if required, ensuring that connections are airtight. Switch on the flow/compressor for the nebuliser. Sample for a predetermined time (T_0). Once determined, this time (T_0) must be defined and used in the analytical method for a particular medicinal product to ensure that mass fraction data can be compared. At the end of the sampling period, switch off the driving gas flow/compressor to the nebuliser, remove the nebuliser from the induction port and switch off the flow from the vacuum source to the impactor.

Dismantle the impactor and, using a suitable method of analysis, determine the mass of active substance collected in the induction port, on each stage and on the back-up filter/external filter as described for Apparatus E in general chapter *2.9.18.* Add the mass of active substance collected in the MOC to that deposited on the back-up filter/external filter and treat as a single sample for the purpose of subsequent calculations.

Calculate the mass fraction ($F_{m,comp}$) of the active substance deposited on each component of the impactor, commencing with the induction port and proceeding in order through the impactor, using the following expression:

$$F_{m,comp} = \frac{m_{comp}}{M}$$

m_{comp} = mass associated with the component under evaluation;
M = total mass collected by the system.

Present $F_{m,comp}$ in order of location within the measurement equipment, beginning at the induction port and ending with the back-up filter of the impactor (see Figure 2.9.44.-3). Where appropriate, $F_{m,comp}$ for adjacent stages of the impactor may be combined in order to report the mass fraction collected on a group of stages as a single value.

Determine the cumulative mass-weighted particle-size distribution of the aerosol size-fractionated by the impactor in accordance with the procedure given in general chapter *2.9.18.* Starting at the filter, derive a cumulative mass versus effective cut-off diameter of the respective stages (see Table 2.9.44.-2 for the appropriate cut-off diameters at 15 L/min). Plot the cumulative fraction of active substance versus cut-off diameter in a suitable format, for example logarithmic or log-probability format. Where appropriate, determine by interpolation the fraction either below a given size or between an upper and a lower size limit.

Table 2.9.44.-2. – *Cut-off sizes for Apparatus E at 15 L/min*

Stage	Cut-off diameter (µm)
1	14.1
2	8.61
3	5.39
4	3.30
5	2.08
6	1.36
7	0.98

If necessary, and where appropriate, determine values for the mass median aerodynamic diameter (MMAD) and the geometric standard deviation (GSD), as appropriate.

9. Demonstration of Uniformity of Dosage Units Using Large Samples Sizes

(Ph. Eur. method 2.9.47)

The procedure is intended for, but not limited to, the evaluation of medicinal products that are manufactured using process analytical technology (PAT) methodology.

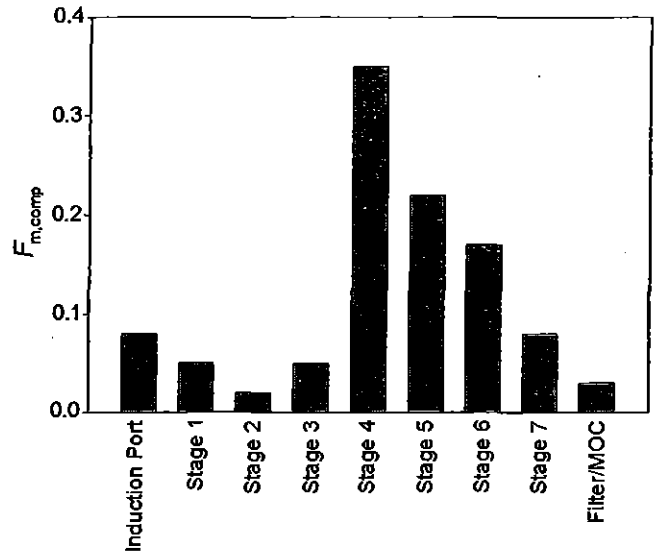


Figure 2.9.44.-3. – *Example of mass fraction of droplets presented in terms of location within the sampling system*

Compliance with general chapter *2.9.40. Uniformity of dosage units* can be demonstrated by the following procedure, when large samples (sample size $n \geq 100$) are evaluated.

Application of this chapter does not constitute a mandatory requirement. It presents 2 alternative tests (Alternative 1 and Alternative 2). Fulfilling the requirements of either of the 2 alternatives is considered as evidence that the medicinal product tested complies with general chapter *2.9.40.* The 2 alternatives are considered equivalent in their demonstration of compliance with general chapter *2.9.40.*

ALTERNATIVE 1 (PARAMETRIC)

Select not fewer than 100 units according to a predefined sampling plan.

The consistency of dosage units is evaluated by content uniformity or mass variation as prescribed in Table 2.9.40.-1. Calculate the acceptance value (*AV*) using the following expression:

$$|M - \overline{X}| + ks$$

for which the terms are defined in Table 2.9.40.-2, but using the sample size-dependent value for k defined in Table 2.9.47.-1.

CRITERIA

Apply the following criteria, unless otherwise specified.

The requirements for dosage form uniformity are met if:

1. the acceptance value (*AV*) is less than or equal to $L1$; and
2. in the calculation of acceptance value (*AV*) under content uniformity or under mass variation, the number of individual dosage units outside $(1 \pm L2 \times 0.01)M$ is less than or equal to $c2$ as defined for a given sample size n in Table 2.9.47.-1.

Unless otherwise specified, $L1$ is 15.0 and $L2$ is 25.0.

Table 2.9.47.-1. is to be interpreted as follows:

— for a sample size of $n = 400$, enter the table at $n \geq 385$: $k = 2.23$ and $c2 = 3$;
— for a sample size of $n = 450$, enter the table at $n \geq 407$: $k = 2.24$ and $c2 = 3$;
— for a sample size of $n = 500$, enter the table at $n \geq 490$: $k = 2.24$ and $c2 = 4$.

ALTERNATIVE 2 (NON-PARAMETRIC)

Select not fewer than 100 units according to a predefined sampling plan.

The consistency of dosage units is evaluated by content uniformity or mass variation as prescribed in Table 2.9.40.-1. Assay individually or weigh the units and calculate individual contents as prescribed in general chapter *2.9.40.* Count the number of individual dosage units with a content outside $(1 \pm L1 \times 0.01)T$ and the number of individual dosage units with a content outside $(1 \pm L2 \times 0.01)T$. Evaluate if the values are within the limits defined in Table 2.9.47.-2.

CRITERIA

Apply the following criteria, unless otherwise specified.

The requirements for dosage form uniformity are met if:

1. the number of individual dosage units outside $(1 \pm L1 \times 0.01)T$ is less than or equal to $c1$; and
2. the number of individual dosage units outside $(1 \pm L2 \times 0.01)T$ is less than or equal to $c2$.

$c1$ and $c2$ for a given sample size n are defined in Table 2.9.47.-2. Unless otherwise specified, $L1$ is 15.0 and $L2$ is 25.0.

Table 2.9.47.-2 is to be interpreted as follows:

— for a sample size of $n = 400$, enter the table at $n \geq 394$: $c1 = 11$ and $c2 = 3$;
— for a sample size of $n = 450$, enter the table at $n \geq 434$: $c1 = 12$ and $c2 = 3$;
— for a sample size of $n = 500$, enter the table at $n \geq 490$: $c1 = 13$ and $c2 = 4$.

10. Content of active ingredient on actuation of the valve test

The following test conditions are for use in Preparations for Inhalation of the British Pharmacopoeia. Specifically the methodology should be applied to Pressurised Inhalation products.

Content of active ingredient delivered by actuation of the valve

Remove the pressurised container from the actuator and remove all labels and markings which may be present on the container with a suitable solvent. Dry the container, replace in its actuator, shake for about 30 seconds and prime the

Table 2.9.47.-1. – *Acceptability constant (k) and acceptable number of dosage units with a content outside (1 ± L2 × 0.01)M (= c2) for a given sample size n*

n (≥)	k	c2
100	2.15	
105	2.16	
120	2.17	0
139	2.18	
161	2.19	
176	2.19	
189	2.20	1
224	2.21	
270	2.22	
280	2.22	2
328	2.23	
385	2.23	3
407	2.24	
490	2.24	4
516	2.25	
594	2.25	5
672	2.26	
699	2.26	6
804	2.26	7
905	2.27	
908	2.27	8
1013	2.27	9
1118	2.27	10
1223	2.27	11
1276	2.28	
1328	2.28	12
1432	2.28	13
1537	2.28	14
1642	2.28	15
1747	2.28	16
1851	2.28	17
1918	2.29	
1956	2.29	18
2061	2.29	19
2166	2.29	20
2270	2.29	21
2375	2.29	22
2480	2.29	23
2585	2.29	24
2690	2.29	25
2794	2.29	26
2899	2.29	27
3004	2.29	28
3109	2.29	29
3171	2.30	
3213	2.30	30
3318	2.30	31
3423	2.30	32
3528	2.30	33
3633	2.30	34
3737	2.30	35
3842	2.30	36
3947	2.30	37
4052	2.30	38
4156	2.30	39
4261	2.30	40
4366	2.30	41
4471	2.30	42
4576	2.30	43
4680	2.30	44
4785	2.30	45
4890	2.30	46
4995	2.30	47
5099	2.30	48
5204	2.30	49
5309	2.30	50
5414	2.30	51
5519	2.30	52
5623	2.30	53
5728	2.30	54
5833	2.30	55
5938	2.30	56
6042	2.30	57
6136	2.31	
6147	2.31	58
6252	2.31	59
6357	2.31	60
6462	2.31	61
6566	2.31	62
6671	2.31	63
6776	2.31	64
6881	2.31	65
6985	2.31	66
7090	2.31	67
7195	2.31	68
7300	2.31	69
7404	2.31	70
7509	2.31	71
7614	2.31	72
7719	2.31	73
7824	2.31	74
7928	2.31	75
8033	2.31	76
8138	2.31	77
8243	2.31	78
8347	2.31	79
8452	2.31	80
8557	2.31	81
8662	2.31	82
8767	2.31	83
8871	2.31	84
8976	2.31	85
9081	2.31	86
9186	2.31	87
9290	2.31	88
9395	2.31	89
9500	2.31	90
9605	2.31	91
9710	2.31	92
9814	2.31	93
9919	2.31	94

metering valve as follows. Discharge once to waste, wait for not less than 5 seconds and discharge again to waste. Remove the pressurised container from its actuator, clean the valve stem (internally and externally) and the valve ferrule by washing with a suitable solvent. Dry the complete valve assembly using an air line fitted with an appropriate narrow jet to ensure that all solvent is removed from the inside of the valve stem.

Place a stainless steel base plate that has three legs and a central circular indentation with a hole about 1.5 mm in diameter in a small vessel suitable for shaking and add the volume of solvent specified in the monograph. The size of the vessel is such that when the pressurised inhalation is discharged into the specified volume of solvent as described below the discharge takes place not less than 25 mm below the surface of the solvent.

Shake the pressurised container for about 30 seconds and place it inverted in the vessel. Discharge 10 deliveries below the surface of the solvent actuating the valve at intervals of not less than 5 seconds, maintaining the pressurised container in the vertical plane and discharging the pressurised inhalation through the hole in the centre of the base plate. (It may be necessary because of the nature of the formulation to shake the pressurised container between each actuation of the valve; where this is the case shaking should be carried out without removing the pressurised container from its inverted position in the vessel.) Remove the pressurised container, wash it with the specified solvent and dilute the combined solution and washings to the volume specified in the monograph. Determine the amount of active ingredient by the method described under the Assay and calculate the amount delivered from each actuation of the valve.

The result lies within the range for the content of active ingredient stated in the monograph.

Table 2.9.47.-2. – *Acceptable number of individual dosage units with a content outside (1 ± L1 × 0.01)T (= c1) and (1 ± L2 × 0.01)T (= c2) respectively, for a given sample size n*

n (≥)	c1	c2
100	3	0
123	4	
159	5	
176	5	1
196	6	
234	7	
273	8	
280	8	2
313	9	
353	10	
385	10	3
394	11	
434	12	
476	13	
490	13	4
517	14	
559	15	
594	15	5
601	16	
644	17	
686	18	
699	18	6
729	19	
772	20	
804	20	7
815	21	
858	22	
902	23	
908	23	8
945	24	
989	25	
1013	25	9
1033	26	
1077	27	
1118	27	10
1121	28	
1165	29	
1209	30	
1223	30	11
1253	31	
1298	32	
1328	32	12
1342	33	
1387	34	
1432	35	13
1476	36	
1521	37	
1537	37	14
1566	38	
1611	39	
1642	39	15
1656	40	
1701	41	
1746	42	
1747	42	16
1791	43	
1836	44	
1851	44	17
1882	45	
1927	46	
1956	46	18
1972	47	
2018	48	
2061	48	19
2063	49	
2109	50	
2154	51	
2166	51	20
2200	52	
2246	53	
2270	53	21
2291	54	
2337	55	
2375	55	22
2383	56	
2429	57	
2475	58	
2480	58	23
2520	59	
2566	60	
2585	60	24
2612	61	
2658	62	
2690	62	25
2704	63	
2750	64	
2794	64	26
2796	65	
2843	66	
2889	67	
2899	67	27
2935	68	
2981	69	
3004	69	28
3027	70	
3073	71	
3109	71	29
3120	72	
3166	73	
3212	74	
3213	74	30
3259	75	
3305	76	
3318	76	31
3351	77	
3398	78	
3423	78	32
3444	79	
3491	80	
3528	80	33
3537	81	
3584	82	
3630	83	
3633	83	34
3677	84	
3723	85	
3737	85	35
3770	86	
3817	87	
3842	87	36
3863	88	
3910	89	
3947	89	37
3956	90	
4003	91	
4050	92	
4052	92	38
4097	93	
4143	94	
4156	94	39
4190	95	
4237	96	
4261	96	40
4284	97	
4330	98	
4366	98	41
4377	99	
4424	100	
4471	101	42
4518	102	
4565	103	
4576	103	43
4612	104	
4658	105	
4680	105	44
4705	106	
4752	107	
4785	107	45
4799	108	
4846	109	
4890	109	46
4893	110	
4940	111	
4987	112	
4995	112	47
5034	113	
5081	114	
5099	114	48
5128	115	
5175	116	
5204	116	49
5222	117	
5269	118	
5309	118	50
5317	119	
5364	120	
5411	121	
5414	121	51
5458	122	
5505	123	
5519	123	52
5552	124	
5599	125	
5623	125	53
5647	126	
5694	127	
5728	127	54
5741	128	
5788	129	
5833	129	55
5835	130	
5883	131	
5930	132	
5938	132	56
5977	133	
6024	134	
6042	134	57
6072	135	
6119	136	
6147	136	58
6166	137	
6214	138	
6252	138	59
6261	139	
6308	140	
6355	141	
6357	141	60
6403	142	
6450	143	
6462	143	61
6498	144	
6545	145	
6566	145	62
6592	146	
6640	147	
6671	147	63
6687	148	
6734	149	
6776	149	64
6782	150	
6829	151	
6877	152	
6881	152	65
6924	153	
6972	154	
6985	154	66
7019	155	
7067	156	
7090	156	67
7114	157	
7161	158	
7195	158	68
7209	159	
7256	160	
7300	160	69
7304	161	
7351	162	
7399	163	
7404	163	70
7447	164	
7494	165	
7509	165	71
7542	166	
7589	167	
7614	167	72
7637	168	
7684	169	
7719	169	73
7732	170	
7779	171	
7824	171	74
7827	172	
7875	173	
7922	174	
7928	174	75
7970	175	
8017	176	
8033	176	76
8065	177	
8113	178	
8138	178	77
8160	179	
8208	180	
8243	180	78
8256	181	
8303	182	
8347	182	79
8351	183	
8399	184	
8446	185	
8452	185	80
8494	186	
8542	187	
8557	187	81
8589	188	
8637	189	
8662	189	82
8685	190	
8732	191	
8767	191	83
8780	192	
8828	193	
8871	193	84
8875	194	
8923	195	
8971	196	
8976	196	85
9019	197	
9066	198	
9081	198	86
9114	199	
9162	200	
9186	200	87
9210	201	
9257	202	
9290	202	88
9305	203	
9353	204	
9395	204	89
9401	205	
9449	206	
9496	207	
9500	207	90
9544	208	
9592	209	
9605	209	91
9640	210	
9688	211	
9710	211	92
9735	212	
9783	213	
9814	213	93
9831	214	
9879	215	
9919	215	94
9927	216	
9975	217	
10023	218	
10070	219	

Appendix XIII

A. Particulate Contamination: Sub-visible Particles[1]

(Ph. Eur. method 2.9.19)

Particulate contamination of injections and infusions consists of mobile, undissolved particles, other than gas bubbles, that are unintentionally present. ◊Particulate contamination may originate from various sources and is to be minimised, independent of its type. The level of particulate contamination in parenteral preparations must be controlled.◊

For the determination of particulate contamination, 2 procedures, Method 1 (light obscuration particle count test) and Method 2 (microscopic particle count test), are specified hereinafter. When examining injections and infusions for sub-visible particles, Method 1 is preferably applied. However, it may be necessary to test some preparations by the light obscuration particle count test followed by the microscopic particle count test to reach a conclusion on conformance to the requirements.

Not all parenteral preparations can be examined for sub-visible particles by one or both of these methods. When Method 1 is not applicable, e.g. in case of preparations having reduced clarity or increased viscosity, the test is carried out according to Method 2. Emulsions, colloids, and liposomal preparations are examples. Similarly, products that produce air or gas bubbles when drawn into the sensor may also require ◊specific precautions during sample preparation and/or◊ microscopic particle count testing. If the viscosity of the preparation to be tested is sufficiently high so as to preclude its examination by either test method, a quantitative dilution with an appropriate ◊particle-free◊ diluent may be made to decrease viscosity, as necessary, to allow the analysis to be performed.

The results obtained in examining a discrete unit or group of units for particulate contamination cannot be extrapolated with certainty to other units that remain untested. Thus, statistically sound sampling plans must be developed if valid inferences are to be drawn from observed data to characterise the level of particulate contamination in a large group of units.

METHOD 1. LIGHT OBSCURATION PARTICLE COUNT TEST

Use a suitable apparatus based on the principle of light blockage which allows an automatic determination of the size of particles and the number of particles according to size.

The apparatus is calibrated using suitable certified reference materials consisting of dispersions of spherical particles of known sizes between 10 µm and 25 µm. These standard particles are dispersed in *particle-free water R*. Care must be taken to avoid aggregation of particles during dispersion.

General precautions

The test is carried out under conditions limiting particulate contamination, preferably in a laminar-flow cabinet.

Very carefully wash the glassware and filtration equipment used, except for the membrane filters, with a warm detergent solution and rinse with abundant amounts of water to remove all traces of detergent. Immediately before use, rinse the equipment from top to bottom, outside and then inside, with *particle-free water R*.

Take care not to introduce air bubbles into the preparation to be examined, especially when fractions of the preparation are being transferred to the container in which the determination is to be carried out.

In order to check that the environment is suitable for the test, that the glassware is properly cleaned and that the water to be used is particle-free, the following test is carried out: determine the particulate contamination of 5 samples of *particle-free water R*, each of 5 mL, according to the method described below. If the number of particles of 10 µm or greater size exceeds 25 for the combined 25 mL, the precautions taken for the test are not sufficient.

The preparatory steps must be repeated until the environment, glassware and water are suitable for the test.

Method

Mix the contents of the sample by slowly inverting the container 20 times successively. If necessary, cautiously remove the sealing closure. Clean the outer surfaces of the container opening using a jet of *particle-free water R* and remove the closure, avoiding any contamination of the contents. Eliminate gas bubbles by appropriate measures such as allowing the sample to stand for 2 min or sonicating.

For large-volume parenterals, single units are tested.

For small-volume parenterals less than 25 mL in volume, the contents of 10 or more units are combined in a cleaned container to obtain a volume of not less than 25 mL; where justified and authorised, the test solution may be prepared by mixing the contents of a suitable number of vials and diluting to 25 mL with *particle-free water R* or with an appropriate solvent without contamination of particles when *particle-free water R* is not suitable. Small-volume parenterals having a volume of 25 mL or more may be tested individually.

Powders for parenteral administration are reconstituted with *particle-free water R* or with an appropriate solvent without contamination of particles when *particle-free water R* is not suitable.

The number of test specimens must be adequate to provide a statistically sound assessment. For large-volume parenterals or for small-volume parenterals having a volume of 25 mL or more, fewer than 10 units may be tested, based on an appropriate sampling plan.

Remove 4 portions, each of not less than 5 mL, and count the number of particles equal to or greater than 10 µm and 25 µm. Disregard the result obtained for the first portion, and calculate the mean number of particles for the preparation to be examined.

◊Alternative method

This method is intended to improve the applicability of the test to biological preparations. However, it can be used for any type of preparation.

Clean the outer surfaces of the container(s) using a jet of *particle-free water R*, avoiding any contamination of the contents. Samples are tested under in-use conditions, as directed in the instructions for use (e.g. expelled syringe contents).

For parenteral preparations that have a sufficient volume (i.e. a volume large enough to permit testing), testing of individual units is often preferred.

If the volume is not sufficient, take a suitable number of units, mix each one carefully and thoroughly and then combine the contents in a separate container to obtain the volume required for a single test, depending on the instrument capability and the properties of the sample.

Powders for parenteral administration are reconstituted with *particle-free water R* or with an appropriate particle-free solvent when *particle-free water R* is not suitable.

[1] *This chapter has undergone pharmacopoeial harmonisation. See chapter 5.8 Pharmacopoeial harmonisation.*

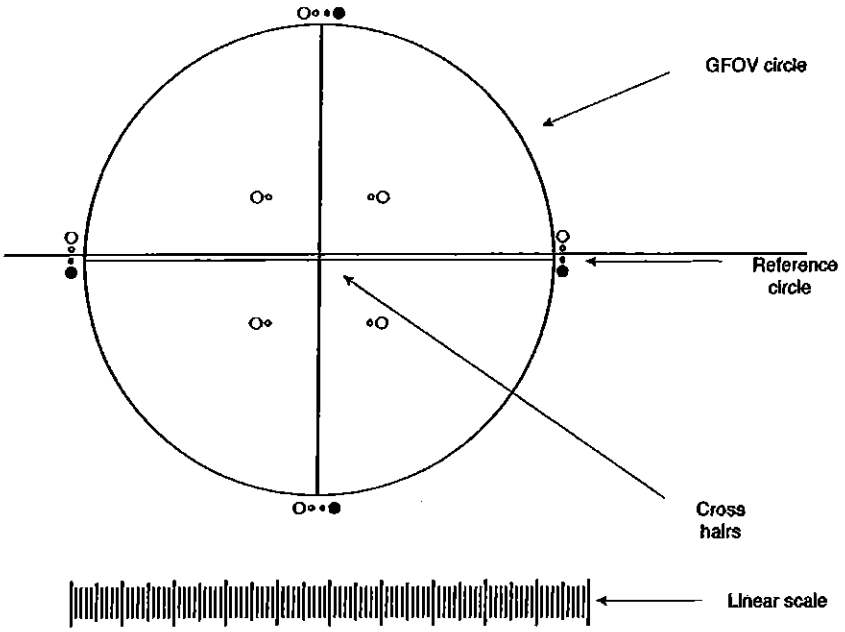


Figure 2.9.19.-1. – *Circular diameter graticule*

Eliminate gas bubbles by appropriate measures such as allowing the sample to stand, applying a gentle vacuum or sonicating. Sonication of preparations containing proteins is not recommended.

The number of test specimens must be adequate to provide a statistically sound assessment. For large- and small-volume parenterals, an adequate volume of sample must be provided for analysis; however, single units may be tested based on a statistically sound sampling plan.

Remove 4 approximately equal portions, typically 5 mL each, and count the number of particles equal to or greater than 10 µm and 25 µm. Disregard the result obtained for the first portion, and calculate the mean number of particles for the preparation to be examined. Volumes smaller than 5 mL can also be tested, provided that this amount is appropriately justified. In general, for parenteral preparations that do not have a sufficient volume (e.g. less than 25 mL), performing the test using a volume of 1 mL to 5 mL may be acceptable if permitted by the instrument.◊

Evaluation

For preparations supplied in containers with a nominal volume of more than 100 mL, apply the criteria of test 1.A.

For preparations supplied in containers with a nominal volume of less than 100 mL, apply the criteria of test 1.B.

♦For preparations supplied in containers with a nominal volume of 100 mL, apply the criteria of test 1.B.♦

If the average number of particles exceeds the limits, test the preparation by the microscopic particle count test.

Test 1.A – Solutions for infusion or solutions for injection supplied in containers with a nominal content of more than 100 mL

The preparation complies with the test if the average number of particles present in the units tested does not exceed 25 per millilitre equal to or greater than 10 µm and does not exceed 3 per millilitre equal to or greater than 25 µm.

Test 1.B – Solutions for infusion or solutions for injection supplied in containers with a nominal content of less than 100 mL

The preparation complies with the test if the average number of particles present in the units tested does not exceed 6000 per container equal to or greater than 10 µm and does not exceed 600 per container equal to or greater than 25 µm.

METHOD 2. MICROSCOPIC PARTICLE COUNT TEST

Use a suitable binocular microscope, filter assembly for retaining particulate contamination and membrane filter for examination.

The microscope is equipped with an ocular micrometer calibrated with an objective micrometer, a mechanical stage capable of holding and traversing the entire filtration area of the membrane filter, 2 suitable illuminators to provide episcopic illumination in addition to oblique illumination, and is adjusted to 100 ± 10 magnifications.

The ocular micrometer is a circular diameter graticule (see Figure 2.9.19.-1.) and consists of a large circle divided by crosshairs into quadrants, transparent and black reference circles 10 µm and 25 µm in diameter at 100 magnifications, and a linear scale graduated in 10 µm increments. It is calibrated using a stage micrometer that is certified by either a domestic or an international standard institution. A relative error of the linear scale of the graticule within ± 2 per cent is acceptable. The large circle is designated the graticule field of view (GFOV).

2 illuminators are required. One is an episcopic brightfield illuminator internal to the microscope, the other is an external, focusable auxiliary illuminator adjustable to give reflected oblique illumination at an angle of 10-20°.

The filter assembly for retaining particulate contamination consists of a filter holder made of glass or other suitable material, and is equipped with a vacuum source and a suitable membrane filter.

The membrane filter is of suitable size, black or dark grey in colour, non-gridded or gridded, and 1.0 µm or finer in nominal pore size.

General precautions

The test is carried out under conditions limiting particulate contamination, preferably in a laminar-flow cabinet.

Very carefully wash the glassware and filter assembly used, except for the membrane filter, with a warm detergent

solution and rinse with abundant amounts of water to remove all traces of detergent. Immediately before use, rinse both sides of the membrane filter and the equipment from top to bottom, outside and then inside, with *particle-free water R*.

In order to check that the environment is suitable for the test, that the glassware and the membrane filter are properly cleaned and that the water to be used is particle-free, the following test is carried out: determine the particulate contamination of a 50 mL volume of *particle-free water R* according to the method described below. If more than 20 particles 10 µm or larger in size or if more than 5 particles 25 µm or larger in size are present within the filtration area, the precautions taken for the test are not sufficient. The preparatory steps must be repeated until the environment, glassware, membrane filter and water are suitable for the test.

Method

Mix the contents of the samples by slowly inverting the container 20 times successively. If necessary, cautiously remove the sealing closure. Clean the outer surfaces of the container opening using a jet of *particle-free water R* and remove the closure, avoiding any contamination of the contents.

For large-volume parenterals, single units are tested. For small-volume parenterals less than 25 mL in volume, the contents of 10 or more units are combined in a cleaned container; where justified and authorised, the test solution may be prepared by mixing the contents of a suitable number of vials and diluting to 25 mL with *particle-free water R* or with an appropriate solvent without contamination of particles when *particle-free water R* is not suitable. Small-volume parenterals having a volume of 25 mL or more may be tested individually.

Powders for parenteral administration are constituted with *particle-free water R* or with an appropriate solvent without contamination of particles when *particle-free water R* is not suitable.

The number of test specimens must be adequate to provide a statistically sound assessment. For large-volume parenterals or for small-volume parenterals having a volume of 25 mL or more, fewer than 10 units may be tested, based on an appropriate sampling plan.

Wet the inside of the filter holder fitted with the membrane filter with several millilitres of *particle-free water R*. Transfer to the filtration funnel the total volume of a solution pool or of a single unit, and apply vacuum. If needed, add stepwise a portion of the solution until the entire volume is filtered. After the last addition of solution, begin rinsing the inner walls of the filter holder by using a jet of *particle-free water R*. Maintain the vacuum until the surface of the membrane filter is free from liquid. Place the filter in a Petri dish and allow the filter to air-dry with the cover slightly ajar. After the filter has been dried, place the Petri dish on the stage of the microscope, scan the entire membrane filter under the reflected light from the illuminating device, and count the number of particles that are equal to or greater than 10 µm and the number of particles that are equal to or greater than 25 µm. Alternatively, partial filter count and determination of the total filter count by calculation is allowed. Calculate the mean number of particles for the preparation to be examined.

The particle sizing process with the use of the circular diameter graticule is carried out by transforming mentally the image of each particle into a circle and then comparing it to the 10 µm and 25 µm graticule reference circles. Thereby the particles are not moved from their initial locations within the graticule field of view and are not superimposed on the reference circles for comparison. The inner diameter of the transparent graticule reference circles is used to size white and transparent particles, while dark particles are sized by using the outer diameter of the black opaque graticule reference circles.

In performing the microscopic particle count test do not attempt to size or enumerate amorphous, semi-liquid, or otherwise morphologically indistinct materials that have the appearance of a stain or discolouration on the membrane filter. These materials show little or no surface relief and present a gelatinous or film-like appearance. In such cases the interpretation of enumeration may be aided by testing a sample of the solution by the light obscuration particle count test.

◊*Alternative method*

This method is intended to improve the applicability of the test to biological preparations. However, it can be used for any type of preparation.

Clean the outer surfaces of the container(s) using a jet of *particle-free water R*, avoiding any contamination of the contents. Samples are tested under in-use conditions, as directed in the instructions for use (e.g. expelled syringe contents).

For parenteral preparations that have a sufficient volume (i.e. a volume large enough to permit testing), testing of individual units is often preferred.

If the volume is not sufficient, take a suitable number of units, mix each one carefully and thoroughly and then combine the contents in a separate container to obtain the volume required for a single test, depending on the instrument capability and the properties of the sample.

Powders for parenteral administration are reconstituted with *particle-free water R* or with an appropriate particle-free solvent when *particle-free water R* is not suitable.

The number of test specimens must be adequate to provide a statistically sound assessment. For large- and small-volume parenterals, an adequate volume of sample must be provided for analysis; however, single units may be tested based on a statistically sound sampling plan.

Continue as described under Method 2 above, starting from "Wet the inside...".◊

Evaluation

For preparations supplied in containers with a nominal volume of more than 100 mL, apply the criteria of test 2.A.

For preparations supplied in containers with a nominal volume of less than 100 mL, apply the criteria of test 2.B.

♦For preparations supplied in containers with a nominal volume of 100 mL, apply the criteria of test 2.B.♦

Test 2.A – Solutions for infusion or solutions for injection supplied in containers with a nominal content of more than 100 mL

The preparation complies with the test if the average number of particles present in the units tested does not exceed 12 per millilitre equal to or greater than 10 µm and does not exceed 2 per millilitre equal to or greater than 25 µm.

Test 2.B – Solutions for infusion or solutions for injection supplied in containers with a nominal content of less than 100 mL

The preparation complies with the test if the average number of particles present in the units tested does not exceed 3000 per container equal to or greater than 10 µm and does not exceed 300 per container equal to or greater than 25 µm.

B. Particulate Contamination: Visible Particles

(Ph. Eur. method 2.9.20)

Particulate contamination consists of mobile undissolved substances, other than gas bubbles, unintentionally present in liquid preparations.

The test is intended to provide a simple procedure for the visual assessment of the quality of liquid preparations, if applicable after reconstitution, as regards visible particles.

EQUIPMENT

The equipment (see Figure 2.9.20.-1) consists of a viewing station comprising:

— a matt black panel (A) of appropriate size held in a vertical position;
— a non-glare white panel (B) of appropriate size held in a vertical position next to the black panel;
— a non-glare white panel (C) of appropriate size held in a horizontal position next to (A) and (B);
— a lampholder (D) fitted with a suitable, shaded, white-light source and with a suitable light diffuser (e.g. a viewing illuminator containing two 13 W fluorescent tubes, each 525 mm in length, or an appropriate light-emitting diode (LED) light source). The intensity of illumination at the viewing point is maintained between 2000 lux and 3750 lux, although higher values may be required for coloured glass or plastic containers and for coloured or turbid preparations.

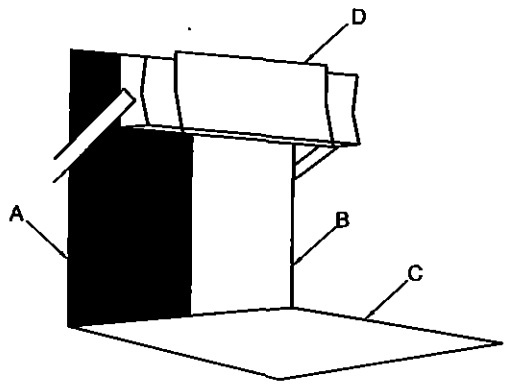


Figure 2.9.20.-1. – *Equipment for visible particles*

METHOD

Adequate visual inspection of the container and contents is necessary, which may require the removal of any adherent labels from the container. Gently swirl or invert the container, ensuring that air bubbles are not introduced, and observe without magnification for about 5 s in front of the white panel (B), although longer observation times may be required for coloured glass or plastic containers and for coloured or turbid preparations. Where inspection in the primary container is not possible, the contents may be transferred for inspection into a sample container that is free from visible particles, taking precautions to prevent contamination during transfer. Repeat the inspection in front of the black panel (A). Record the presence of any visible particles.

Appendix XIV

Biological Assays and Tests

General guidance concerning biological assays and tests is provided in Supplementary Chapter I H.

A. Microbiological Assay of Antibiotics

(Ph. Eur. method 2.7.2)

The potency of an antibiotic is estimated by comparing the inhibition of growth of sensitive micro-organisms produced by known concentrations of the antibiotic to be examined and a reference substance.

The reference substances used in the assays are substances whose activity has been precisely determined with reference to the corresponding international standard or international reference preparation.

The assay must be designed in a way that will permit examination of the validity of the mathematical model on which the potency equation is based. If a parallel-line model is chosen, the 2 log dose-response (or transformed response) lines of the preparation to be examined and the reference preparation must be parallel; they must be linear over the range of doses used in the calculation. These conditions must be verified by validity tests for a given probability, usually $P = 0.05$. Other mathematical models, such as the slope ratio model, may be used provided that proof of validity is demonstrated.

Unless otherwise stated in the monograph, the confidence limits ($P = 0.95$) of the assay for potency are not less than 95 per cent and not more than 105 per cent of the estimated potency.

Carry out the assay by method A or method B.

A. DIFFUSION METHOD

Liquefy a medium suitable for the conditions of the assay and inoculate it at a suitable temperature, for example 48 °C to 50 °C for vegetative forms, with a known quantity of a suspension of micro-organisms sensitive to the antibiotic to be examined, such that clearly defined zones of inhibition of suitable diameter are produced with the concentrations of the antibiotic used for the assay. Immediately pour into Petri dishes or large rectangular dishes a quantity of the inoculated medium to form a uniform layer 2-5 mm thick. Alternatively, the medium may consist of 2 layers, only the upper layer being inoculated.

Store the dishes so that no appreciable growth or death of the micro-organisms occurs before the dishes are used and so that the surface of the medium is dry at the time of use.

Using the solvent and the buffer solution indicated in Table 2.7.2.-1, prepare solutions of the reference substance and of the antibiotic to be examined having known concentrations and presumed to be of equal activity. Apply the solutions to the surface of the medium, for example, in sterile cylinders of porcelain, stainless steel or other suitable material, or in cavities prepared in the agar. The same volume of solution must be added to each cylinder or cavity. Alternatively, use sterile absorbent paper discs of suitable quality; impregnate the discs with the solutions of the reference substance or the solutions of the antibiotic to be examined and place on the surface of the agar.

In order to assess the validity of the assay, use not fewer than 3 doses of the reference substance and 3 doses of the antibiotic to be examined having the same presumed activity as the doses of the reference substance. It is preferable to use a series of doses in geometric progression. In routine assays when the linearity of the system has been demonstrated over an adequate number of experiments using a three-point assay, a two-point assay may be sufficient, subject to agreement by the competent authority. However, in all cases of dispute, a three-point assay as described above must be applied.

Arrange the solutions on each Petri dish or on each rectangular dish according to a statistically suitable design, except for small Petri dishes that cannot accommodate more than 6 solutions, arrange the solutions of the antibiotic to be examined and the solutions of the reference substance in an alternate manner to avoid interaction of the more concentrated solutions.

Incubate at a suitable temperature for about 18 h. A period of diffusion prior to incubation, usually 1-4 h, at room temperature or at about 4 °C, as appropriate, may be used to minimise the effects of the variation in time between the application of the solutions and to improve the regression slope.

Measure the diameters to the nearest 0.1 mm or the areas of the circular inhibition zones to the nearest 0.01 and calculate the potency using appropriate statistical methods.

Use in each assay the number of replications per dose sufficient to ensure the required accuracy and precision. The assay may be repeated and the results combined statistically to obtain the required accuracy and precision and to ascertain whether the potency of the antibiotic to be examined is not less than the minimum required.

B. TURBIDIMETRIC METHOD

Inoculate a suitable medium with a suspension of the chosen micro-organism having a sensitivity to the antibiotic to be examined such that a sufficiently large inhibition of microbial growth occurs in the conditions of the test. Use a known quantity of the suspension chosen so as to obtain a readily measurable opacity after an incubation period of about 4 h.

Use the inoculated medium immediately after its preparation.

Using the solvent and the buffer solution indicated in Table 2.7.2.-2 prepare solutions of the reference substance and of the antibiotic to be examined having known concentrations presumed to be of equal activity.

In order that the validity of the assay may be assessed, use not fewer than 3 doses of the reference substance and 3 doses of the antibiotic to be examined having the same presumed activity as the doses of the reference substance. It is preferable to use a series of doses in geometric progression. In order to obtain the required linearity, it may be necessary to select from a large number 3 consecutive doses, using corresponding doses for the reference substance and the antibiotic to be examined.

Distribute an equal volume of each of the solutions into identical test-tubes and add to each tube an equal volume of inoculated medium (for example, 1 mL of the solution and 9 mL of the medium). For the assay of tyrothricin add 0.1 mL of the solution to 9.9 mL of inoculated medium.

Prepare at the same time 2 control tubes without antibiotic, both containing the inoculated medium and to one of which is added immediately 0.5 mL of *formaldehyde R*. These tubes are used to set the optical apparatus used to measure the growth.

Place all the tubes, randomly distributed or in a Latin square or randomised block arrangement, in a water-bath or other suitable apparatus fitted with a means of bringing all the tubes rapidly to the appropriate incubation temperature and maintain them at that temperature for 3-4 h, taking precautions to ensure uniformity of temperature and identical incubation time.

After incubation, stop the growth of the micro-organisms by adding 0.5 mL of *formaldehyde R* to each tube or by heat treatment and measure the opacity to 3 significant figures using suitable optical apparatus. Alternatively use a method which allows the opacity of each tube to be measured after exactly the same period of incubation.

Calculate the potency using appropriate statistical methods.

Linearity of the dose-response relationship, transformed or untransformed, is often obtained only over a very limited range. It is this range which must be used in calculating the activity and it must include at least 3 consecutive doses in order to permit linearity to be verified. In routine assays when the linearity of the system has been demonstrated over an adequate number of experiments using a three-point assay, a two-point assay may be sufficient, subject to agreement by the competent authority. However, in all cases of dispute, a three-point assay must be applied.

Use in each assay the number of replications per dose sufficient to ensure the required accuracy and precision. The assay may be repeated and the results combined statistically to obtain the required accuracy and precision and to ascertain whether the potency of the antibiotic to be examined is not less than the minimum required.

The following section is published for information.

RECOMMENDED MICRO-ORGANISMS

The following text details the recommended micro-organisms and the conditions of use. Other micro-organisms may be used provided that they are shown to be sensitive to the antibiotic to be examined and are used in appropriate media and appropriate conditions of temperature and pH.
The concentrations of the solutions used should be chosen so as to ensure that a linear relationship exists between the logarithm of the dose and the response in the conditions of the test.

Preparation of inocula

Bacillus cereus var. *mycoides*; *Bacillus subtilis*; *Bacillus pumilus*.
Spore suspensions of the organisms to be used as inocula are prepared as follows.

Grow the organism at 35-37 °C for 7 days on the surface of a suitable medium to which has been added 0.001 g/L of *manganese sulfate R*. Using sterile *water R*, wash off the growth, which consists mainly of spores. Heat the suspension at 70 °C for 30 min and dilute to give an appropriate concentration of spores, usually 10×10^6 to 100×10^6 per millilitre. The spore suspensions may be stored for long periods at a temperature not exceeding 4 °C.

Alternatively, spore suspensions may be prepared by cultivating the organisms in medium C at 26 °C for 4-6 days, then adding, aseptically, sufficient *manganese sulfate R* to give a concentration of 0.001 g/L and incubating for a further 48 h. Examine the suspension microscopically to ensure that adequate spore formation has taken place (about 80 per cent) and centrifuge. Re-suspend the sediment in sterile *water R* to give a concentration of 10×10^6 to 100×10^6 spores per millilitre, and then heat to 70 °C for 30 min. Store the suspension at a temperature not exceeding 4 °C.

Table 2.7.2.-1. – *Diffusion assay*

Antibiotic	Reference substance	Solvent to be used in preparing the stock solution	Buffer solution (pH)	Micro-organism	Medium and final pH (± 0.1 pH unit)	Incubation temperature
Amphotericin B	*Amphotericin B for microbiological assay CRS*	*Dimethyl sulfoxide R*	pH 10.5 (0.2 M)	*Saccharomyces cerevisiae* ATCC 9763 IP 1432-83	F - pH 6.1	35-37 °C
Bacitracin zinc	*Bacitracin zinc CRS*	*0.01 M hydrochloric acid*	pH 7.0 (0.05 M)	*Micrococcus luteus* NCTC 7743 CIP 53.160 ATCC 10240	A - pH 7.0	35-39 °C
Bleomycin sulfate	*Bleomycin sulfate CRS*	*Water R*	pH 6.8 (0.1 M)	*Mycobacterium smegmatis* ATCC 607	G - pH 7.0	35-37 °C
Colistimethate sodium	*Colistimethate sodium CRS*	*Water R*	pH 6.0 (0.05 M)	*Bordetella bronchiseptica* NCTC 8344 CIP 53.157 ATCC 4617	B - pH 7.3	35-39 °C
				Escherichia coli NCIMB 8879 CIP 54.127 ATCC 10536	B - pH 7.3	35-39 °C
Colistin sulfate	*Colistin sulfate for microbiological assay CRS*	*Water R*	pH 6.0 (0.05 M)	*Bordetella bronchiseptica* NCTC 8344 CIP 53.157 ATCC 4617	B - pH 7.3	35-39 °C
				Escherichia coli NCIMB 8879 CIP 54.127 ATCC 10536	B - pH 7.3	35-39 °C
Framycetin sulfate	*Framycetin sulfate CRS*	*Water R*	pH 8.0 (0.05 M)	*Bacillus subtilis* NCTC 10400 CIP 52.62 ATCC 6633	E - pH 7.9	30-37 °C
				Bacillus pumilus NCTC 8241 CIP 76.18	E - pH 7.9	30-37 °C
Gentamicin sulfate	*Gentamicin sulfate CRS*	*Water R*	pH 8.0 (0.05 M)	*Bacillus pumilus* NCTC 8241 CIP 76.18	A - pH 7.9	35-39 °C
				Staphylococcus epidermidis NCIMB 8853 CIP 68.21 ATCC 12228	A - pH 7.9	35-39 °C
Josamycin	*Josamycin CRS*	*Methanol R* (see the monograph)	pH 5.6	*Bacillus subtilis* CIP 52.62 ATCC 6633 NCTC 10400	A - pH 6.6	35-37 °C
Josamycin propionate	*Josamycin propionate CRS*	*Methanol R* (see the monograph)	pH 5.6	*Bacillus subtilis* CIP 52.62 ATCC 6633 NCTC 10400	A - pH 6.6	35-37 °C
Kanamycin monosulfate Kanamycin acid sulfate	*Kanamycin monosulfate CRS*	*Water R*	pH 8.0 (0.05 M)	*Bacillus subtilis* NCTC 10400 CIP 52.62 ATCC 6633	A - pH 7.9	30-37 °C
				Staphylococcus aureus NCTC 7447 CIP 53.156 ATCC 6538 P	A - pH 7.9	35-39 °C

Antibiotic	Reference substance	Solvent to be used in preparing the stock solution	Buffer solution (pH)	Micro-organism	Medium and final pH (± 0.1 pH unit)	Incubation temperature
Neomycin sulfate	*Neomycin sulfate for microbiological assay CRS*	*Water R*	pH 8.0 (0.05 M)	*Bacillus pumilus* NCTC 8241 CIP 76.18	E - pH 7.9	30-37 °C
				Bacillus subtilis NCTC 10400 CIP 52.62 ATCC 6633	E - pH 7.9	30-37 °C
Netilmicin sulfate	*Netilmicin sulfate CRS*	*Water R*	pH 8.0 ± 0.1	*Staphylococcus aureus* ATCC 6538 P CIP 53.156	A - pH 7.9	32-35 °C
Nystatin	*Nystatin CRS*	*Dimethylformamide R*	pH 6.0 (0.05 M) containing 5 per cent *V/V* of *dimethylformamide R*	*Candida tropicalis* CIP 1433-83 NCYC 1393	F - pH 6.0	30-37 °C
				Saccharomyces cerevisiae NCYC 87 CIP 1432-83 ATCC 9763	F - pH 6.0	30-32 °C
Polymyxin B sulfate	*Polymyxin B sulfate for microbiological assay CRS*	*Water R*	pH 6.0 (0.05 M)	*Bordetella bronchiseptica* NCTC 8344 CIP 53.157 ATCC 4617	B - pH 7.3	35-39 °C
Rifamycin sodium	*Rifamycin sodium CRS*	*Methanol R*	pH 7.0 (0.05 M)	*Micrococcus luteus* NCTC 8340 CIP 53.45 ATCC 9341	A - pH 6.6	35-39 °C
Spiramycin	*Spiramycin CRS*	*Methanol R*	pH 8.0 (0.05 M)	*Bacillus subtilis* NCTC 10400 CIP 52.62 ATCC 6633	A - pH 7.9	30-32 °C
Streptomycin sulfate	*Streptomycin sulfate CRS*	*Water R*	pH 8.0 (0.05 M)	*Bacillus subtilis* NCTC 8236 CIP 1.83	A - pH 7.9	30-37 °C
				Bacillus subtilis NCTC 10400 CIP 52.62 ATCC 6633	A - pH 7.9	30-37 °C
Teicoplanin	*Teicoplanin CRS*	pH 6.0 (0.05 M)	pH 6.0 (0.05 M)	*Bacillus subtilis* NCTC 10400 CIP 52.62 ATCC 6633	H - pH 7.8-8.0	35-37 °C
Tylosin for veterinary use Tylosin phosphate for veterinary use Tylosin tartrate for veterinary use	*Tylosin CRS*	2.5 per cent *V/V* solution of *methanol R* in *0.1 M phosphate buffer solution pH 7.0 R*	A mixture of 40 volumes of *methanol R* and 60 volumes of *0.1 M phosphate buffer solution pH 8.0 R*	*Micrococcus luteus* NCTC 8340 CIP 53.45 ATCC 9341	A - pH 8.0	32-35 °C
Vancomycin hydrochloride	*Vancomycin hydrochloride CRS*	*Water R*	pH 8.0	*Bacillus subtilis* NCTC 10400 CIP 52.62 ATCC 6633	A - pH 8.0	37-39 °C

Bordetella bronchiseptica. Grow the test organism on medium B at 35-37 °C for 16-18 h. Wash off the bacterial growth with sterile *water R* and dilute to a suitable opacity.

Staphylococcus aureus; *Klebsiella pneumoniae*; *Escherichia coli*; *Micrococcus luteus*; *Staphylococcus epidermidis*. Prepare as described above for *B. bronchiseptica* but using medium A and adjusting the opacity to one which has been shown to produce a satisfactory dose-response relationship in the turbidimetric assay, or to produce clearly defined zones of inhibition of convenient diameter in the diffusion assay, as appropriate.

Saccharomyces cerevisiae; *Candida tropicalis*. Grow the test organism on medium F at 30-37 °C for 24 h. Wash off the growth with a sterile 9 g/L solution of *sodium chloride R*. Dilute to a suitable opacity with the same solution.

Table 2.7.2.-2. – *Turbidimetric assay*

Antibiotic	Reference substance	Solvent to be used in preparing the stock solution	Buffer solution (pH)	Micro-organism	Medium and final pH (± 0.1 pH unit)	Incubation temperature
Colistimethate sodium	*Colistimethate sodium CRS*	*Water R*	pH 7.0	*Escherichia coli* NCIMB 8666 CIP 2.83 ATCC 9637	C - pH 7.0	35-37 °C
Colistin sulfate	*Colistin sulfate for microbiological assay CRS*	*Water R*	pH 7.0	*Escherichia coli* NCIMB 8666 CIP 2.83 ATCC 9637	C - pH 7.0	35-37 °C
Framycetin sulfate	*Framycetin sulfate CRS*	*Water R*	pH 8.0	*Staphylococcus aureus* NCTC 7447 CIP 53.156 ATCC 6538 P	C - pH 7.0	35-37 °C
Gentamicin sulfate	*Gentamicin sulfate CRS*	*Water R*	pH 7.0	*Staphylococcus aureus* NCTC 7447 CIP 53.156 ATCC 6538 P	C - pH 7.0	35-37 °C
Gramicidin	*Gramicidin CRS*	*Methanol R*	pH 7.0*	*Enterococcus hirae* CIP 58.55 ATCC 10541 *Staphylococcus aureus* ATCC 6538 P	C - pH 7.0	35-37 °C
	*Addition of a detergent may be necessary to avoid adsorption on the material during the dilutions, for example 0.1 mg/mL of *polysorbate 80 R*					
Josamycin	*Josamycin CRS*	*Methanol R* (see the monograph)	pH 5.6	*Staphylococcus aureus* CIP 53.156 ATCC 6538 P NCTC 7447	C - pH 8.0	35-37 °C
Josamycin propionate	*Josamycin propionate CRS*	*Methanol R* (see the monograph)	pH 5.6	*Staphylococcus aureus* CIP 53.156 ATCC 6538 P NCTC 7447	C - pH 8.0	35-37 °C
Kanamycin monosulfate Kanamycin acid sulfate	*Kanamycin monosulfate CRS*	*Water R*	pH 8.0	*Staphylococcus aureus* NCTC 7447 CIP 53.156 ATCC 6538 P	C - pH 7.0	35-37 °C
Neomycin sulfate	*Neomycin sulfate for microbiological assay CRS*	*Water R*	pH 8.0	*Staphylococcus aureus* NCTC 7447 CIP 53.156 ATCC 6538 P	C - pH 7.0	35-37 °C
Rifamycin sodium	*Rifamycin sodium CRS*	*Methanol R*	pH 7.0	*Escherichia coli* NCIMB 8879 CIP 54.127 ATCC 10536	C - pH 7.0	35-37 °C
Spiramycin	*Spiramycin CRS*	*Methanol R*	pH 7.0	*Staphylococcus aureus* NCTC 7447 CIP 53.156 ATCC 6538 P	C - pH 7.0	35-37 °C
Streptomycin sulfate	*Streptomycin sulfate CRS*	*Water R*	pH 8.0	*Klebsiella pneumoniae* NCTC 7427 CIP 53.153 ATCC 10031	C - pH 7.0	35-37 °C
Tylosin for veterinary use Tylosin tartrate for veterinary use	*Tylosin CRS*	2.5 per cent *V/V* solution of *methanol R* in *0.1 M phosphate buffer solution pH 7.0 R*	pH 7.0	*Staphylococcus aureus* NCTC 6571 ATCC 9144 CIP 53.154	C - pH 7.0	37 °C
Tyrothricin	*Gramicidin CRS*	*Alcohol R*	*Alcohol R*	*Enterococcus hirae* ATCC 10541	C - pH 7.0	37 °C
Vancomycin hydrochloride	*Vancomycin hydrochloride CRS*	*Water R*	pH 8.0	*Staphylococcus aureus* CIP 53.156 ATCC 6538 P	C - pH 7.0	37-39 °C

Buffer solutions

Buffer solutions having a pH between 5.8 and 8.0 are prepared by mixing 50.0 mL of *0.2 M potassium dihydrogen phosphate R* with the quantity of *0.2 M sodium hydroxide* indicated in Table 2.7.2.-3. Dilute with freshly prepared *distilled water R* to produce 200.0 mL.

Table 2.7.2.-3

pH	*0.2 M Sodium hydroxide* (mL)
5.8	3.72
6.0	5.70
6.2	8.60
6.4	12.60
6.6	17.80
6.8	23.65
7.0	29.63
7.2	35.00
7.4	39.50
7.6	42.80
7.8	45.20
8.0	46.80

These buffer solutions are used for all microbiological assays shown in Table 2.7.2.-1 with the exception of bleomycin sulfate and amphotericin B.

For bleomycin sulfate, prepare the buffer solution pH 6.8 as follows: dissolve 6.4 g of *potassium dihydrogen phosphate R* and 18.9 g of *disodium hydrogen phosphate dodecahydrate R* in *water R* and dilute to 1000 mL with *water R*.

For amphotericin B, prepare the 0.2 M phosphate buffer solution pH 10.5 as follows: dissolve 35 g of *dipotassium hydrogen phosphate R* in 900 mL of *water R*, add 20 mL of *1 M sodium hydroxide* and dilute to 1000.0 mL with *water R*.

Culture media

The following media or equivalent media may be used.

Medium A

Peptone	6 g
Pancreatic digest of casein	4 g
Beef extract	1.5 g
Yeast extract	3 g
Glucose monohydrate	1 g
Agar	15 g
Water	to 1000 mL

Medium B

Pancreatic digest of casein	17 g
Papaic digest of soya bean	3 g
Sodium chloride	5 g
Dipotassium hydrogen phosphate	2.5 g
Glucose monohydrate	2.5 g
Agar	15 g
Polysorbate 80	10 g
Water	to 1000 mL

The polysorbate 80 is added to the hot solution of the other ingredients after boiling, and immediately before adjusting to volume.

Medium C

Peptone	6 g
Beef extract	1.5 g
Yeast extract	3 g
Sodium chloride	3.5 g
Glucose monohydrate	1 g
Dipotassium hydrogen phosphate	3.68 g
Potassium dihydrogen phosphate	1.32 g
Water	to 1000 mL

Medium D

Heart extract	1.5 g
Yeast extract	1.5 g
Peptone-casein	5 g
Glucose monohydrate	1 g
Sodium chloride	3.5 g
Dipotassium hydrogen phosphate	3.68 g
Potassium dihydrogen phosphate	1.32 g
Potassium nitrate	2 g
Water	to 1000 mL

Medium E

Peptone	5 g
Meat extract	3 g
Disodium hydrogen phosphate,12H_2O	26.9 g
Agar	10 g
Water	to 1000 mL

The disodium hydrogen phosphate is added as a sterile solution after sterilisation of the medium.

Medium F

Peptone	9.4 g
Yeast extract	4.7 g
Beef extract	2.4 g
Sodium chloride	10.0 g
Glucose monohydrate	10.0 g
Agar	23.5 g
Water	to 1000 mL

Medium G

Glycerol	10 g
Peptone	10 g
Meat extract	10 g
Sodium chloride	3 g
Agar	15 g
Water	to 1000 mL

pH 7.0 ± 0.1 after sterilisation.

Medium H

Peptone	5.0 g
Agar	15.0 g
Beef extract powder	3.0 g
Water	to 1000 mL

pH 7.8 - 8.0 adjusted with *0.1 M sodium hydroxide.*

Monographs of the British Pharmacopoeia

The following additional information and guidance apply to monographs of the British Pharmacopoeia.

The required minimum precision for an acceptable assay of any particular antibiotic or preparation is defined in the appropriate monograph in the paragraph on the Assay. This degree of precision is the minimum acceptable for determining that the final product complies with the official requirements. It may be inadequate for a decision about the potency that should be stated on the label or used as the basis for calculating the quantity of an antibiotic to be incorporated in a preparation. In such circumstances, assays of greater precision may be desirable with, for instance, fiducial limits of error of the order of 98 to 102%. With this degree of precision, the lower fiducial limit lies close to the estimated potency. By using this limit, instead of the estimated potency, to assign a potency to the antibiotic either for labelling or for calculating the quantity to be included in a preparation, there is less likelihood of the final preparation subsequently failing to comply with the official requirements for potency.

Culture Media The following media or equivalent media may be used.

Medium I	
D-Glucose	10 g
Tryptone	6 g
Yeast extract*	2 g
Water to produce	1000 mL

Adjust to pH 8.0 with 1M *sodium hydroxide* or 0.1M *orthophosphoric acid.*

(*Ardamine yeast extract supplied by Champlain Industries Inc., Clifton, NJ 07012, USA is suitable.)

Method A. Diffusion Method

Table 2.7.2.-1 –*Diffusion Assay; Additional Section for Monographs of the British Pharmacopoeia*

Antibiotic	Reference substance	Solvent to be used in preparing the stock Solution	Buffer solution (pH)	Micro-organism	Medium and final pH (± 0.1 pH unit)	Incubation temperature
Erythromycin Estolate Erythromycin Ethyl Succinate Erythromycin Stearate	*Erythromycin EPCRS*	*Methanol*	pH 8.0 (0.05M)	*Bacillus pumilus* NCTC 8241 CIP 76.18 *Bacillus subtilis* NCTC 10400 CIP 52.62 ATCC 6633	A; 7.9 A; 7.9	30 - 37° 30 - 37°
Lymecycline	Lymecycline 2nd Int. Ref., 1971		pH 5.8	*Bacillus pumilus* NCTC 8241 CIP 76.18	A; 6.6	37 - 39°
Polymyxin B Sulphate	*Polymyxin B Sulphate EPCRS*	*Water*	pH 6.0 (0.05M)	*Bordetella bronchiseptica* NCTC 8344 CIP 53.157 ATCC 4617	B; 7.3	35 - 39°

Method B. Turbidimetric Method

Table 2.7.2.-2 –*Turbidimetric Assay; Additional Section for Monographs of the British Pharmacopoeia*

Antibiotic	Reference substance	Solvent to be used in preparing the stock Solution	Buffer solution (pH)	Micro-organism	Medium and final pH (± 0.1 pH unit)	Incubation temperature
Apramycin	*Apramycin BPCRS*		*	*Salmonella cholerasuis*	I; 8.0	37°
Erythromycin Estolate Erythromycin Ethyl Succinate Erythromycin Stearate	*Erythromycin EPCRS*	*Methanol*	pH 8.0	*Klebsiella pneumoniae* NCTC 7427 CIP 53.153 ATCC 10031 *Staphylococcus aureus* NCTC 7447 CIP 53.156 ATCC 6538 P	D; 7.0 C; 7.0	35 - 37° 35 - 37°

* Solution prepared by dissolving 16.73 g of *dipotassium hydrogen orthophosphate* and 0.523 g of *potassium dihydrogen orthophosphate* in about 750 ml of *water*, if necessary adjusting to pH 8.0 with 0.1M *sodium hydroxide* or 0.1M *orthophosphoric acid*, and diluting to 1000 ml with *water*.

B. Immunochemical Methods

(Ph. Eur. method 2.7.1)

Immunochemical methods are based on the selective, reversible and non-covalent binding of antigens by antibodies. These methods are employed to detect or quantify either antigens or antibodies. The formation of an antigen-antibody complex may be detected, and the amount of complex formed may be measured by a variety of techniques. The provisions of this general method apply to immunochemical methods using labelled or unlabelled reagents, as appropriate.

The results of immunochemical methods depend on the experimental conditions and the nature and quality of the reagents used. It is essential to standardise the components of an immunoassay and to use, wherever available, international reference preparations for immunoassays.

The reagents necessary for many immunochemical methods are available as commercial assay kits, that is, a set including reagents (particularly the antigen or the antibody) and materials intended for the *in vitro* estimation of a specified substance as well as instructions for their proper use. The kits are used in accordance with the manufacturers' instructions; it is important to ascertain that the kits are suitable for the analysis of the substance to be examined, with particular reference to selectivity and sensitivity. Guidance concerning immunoassay kits is provided by the World Health Organization, Technical Report Series 658 (1981).

METHODS IN WHICH A LABELLED ANTIGEN OR A LABELLED ANTIBODY IS USED

Methods using labelled substances may employ suitable labels such as enzymes, fluorophores, luminophores and radioisotopes. Where the label is a radioisotope, the method is described as a "radio-immunoassay".

The recommendations for the measurement of radioactivity given in the monograph on *Radiopharmaceutical Preparations (0125)* are applicable to immunoassays involving radioisotopes. All work with radioactive materials must be carried out in conformity with national legislation and internationally accepted codes of practice for protection against radiation hazards.

METHODS IN WHICH AN UNLABELLED ANTIGEN OR ANTIBODY IS USED

Immunoprecipitation methods

Immunoprecipitation methods include flocculation and precipitation reactions. When a solution of an antigen is mixed with its corresponding antibody under suitable conditions, the reactants form flocculating or precipitating aggregates. The ratio of the reactants which gives the shortest flocculation time or the most marked precipitation is called the optimal ratio, and is usually produced by equivalent amounts of antigen and antibody. Immunoprecipitation can be assessed visually or by light-scattering techniques (nephelometric or turbidimetric assay). An increase in sensitivity can be obtained by using antigen- or antibody-coated particles (e.g. latex) as reactants.

In flocculation methods, stepwise dilutions of one of the reactants is usually used whereas, in immunodiffusion (ID) methods, the dilution is obtained by diffusion in a gel medium: concentration gradients of one or both of the reactants are obtained, thus creating zones in the gel medium where the ratio of the reactants favours precipitation. While flocculation methods are performed in tubes, immunodiffusion methods may be performed using different supports such as tubes, plates, slides, cells or chambers.

Where the immunoprecipitating system consists of one antigen combining with its corresponding antibody, the system is referred to as *simple*; when it involves related but not serologically identical reactants, the system is *complex* and where several serologically unrelated reactants are involved, the system is *multiple*.

In *simple diffusion methods*, a concentration gradient is established for only one of the reactants diffusing from an external source into the gel medium containing the corresponding reactant at a comparatively low concentration.

Single radial immunodiffusion (SRID) is a simple quantitative immunodiffusion technique. When the equilibrium between the external and the internal reactant has been established, the circular precipitation area, originating from the site of the external reactant, is directly proportional to the amount of the antigen applied and inversely proportional to the concentration of the antibody in the gel.

In *double diffusion methods*, concentration gradients are established for both reactants. Both antigen and antibody diffuse from separate sites into an initially immunologically neutral gel.

Comparative double diffusion methods Are used for qualitatively comparing various antigens versus a suitable antibody or vice versa. The comparison is based on the presence or absence of interaction between the precipitation patterns. Reactions of identity, non-identity or partial identity of antigens/antibodies can be distinguished.

Immunoelectrophoretic methods

Immunoelectrophoresis (IE) is a qualitative technique combining 2 methods: gel electrophoresis followed by immunodiffusion.

Crossed immunoelectrophoresis Is a modification of the IE method. It is suitable both for qualitative and quantitative analysis. The first part of the procedure is an ordinary gel electrophoresis, after which a longitudinal gel strip, containing the separated fractions to be determined, is cut out and transferred to another plate. The electrophoresis in the second direction is carried out perpendicular to the previous electrophoretic run in a gel containing a comparatively low concentration of antibodies corresponding to the antigens. For a given antibody concentration and gel thickness, the relationship between the area of the respective precipitation peaks and the amount of the corresponding antigen is linear.

Electroimmunoassay Often referred to as *rocket immunoelectrophoresis* is a rapid quantitative method for determining antigens with a charge differing from that of the antibodies or vice versa. The electrophoresis of the antigen to be determined is carried out in a gel containing a comparatively lower concentration of the corresponding antibody. The test material and dilutions of a standard antigen used for calibration are introduced into different wells in the gel. During electrophoresis, migrating peak-shaped precipitation zones originating from the wells are developed. The front of the precipitate becomes stationary when the antigen is no longer in excess. For a given antibody concentration, the relationship between the distance travelled by the precipitate and the amount of antigen applied is linear.

Counter-immunoelectrophoresis Is a rapid quantitative method allowing concentration gradients of external antigen and external antibody to be established in an electric field depending on the different charges. Dilutions of a standard

for calibration and dilutions of the test material are introduced into a row of wells in a gel and a fixed amount of the corresponding reactant is introduced into an opposite row of wells. The titre of the test material may be determined as the highest dilution showing a precipitation line.

A number of modifications of crossed immunoelectrophoresis and electroimmunoassay methods exist.

Other techniques combine separation of antigens by molecular size and serological properties.

Visualisation and characterisation of immunoprecipitation lines

These may be performed by selective or non-selective stains, by fluorescence, by enzyme or isotope labelling or other relevant techniques. Selective staining methods are usually performed for characterisation of non-protein substances in the precipitates.

In translucent gels such as agar or agarose, the precipitation line becomes clearly visible in the gel, provided that the concentration of each of the reactants is appropriate.

VALIDATION OF THE METHOD

Validation criteria

A quantitative immunochemical method is not valid unless:

1) The antibody or antigen does not significantly discriminate between the test and standard. For a labelled reactant, the corresponding reactant does not significantly discriminate between the labelled and unlabelled compound,

2) The method is not affected by the assay matrix, that is, any component of the test sample or its excipients, which can vary between samples. These may include high concentrations of other proteins, salts, preservatives or contaminating proteolytic activity,

3) The limit of quantitation is below the acceptance criteria stated in the individual monograph,

4) The precision of the assay is such that the variance of the results meets the requirements stated in the individual monographs,

5) The order in which the assay is performed does not give rise to systematic errors.

Validation methods

In order to verify these criteria, the validation design includes the following elements:

1) The assay is performed at least in triplicate,

2) The assay includes at least 3 different dilutions of the standard preparation and 3 dilutions of sample preparations of presumed activity similar to the standard preparation,

3) The assay layout is randomised,

4) If the test sample is presented in serum or formulated with other components, the standard is likewise prepared,

5) The test includes the measurement of non-specific binding of the labelled reactant,

6) For displacement immunoassay:

(a) maximum binding (zero displacement) is determined,

(b) dilutions cover the complete response range from values close to non-specific binding to maximum binding, preferably for both standard and test preparations.

STATISTICAL CALCULATION

To analyse the results, response curves for test and standard may be analysed by the methods described in *5.3. Statistical Analysis of Results of Biological Assays and Tests.*

Significant non-parallelism indicates that the antibody or antigen discriminates between test and standard, and the results are not valid.

In displacement immunoassays, the values for non-specific binding and maximum displacement at high test or standard concentration must not be significantly different. Differences may indicate effects due to the matrix, either inhibition of binding or degradation of tracer.

C. Test for Bacterial Endotoxins (LAL Test)[1]

(Bacterial Endotoxins, Ph. Eur. method 2.6.14)

The test for bacterial endotoxins (BET) is used to detect or quantify endotoxins from gram-negative bacteria using amoebocyte lysate from the horseshoe crab (*Limulus polyphemus* or *Tachypleus tridentatus*). There are 3 techniques for this test: the gel-clot technique, which is based on gel formation; the turbidimetric technique, based on the development of turbidity after cleavage of an endogenous substrate; and the chromogenic technique, based on the development of colour after cleavage of a synthetic peptide-chromogen complex.

The following 6 methods are described in the present chapter:

Method A.	Gel-clot method: limit test
Method B.	Gel-clot method: quantitative test
Method C.	Turbidimetric kinetic method
Method D.	Chromogenic kinetic method
Method E.	Chromogenic end-point method
Method F.	Turbidimetric end-point method

Proceed by any of the 6 methods for the test. In the event of doubt or dispute, the final decision is made based upon method A unless otherwise indicated in the monograph.

The test is carried out in a manner that avoids endotoxin contamination.

1. APPARATUS

Depyrogenate all glassware and other heat-stable apparatus in a hot-air oven using a validated process. A commonly used minimum time and temperature is 30 min at 250 °C.

If employing plastic apparatus, such as microtitre plates and pipette tips for automatic pipetters, use apparatus shown to be free of detectable endotoxin and which does not interfere in the test.

NOTE: in this chapter, the term 'tube' includes all types of receptacles, for example microtitre plate wells.

2. REAGENTS, TEST SOLUTIONS

(1) Amoebocyte lysate

Amoebocyte lysate is a lyophilised product obtained from amoebocyte lysate from the horseshoe crab (*Limulus polyphemus* or *Tachypleus tridentatus*). This reagent refers only to a product manufactured in accordance with the regulations of the competent authority.

NOTE: amoebocyte lysate reacts with some β-glucans in addition to endotoxins. Amoebocyte lysate preparations which do not react with glucans are available; they are prepared by removing from amoebocyte lysate the G factor, which reacts with glucans, or by inhibiting the G factor reacting system of amoebocyte lysate. These

[1] *This chapter has undergone pharmacopoeial harmonisation. See chapter 5.8 Pharmacopoeial harmonisation.*

preparations may be used for endotoxin testing in the presence of glucans.

(2) Lysate solution
Dissolve amoebocyte lysate in water for BET or in a buffer, as recommended by the lysate manufacturer, by gentle stirring. Store the reconstituted lysate, refrigerated or frozen, as indicated by the manufacturer.

(3) Water for BET (water for bacterial endotoxins test)
Water for injections R or water produced by other procedures that shows no reaction with the lysate employed at the detection limit of the reagent.

3. PREPARATION OF THE STANDARD ENDOTOXIN STOCK SOLUTION

The standard endotoxin stock solution is prepared from an endotoxin reference standard that has been calibrated against the International Standard, for example *endotoxin standard BRP.*

Endotoxin is expressed in International Units (IU).
The equivalence in IU of the International Standard is stated by the World Health Organization.

NOTE: one International Unit (IU) of endotoxin is equal to one Endotoxin Unit (E.U.).

Follow the specifications in the package leaflet and on the label for preparation and storage of the standard endotoxin stock solution.

4. PREPARATION OF THE STANDARD ENDOTOXIN SOLUTIONS

After vigorously mixing the standard endotoxin stock solution, prepare appropriate serial dilutions of this solution using water for BET.

Use the solutions as soon as possible to avoid loss of activity by adsorption.

5. PREPARATION OF THE TEST SOLUTIONS

Prepare the test solutions by dissolving or diluting active substances or medicinal products using water for BET. Some substances or preparations may be more appropriately dissolved or diluted in other aqueous solutions. If necessary, adjust the pH of the test solution (or dilution thereof) so that the pH of the mixture of the lysate and test solution falls within the pH range specified by the lysate manufacturer, usually 6.0 to 8.0. The pH may be adjusted by the use of acid, base or a suitable buffer, as recommended by the lysate manufacturer. Acids and bases may be prepared from concentrates or solids with water for BET in containers free of detectable endotoxin. Buffers must be validated to be free of detectable endotoxin and interfering factors.

6. DETERMINATION OF THE MAXIMUM VALID DILUTION

The Maximum Valid Dilution (MVD) is the maximum allowable dilution of a sample at which the endotoxin limit can be determined. Determine the MVD using the following formulae:

$$MVD = \frac{\text{endotoxin limit} \times \text{concentration of test solution}}{\lambda}$$

Endotoxin limit The endotoxin limit for active substances administered parenterally, defined on the basis of dose, is equal to:

$$\frac{K}{M}$$

K = threshold pyrogenic dose of endotoxin per kilogram of body mass,
M = maximum recommended bolus dose of product per kilogram of body mass.

When the product is to be injected at frequent intervals or infused continuously, M is the maximum total dose administered in a single hour period.

The endotoxin limit for active substances administered parenterally is specified in units such as IU/mL, IU/mg, IU/Unit of biological activity, etc., in monographs.

Concentration of test solution:
— mg/mL if the endotoxin limit is specified by mass (IU/mg),
— Units/mL if the endotoxin limit is specified by unit of biological activity (IU/Unit),
— mL/mL if the endotoxin limit is specified by volume (IU/mL).

λ = the labelled lysate sensitivity in the gel-clot technique (IU/mL) or the lowest concentration used in the standard curve of the turbidimetric or chromogenic techniques.

7. GEL-CLOT TECHNIQUE (METHODS A AND B)

The gel-clot technique allows detection or quantification of endotoxins and is based on clotting of the lysate in the presence of endotoxins. The minimum concentration of endotoxins required to cause the lysate to clot under standard conditions is the labelled lysate sensitivity.
To ensure both the precision and validity of the test, confirm the labelled lysate sensitivity and perform the test for interfering factors as described under 1. Preparatory testing.

1. PREPARATORY TESTING

(i) Confirmation of the labelled lysate sensitivity
Confirm in 4 replicates the labelled sensitivity λ, expressed in IU/mL, of the lysate solution prior to use in the test. Confirmation of the lysate sensitivity is carried out when a new lot of lysate is used or when there is any change in the test conditions which may affect the outcome of the test.

Prepare standard solutions of at least 4 concentrations equivalent to 2λ, λ, 0.5λ and 0.25λ by diluting the standard endotoxin stock solution with water for BET.

Mix a volume of the lysate solution with an equal volume of 1 of the standard solutions (such as 0.1 mL aliquots) in each tube. When single test vials or ampoules containing lyophilised lysate are employed, add solutions of standards directly to the vial or ampoule. Incubate the reaction mixture for a constant period according to the recommendations of the lysate manufacturer (usually at 37 ± 1 °C for 60 ± 2 min), avoiding vibration. Test the integrity of the gel: for tubes, take each tube in turn directly from the incubator and invert it through approximately 180° in one smooth motion. If a firm gel has formed that remains in place upon inversion, record the result as positive. A result is negative if an intact gel is not formed.

The test is considered valid when the lowest concentration of the standard solutions shows a negative result in all replicate tests.

Table 2.6.14.-1

Solution	Endotoxin concentration/Solution to which endotoxin is added	Diluent	Dilution factor	Endotoxin concentration	Number of replicates
A	None/Test solution	-	-	-	4
B	2λ/Test solution	Test solution	1	2λ	4
			2	1λ	4
			4	0.5λ	4
			8	0.25λ	4
C	2λ/Water for BET	Water for BET	1	2λ	2
			2	1λ	2
			4	0.5λ	2
			8	0.25λ	2
D	None/Water for BET	-	-	-	2

Solution A = solution of the preparation being examined that is free of detectable endotoxins.
Solution B = test for interference.
Solution C = control of the labelled lysate sensitivity.
Solution D = negative control (water for BET).

The end-point is the lowest concentration in the series of decreasing concentrations of standard endotoxin that clots the lysate. Determine the geometric mean end-point concentration by calculting the mean of the logarithms of the end-point concentrations of the 4 dilution series, take the antilogarithm of this value, as indicated by the following expression:

$$\text{Geometric mean end-point concentration} = \text{antilog}\frac{\sum e}{f}$$

$\sum e$ = sum of the $\log_{10}$ end-point concentrations of the dilution series used,

f = number of replicates.

The geometric mean end-point concentration is the measured sensitivity of the lysate solution (IU/mL). If this is not less than 0.5λ and not more than 2λ, the labelled sensitivity is confirmed and is used in the tests performed with this lysate.

(ii) Test for interfering factors

Prepare solutions A, B, C and D as shown in Table 2.6.14.-1, and use the test solutions at a dilution less than the MVD, not containing any detectable endotoxins, operating as described under 1. Preparatory testing, (i) Confirmation of the labelled lysate sensitivity.

The geometric mean end-point concentrations of solutions B and C are determined using the expression described in 1. Preparatory testing, (i) Confirmation of the labelled lysate sensitivity.

The test for interfering factors must be repeated when any changes are made to the experimental conditions that are likely to influence the result of the test.

The test is considered valid when all replicates of solutions A and D show no reaction and the result of solution C confirms the labelled lysate sensitivity.

If the sensitivity of the lysate determined with solution B is not less than 0.5λ and not greater than 2λ, the test solution does not contain interfering factors under the experimental conditions used. Otherwise, the test solution interferes with the test.

If the preparation being examined interferes with the test at a dilution less than the MVD, repeat the test for interfering factors using a greater dilution, not exceeding the MVD. The use of a more sensitive lysate permits a greater dilution of the preparation being examined and this may contribute to the elimination of interference.

Interference may be overcome by suitable validated treatment, such as filtration, neutralisation, dialysis or heat treatment. To establish that the treatment chosen effectively eliminates interference without loss of endotoxins, repeat the test for interfering factors using the preparation being examined to which the standard endotoxin has been added and which has then been submitted to the chosen treatment.

2. LIMIT TEST (METHOD A)

(i) Procedure

Prepare solutions A, B, C and D as shown in Table 2.6.14.-2, and perform the test on these solutions following the procedure described under 1. Preparatory testing, (i) Confirmation of the labelled lysate sensitivity.

Table 2.6.14.-2

Solution	Endotoxin concentration/Solution to which endotoxin is added	Number of replicates
A	None/Diluted test solution	2
B	2λ/Diluted test solution	2
C	2λ/Water for BET	2
D	None/Water for BET	2

Prepare solution A and solution B (positive product control) using a dilution not greater than the MVD and treatments as described in 1. Preparatory testing, (ii) Test for interfering factors. Solutions B and C (positive controls) contain the standard endotoxin at a concentration corresponding to twice the labelled lysate sensitivity. Solution D (negative control) consists of water for BET.

(ii) Interpretation

The test is considered valid when both replicates of solution B and C are positive and those of solution D are negative.

When a negative result is found for both replicates of solution A, the preparation being examined complies with the test.

When a positive result is found for both replicates of solution A, the preparation being examined does not comply with the test.

When a positive result is found for one replicate of solution A and a negative result is found for the other, repeat the test. In the repeat test, the preparation being examined complies with the test if a negative result is found for both replicates of solution A. The preparation does not comply with the test if a positive result is found for one or both replicates of solution A.

Table 2.6.14.-3

Solution	Endotoxin concentration/Solution to which endotoxin is added	Diluent	Dilution factor	Endotoxin concentration	Number of replicates
A	None/Test solution	Water for BET	1	-	2
			2	-	2
			4	-	2
			8	-	2
B	2λ/Test solution		1	2λ	2
C	2λ/Water for BET	Water for BET	1	2λ	2
			2	1λ	2
			4	0.5λ	2
			8	0.25λ	2
D	None/Water for BET	-	-	-	2

Solution A = test solution at the dilution, not exceeding the MVD, with which the test for interfering factors was carried out. Subsequent dilution of the test solution must not exceed the MVD. Use water for BET to make a dilution series of 4 tubes containing the test solution at concentrations of 1, 1/2, 1/4 and 1/8, relative to the dilution used in the test for interfering factors. Other dilutions up to the MVD may be used as appropriate.
Solution B = solution A containing standard endotoxin at a concentration of 2λ (positive product control).
Solution C = a dilution series of 4 tubes of water for BET containing the standard endotoxin at concentrations of 2λ, λ, 0.5λ and 0.25λ.
Solution D = water for BET (negative control).

However, if the preparation does not comply with the test at a dilution less than the MVD, the test may be repeated using a greater dilution, not exceeding the MVD.

3. QUANTITATIVE TEST (METHOD B)

(i) Procedure

The test quantifies bacterial endotoxins in the test solution by titration to an end-point. Prepare solutions A, B, C and D as shown in Table 2.6.14.-3, and test these solutions according to the procedure described under 1. Preparatory testing, (i) Confirmation of the labelled lysate sensitivity.

(ii) Calculation and interpretation

The test is considered valid when the following 3 conditions are met:

(a) both replicates of solution D (negative control) are negative,

(b) both replicates of solution B (positive product control) are positive,

(c) the geometric mean end-point concentration of solution C is in the range of 0.5λ to 2λ.

To determine the endotoxin concentration of solution A, calculate the end-point concentration for each replicate, by multiplying each end-point dilution factor by λ.

The endotoxin concentration in the test solution is the end-point concentration of the replicates. If the test is conducted with a diluted test solution, calculate the concentration of endotoxin in the original solution by multiplying the result by the dilution factor.

If none of the dilutions of the test solution is positive in a valid test, report the endotoxin concentration as less than λ (or, if a diluted sample was tested, report as less than the lowest dilution factor of the sample × λ). If all dilutions are positive, the endotoxin concentration is reported as equal to or greater than the largest dilution factor multiplied by λ (e.g. in Table 2.6.14.-3, the initial dilution factor × 8 × λ).

The preparation being examined meets the requirements of the test if the endotoxin concentration in both replicates is less than that specified in the monograph.

8. PHOTOMETRIC QUANTITATIVE TECHNIQUES (METHODS C, D, E AND F)

1. TURBIDIMETRIC TECHNIQUE (METHODS C AND F)

This technique is a photometric test to measure the increase in turbidity. Based on the test principle employed, this technique may be classified as being either the end-point-turbidimetric test or the kinetic-turbidimetric test.

The end-point-turbidimetric test (Method F) is based on the quantitative relationship between the endotoxin concentration and the turbidity (absorbance or transmission) of the reaction mixture at the end of an incubation period.

The kinetic-turbidimetric test (Method C) is a method to measure either the time (onset time) needed for the reaction mixture to reach a predetermined absorbance or transmission, or the rate of turbidity development.

The test is carried out at the incubation temperature recommended by the lysate manufacturer (usually 37 ± 1 °C).

2. CHROMOGENIC TECHNIQUE (METHODS D AND E)

This technique is used to measure the chromophore released from a suitable chromogenic peptide by the reaction of endotoxins with the lysate. Depending on the test principle employed, this technique may be classified as being either the end-point-chromogenic test or the kinetic-chromogenic test.

The end-point-chromogenic test (Method E) is based on the quantitative relationship between the endotoxin concentration and the quantity of chromophore released at the end of an incubation period.

The kinetic-chromogenic test (Method D) measures either the time (onset time) needed for the reaction mixture to reach a predetermined absorbance, or the rate of colour development.

The test is carried out at the incubation temperature recommended by the lysate manufacturer (usually 37 ± 1 °C).

3. PREPARATORY TESTING

To assure the precision or validity of the turbidimetric and chromogenic techniques, preparatory tests are conducted to show that the criteria for the standard curve are satisfied and that the test solution does not interfere with the test.

Validation of the test method is required when any changes are made to the experimental conditions that are likely to influence the result of the test.

(i) Assurance of criteria for the standard curve

The test must be carried out for each lot of lysate reagent.

Using the standard endotoxin solution, prepare at least 3 endotoxin concentrations within the range indicated by the lysate manufacturer to generate the standard curve. Perform

the test using at least 3 replicates of each standard endotoxin solution as recommended by the lysate manufacturer (volume ratios, incubation time, temperature, pH, etc.).

If the desired range is greater than 2 $\log_{10}$ in the kinetic methods, additional standards must be included to bracket each $\log_{10}$ increase in the range of the standard curve.

The absolute value of the correlation coefficient, $|r|$, must be greater than or equal to 0.980, for the range of endotoxin concentrations set up.

(ii) Test for interfering factors

Select an endotoxin concentration at or near the middle of the endotoxin standard curve.

Prepare solutions A, B, C and D as shown in Table 2.6.14.-4. Perform the test on at least 2 replicates of these solutions as recommended by the lysate manufacturer (volume of test solution and lysate solution, volume ratio of test solution to lysate solution, incubation time, etc.).

Table 2.6.14.-4

Solution	Endotoxin concentration	Solution to which endotoxin is added	Number of replicates
A	None	Test solution	Not less than 2
B	Middle concentration of the standard curve	Test solution	Not less than 2
C	At least 3 concentrations (lowest concentration is designated λ)	Water for BET	Each concentration not less than 2
D	None	Water for BET	Not less than 2

Solution A = test solution, that may be diluted not to exceed the MVD.
Solution B = preparation to be examined at the same dilution as solution A, containing added endotoxin at a concentration equal to or near the middle of the standard curve.
Solution C = standard endotoxin solution at the concentrations used in the validation of the method as described under 3. Preparatory testing, (i) Assurance of criteria for the standard curve (positive controls).
Solution D = water for BET (negative control).

The test is considered valid when the following conditions are met:

- the absolute value of the correlation coefficient of the standard curve generated using solution C is greater than or equal to 0.980;
- the result with solution D does not exceed the limit of the blank value required in the description of the lysate reagent employed, or it is less than the endotoxin detection limit of the lysate reagent employed.

Calculate the mean recovery of the added endotoxin by subtracting the mean endotoxin concentration in the solution (if any) (solution A, Table 2.6.14.-4) from that in the solution containing the added endotoxin (solution B, Table 2.6.14.-4).

The test solution is considered free of interfering factors if under the conditions of the test, the measured concentration of the endotoxin added to the test solution is within 50-200 per cent of the known added endotoxin concentration, after subtraction of any endotoxin detected in the solution without added endotoxin.

When the endotoxin recovery is out of the specified range, the test solution is considered to contain interfering factors. Repeat the test using a greater dilution, not exceeding the MVD. Furthermore, interference of the test solution or diluted test solution not to exceed the MVD may be eliminated by suitable validated treatment, such as filtration, neutralisation, dialysis or heat treatment. To establish that the treatment chosen effectively eliminates interference without loss of endotoxins, repeat the test for interfering factors using the preparation being examined to which the standard endotoxin has been added and which has then been submitted to the chosen treatment.

4. TEST

(i) Procedure

Follow the procedure described in 3. Preparatory testing, (ii) Test for interfering factors.

(ii) Calculation

Calculate the endotoxin concentration of each replicate of solution A using the standard curve generated by the positive control solution C.

The test is considered valid when the following 3 requirements are met:

(1) the results obtained with solution C comply with the requirements for validation defined under 3. Preparatory testing, (i) Assurance of criteria for the standard curve,

(2) the endotoxin recovery, calculated from the endotoxin concentration found in solution B after subtracting the endotoxin concentration found in solution A, is within the range of 50-200 per cent,

(3) the result obtained with solution D (negative control) does not exceed the limit of the blank value required in the description of the lysate employed, or it is less than the endotoxin detection limit of the lysate reagent employed.

(iii) Interpretation

The preparation being examined complies with the test if the mean endotoxin concentration of the replicates of solution A, after correction for dilution and concentration, is less than the endotoxin limit for the product.

Guidelines on the test for bacterial endotoxins are given in general chapter 5.1.10.

2.6.32. Test for Bacterial Endotoxins using Recombinant Factor C

(Ph. Eur. general text 2.6.32)

The test for bacterial endotoxins using recombinant factor C (rFC) is carried out to quantify endotoxins from gram-negative bacteria. It is performed using rFC based on the gene sequence of the horseshoe crab (*Limulus polyphemus, Tachypleus tridentatus, Tachypleus gigas* or *Carcinoscorpius rotundicauda*), using a fluorimetric method.

The test is carried out in a manner that avoids bacterial endotoxin contamination.

1. EQUIPMENT

Depyrogenate all glassware and other heat-stable equipment in a dry-heat oven using a validated process. A commonly used minimum time and temperature is 30 min at 250 °C. Where plastic equipment (such as microtitre plates and pipette tips for automatic pipettes) is employed, it must be shown to be free of detectable endotoxin and not to interfere with the test.

2. REAGENTS

Reagents

Recombinant factor C is based on the gene sequence of the horseshoe crab (*Limulus polyphemus, Tachypleus tridentatus, Tachypleus gigas* or *Carcinoscorpius rotundicauda*). All reagents, including the fluorogenic substrate and assay buffer, must be free of detectable endotoxin.

Reagent solutions
If necessary, prepare the reagents according to the test kit manufacturer's instructions. Store the reagents, refrigerated or frozen, as indicated by the manufacturer.

Water for BET (water for bacterial endotoxins test)
Water for injections R or water produced by other procedures that shows no reaction with the reagent employed at the detection limit of the reagent.

3. PREPARATION OF THE STANDARD ENDOTOXIN STOCK SOLUTION

The standard endotoxin stock solution is prepared from an endotoxin reference standard that has been calibrated against the International Standard, for example *endotoxin standard BRP.*

Endotoxin is expressed in International Units (IU).
The equivalence in IU of the International Standard is stated by the World Health Organization.

NOTE: 1 International Unit (IU) of endotoxin is equal to 1 Endotoxin Unit (EU).

Follow the specifications in the package leaflet and on the label for preparation and storage of the standard endotoxin stock solution.

4. PREPARATION OF THE STANDARD ENDOTOXIN SOLUTIONS

After vigorously mixing the standard endotoxin stock solution, prepare appropriate serial dilutions of this solution using water for BET.

Use the solutions as soon as possible to avoid loss of activity by adsorption.

5. PREPARATION OF THE TEST SOLUTIONS

Prepare the test solutions by dissolving or diluting active substances or medicinal products using water for BET. Some substances or preparations may be more appropriately dissolved or diluted in other aqueous solutions. If necessary, adjust the pH of the test solution (or dilution thereof) so that the pH of the mixture of the reagent(s) and test solution falls within the pH range specified by the test kit manufacturer, usually 6.0 to 8.0. The pH may be adjusted by the use of acid, base or a suitable buffer, as recommended by the test kit manufacturer. Acids and bases may be prepared from concentrates or solids with water for BET in containers free of detectable endotoxin. Buffers must be validated to be free of detectable endotoxin and interfering factors.

6. DETERMINATION OF THE MAXIMUM VALID DILUTION

The maximum valid dilution (MVD) is the maximum allowable dilution of a sample at which the endotoxin limit can be determined. Determine the MVD using the following formula:

$$\frac{\textit{endotoxin limit} \times \textit{concentration of test solution}}{\lambda}$$

Endotoxin limit The endotoxin limit for active substances administered parenterally, defined on the basis of dose, is equal to:

$$\frac{K}{M}$$

K = threshold pyrogenic dose of endotoxin per kilogram of body mass;
M = maximum recommended bolus dose of product per kilogram of body mass.

When the product is to be injected at frequent intervals or infused continuously, M is the maximum total dose administered in a single hour period.

The endotoxin limit for active substances administered parenterally is specified in units such as IU/mL, IU/mg, IU/Unit of biological activity, etc., in monographs.

Concentration of test solution:
- mg/mL if the endotoxin limit is specified by mass (IU/mg);
- Units/mL if the endotoxin limit is specified by unit of biological activity (IU/Unit);
- mL/mL if the endotoxin limit is specified by volume (IU/mL).

λ = the lowest concentration used in the standard curve.

7. FLUORIMETRIC QUANTITATIVE TECHNIQUE

This technique is used to measure the fluorescence (relative fluorescence units; RFU) emitted by a fluorescent substrate (reagent) after cleavage by endotoxin-activated factor C. It is used as an end-point-fluorescent test.

The end-point-fluorescent test is based on the quantitative relationship between the endotoxin concentration and the fluorescence of the reagent mixture at the end of the incubation period, expressed for example as ΔRFU:

$$\Delta RFU = RFU_{t_{end\text{-}point}} - RFU_{t_0}$$

$RFUt_{end\text{-}point}$ = fluorescence of the reagent mixture at the end of the incubation period;
$RFUt_0$ = fluorescence of the reagent mixture at the start of the incubation period.

The test is carried out at the incubation temperature recommended by the test kit manufacturer (usually 37 ± 1 °C).

8. PREPARATORY TESTING

Preparatory tests are conducted to ensure that the fluorometric technique is valid. These tests demonstrate that the criteria for the standard curve are satisfied (8-1) and that the test solution does not interfere with the test (8-2).

Validation of the test method is required when any changes are made to the experimental conditions that are likely to influence the result of the test.

8-1 ASSURANCE OF CRITERIA FOR THE STANDARD CURVE

The test must be carried out for each lot of recombinant factor C reagent.

Instrument sensitivity must be adjusted in accordance with the recommendations of the test kit manufacturer.

Using the standard endotoxin solution, prepare at least 3 endotoxin concentrations within the range indicated by the test kit manufacturer to generate the standard curve. If the desired range exceeds the range indicated by the manufacturer by more than 2 $\log_{10}$, additional standards must be included to bracket each log increase in the range. Perform the test using at least 3 replicates of each standard endotoxin solution as recommended by the manufacturer (volume ratios, incubation time, temperature, pH, etc.).

The absolute value of the correlation coefficient, $|r|$, must be greater than or equal to 0.980, for the range of endotoxin concentrations prepared.

8-2 INTERFERING FACTORS

As factor G is absent from the test kit, false-positive results due to β-glucan activation are not expected to occur. This must be taken into account when the method is compared to other bacterial endotoxin quantification methods.

Select an endotoxin concentration at or near the middle of the endotoxin standard curve.

Prepare solutions A, B, C and D as shown in Table 2.6.32.-1. Perform the test on at least 2 replicates of these solutions as recommended by the test kit manufacturer (volume of test solution and reagent test kit mixture, volume ratio of test solution to reagent test kit mixture, incubation time, etc.).

Table 2.6.32.-1

Solution	Endotoxin concentration	Solution to which endotoxin is added	Number of replicates
A	None	Test solution	Not less than 2
B	Middle concentration of the standard curve	Test solution	Not less than 2
C	At least 3 concentrations (lowest concentration is designated λ)	Water for BET	Each concentration not less than 2
D	None	Water for BET	Not less than 2

Solution A = test solution, which may be diluted but not exceeding the MVD.
Solution B (positive product control) = preparation to be examined at the same dilution as solution A, containing added endotoxin at a concentration equal to or near the middle of the standard curve
Solution C = standard endotoxin solution at the concentrations used in the validation of the method as described in section 8-1.
Solution D (negative control) = water for BET.

The test is considered valid when the following conditions are met:

- the absolute value of the correlation coefficient of the standard curve generated using solution C is greater than or equal to 0.980;
- the result with solution D does not exceed the limit of the blank value required in the description of the reagent mixture employed, or it is less than the endotoxin detection limit of the rFC employed.

Calculate the mean recovery of the added endotoxin by subtracting the mean endotoxin concentration in the solution (if any) (solution A, Table 2.6.32.-1) from that in the solution containing the added endotoxin (solution B, Table 2.6.32.-1).

The test solution is considered free of interfering factors if, under the conditions of the test, the measured concentration of the endotoxin added to the test solution is within 50-200 per cent of the known added endotoxin concentration, after subtraction of any endotoxin detected in the solution without added endotoxin.

When the endotoxin recovery is outside the specified range, the test solution is considered to contain interfering factors. Repeat the test using a greater dilution, not exceeding the MVD. Furthermore, interference of the test solution or diluted test solution (not exceeding the MVD) may be eliminated by suitable validated treatment, such as filtration, neutralisation, dialysis, heat treatment or endotoxin-specific binding steps (enrichment of endotoxin from the test solution prior to detection in the absence of the interfering matrix). To establish that the treatment chosen effectively eliminates interference without loss of endotoxins, repeat the test for interfering factors using the preparation being examined to which the standard endotoxin has been added and which has then been submitted to the chosen treatment.

9. TEST

9-1 PROCEDURE

Follow the procedure described in section 8-2.

9-2 CALCULATION

Calculate the endotoxin concentration of each replicate of solution A using the standard curve generated by the standard endotoxin solution C.

The test is considered valid when the following 3 requirements are met:

(1) the results obtained with solution C comply with the requirements for validation defined in section 8-1;

(2) the endotoxin recovery, calculated from the endotoxin concentration found in solution B after subtracting the endotoxin concentration found in solution A, is within the range of 50-200 per cent;

(3) the result obtained with solution D (negative control) does not exceed the limit of the blank value required in the description of the reagent mixture employed, or it is less than the endotoxin detection limit of the rFC employed.

9-3 INTERPRETATION

The preparation being examined complies with the test if the mean endotoxin concentration of the replicates of solution A, after correction for dilution and concentration, is less than the endotoxin limit for the product.

Guidelines on the test for bacterial endotoxins are given in general chapter 5.1.10.

D. Test for Pyrogens

(Ph. Eur. method 2.6.8)

The test consists of measuring the rise in body temperature evoked in rabbits by the intravenous injection of a sterile solution of the substance to be examined.

Selection of animals

Use healthy, adult rabbits of either sex weighing not less than 1.5 kg, fed a complete and balanced diet not containing antibiotics, and not showing loss of body mass during the week preceding the test. A rabbit is not to be used in a pyrogen test if:

a) it has been used in a negative pyrogen test in the preceding 3 days; or

b) it has been used in the preceding 3 weeks in a pyrogen test in which the substance under examination failed to pass the test.

Animals' quarters

Keep the rabbits individually in a quiet area with a uniform appropriate temperature. Withhold food from the rabbits overnight and until the test is completed; withhold water during the test. Carry out the test in a quiet room where there is no risk of disturbance exciting the animals and in which the room temperature is within 3 °C of that of the rabbits' living quarters, or in which the rabbits have been kept for at least 18 h before the test.

Materials

Thoroughly wash all glassware, syringes and needles with water for injections and heat in a hot-air oven at 250 °C for 30 min or at 200 °C for 1 h.

Retaining boxes
The retaining boxes for rabbits whose temperature is being measured by an electrical device are made in such a way that the animals are retained only by loosely fitting neck-stocks; the rest of the body remains relatively free so that the rabbits may sit in a normal position. They are not restrained by straps or other similar methods that may harm the animal. The animals are put into the boxes not less than 1 h before the 1st recording of the temperature and remain in them throughout the test.

Thermometers
Use a thermometer or electrical device that indicates the temperature with a resolution of 0.1 °C and insert into the rectum of the rabbit to a depth of about 5 cm. The depth of insertion is constant for any 1 rabbit in any 1 test. When an electrical device is used it may be left in position throughout the test.

Preliminary test
After selection of the animals, 1-3 days before testing the product to be examined, treat those animals that have not been used during the previous 2 weeks by intravenous injection of 10 mL per kilogram of body mass of a pyrogen-free 9 g/L solution of *sodium chloride R* warmed to about 38.0 °C. Record the temperatures of the animals, beginning at least 90 min before injection and continuing for 3 h after the injection of the solution. Any animal showing a temperature variation greater than 0.6 °C is not used in the main test.

Main test
Carry out the test using a group of 3 rabbits.

Preparation and injection of the product Warm the liquid to be examined to approximately 38.0 °C before the injection. The product to be examined may be dissolved in, or diluted with, a pyrogen-free 9 g/L solution of *sodium chloride R* or another prescribed liquid. Inject the solution slowly into the marginal vein of the ear of each rabbit over a period not exceeding 4 min, unless otherwise prescribed in the monograph. The amount of the product to be injected varies according to the product to be examined and is prescribed in the monograph. The volume injected is not less than 0.5 mL per kilogram and not more than 10 mL per kilogram of body mass.

Determination of the initial and maximum temperatures The 'initial temperature' of each rabbit is the mean of 2 temperature readings recorded for that rabbit at an interval of 30 min in the 40 min immediately preceding the injection of the product to be examined. The 'maximum temperature' of each rabbit is the highest temperature recorded for that rabbit in the 3 h after the injection. Record the temperature of each rabbit at intervals of not more than 30 min, beginning at least 90 min before the injection of the product to be examined and continuing 3 h after the injection. The difference between the maximum temperature and the initial temperature of each rabbit is taken to be its response. When this difference is negative, the result is counted as a zero response.

Rabbits showing a temperature variation greater than 0.2 °C between 2 successive readings in the determination of the initial temperature are withdrawn from the test. In any 1 test, only rabbits having initial temperatures that do not differ from one another by more than 1 °C are used. All rabbits having an initial temperature higher than 39.8 °C or less than 38.0 °C are withdrawn from the test.

Interpretation of results
Having carried out the test 1st on a group of 3 rabbits, repeat if necessary on further groups of 3 rabbits to a total of 4 groups, depending on the results obtained. If the summed response of the 1st group does not exceed the figure given in the 2nd column of Table 2.6.8.-1, the substance passes the test. If the summed response exceeds the figure given in the 2nd column of the table but does not exceed the figure given in the 3rd column of the table, repeat the test as indicated above. If the summed response exceeds the figure given in the 3rd column of the table, the product fails the test.

Table 2.6.8.-1

Number of rabbits	Product passes if summed response does not exceed	Product fails if summed response exceeds
3	1.15 °C	2.65 °C
6	2.80 °C	4.30 °C
9	4.45 °C	5.95 °C
12	6.60 °C	6.60 °C

Rabbits used in a test for pyrogens where the mean rise in the rabbits' temperature has exceeded 1.2 °C are permanently excluded.

In accordance with the provisions of the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes, tests must be carried out in such a way as to use the minimum number of animals and to cause the least pain, suffering, distress or lasting harm. Wherever possible and after product-specific validation, the pyrogen test is replaced by the monocyte-activation test (*2.6.30*).

F. Test for Depressor Substances

(Depressor Substances, Ph. Eur. method 2.6.11)

Carry out the test on a cat weighing not less than 2 kg and anaesthetised with chloralose or with a barbiturate that allows the maintenance of uniform blood pressure. Protect the animal from loss of body heat and maintain it so that the rectal temperature remains within physiological limits. Introduce a cannula into the trachea. Insert a cannula filled with a heparinised 9 g/L solution of sodium chloride into the common carotid artery and connect it to a device capable of giving a continuous record of the blood pressure. Insert into the femoral vein another cannula, filled with a heparinised 9 g/L solution of sodium chloride, through which can be injected the solutions of histamine and of the substance to be examined. Determine the sensitivity of the animal to histamine by injecting intravenously at regular intervals, doses of *histamine solution R* corresponding to 0.1 µg and 0.15 µg of histamine base per kilogram of body mass. Repeat the lower dose at least 3 times. Administer the second and subsequent injections not less than 1 min after the blood pressure has returned to the level it was at immediately before the previous injection. The animal is used for the test only if a readily discernible decrease in blood pressure that is constant for the lower dose is obtained and if the higher dose causes greater responses. Dissolve the substance to be examined in sufficient of a 9 g/L solution of sodium chloride or other prescribed solvent, to give the prescribed concentration. Inject intravenously per kilogram of body mass 1.0 mL of *histamine solution R*, followed by 2 successive injections of the prescribed amount of the solution to be examined and,

finally, 1.0 mL of *histamine solution R*. The second, third and fourth injections are given not less than 1 min after the blood pressure has returned to the level it was at immediately before the preceding injection. Repeat this series of injections twice and conclude the test by giving 1.5 mL of *histamine solution R* per kilogram of body mass.

If the response to 1.5 mL of *histamine solution R* per kilogram of body mass is not greater than that to 1.0 mL the test is invalid. The substance to be examined fails the test if the mean of the series of responses to the substance is greater than the mean of the responses to 1.0 mL of *histamine solution R* per kilogram of body mass or if any one dose of the substance causes a greater depressor response than the concluding dose of the histamine solution. The test animal must not be used in another test for depressor substances if the second criterion applies or if the response to the high dose of histamine given after the administration of the substance to be examined is less than the mean response to the low doses of histamine previously injected.

G. Test for Histamine

(Histamine, Ph. Eur. method 2.6.10)

Euthanise a guinea-pig weighing 250 g to 350 g that has been deprived of food for the preceding 24 h. Remove a portion of the distal small intestine 2 cm in length and empty the isolated part by rinsing carefully with solution B described below using a syringe. Attach a fine thread to each end and make a small transverse incision in the middle of the piece of intestine. Place it in an organ bath with a capacity of 10 mL to 20 mL, containing solution B maintained at a constant temperature (34 °C to 36 °C) and pass through the solution a current of a mixture of 95 parts of oxygen and 5 parts of carbon dioxide. Attach one of the threads near to the bottom of the organ bath. Attach the other thread to an isotonic myograph and record the contractions of the organ on a kymograph or other suitable means of giving a permanent record. If a lever is used, its length is such that the movements of the organ are amplified about 20 times. The tension on the intestine should be about 9.8 mN (1 g) and it should be adjusted to the sensitivity of the organ. Flush out the organ bath with solution B. Allow it to stand for 10 min. Flush 2 or 3 times more with solution B. Stimulate a series of contractions by the addition of measured volumes between 0.2 mL and 0.5 mL of a solution of *histamine dihydrochloride R* having a strength which produces reproducible submaximal responses. This dose is termed the "high dose". Flush the organ bath (preferably by overflow without emptying the bath) 3 times with solution B before each addition of histamine. The successive additions should be made at regular intervals allowing a complete relaxation between additions (about 2 min). Add equal volumes of a weaker dilution of *histamine dihydrochloride R* which produces reproducible responses approximately half as great as the "high dose". This dose is termed the "low dose". Continue the regular additions of "high" and "low" doses of histamine solution as indicated above, and alternate each addition with an equal volume of a dilution of the solution to be examined, adjusting the dilution so that the contraction of the intestine, if any, is smaller than that due to the "high dose" of histamine. Determine whether the contraction, if any, is reproducible and that the responses to the "high" and "low" doses of histamine are unchanged. Calculate the activity of the substance to be examined in terms of its equivalent in micrograms of histamine base from the dilution determined as above.

The quantity so determined does not exceed the quantity prescribed in the monograph.

If the solution to be examined does not produce a contraction, prepare a fresh solution adding a quantity of histamine corresponding to the maximum tolerated in the monograph and note whether the contractions produced by the preparation with the added histamine correspond to the amount of histamine added. If this is not the case, or if the contractions caused by the substance to be examined are not reproducible or if subsequent responses to "high" and "low" doses of histamine are diminished, the results of the tests are invalid and the test for depressor substances (*2.6.11*) must be carried out.

Solution A

Sodium chloride	160.0 g
Potassium chloride	4.0 g
Calcium chloride, anhydrous	2.0 g
Magnesium chloride, anhydrous	1.0 g
Disodium hydrogen phosphate dodecahydrate	0.10 g
Water for injections R	to 1000 mL

Solution B

Solution A	50.0 mL
Atropine sulfate	0.5 mg
Sodium hydrogen carbonate	1.0 g
Glucose monohydrate	0.5 g
Water for injections R	to 1000 mL

Solution B should be freshly prepared and used within 24 h.

H. Monocyte-Activation Test

(Ph. Eur. method 2.6.30)

1 INTRODUCTION

The monocyte-activation test (MAT) is used to detect or quantify substances that activate human monocytes or monocytic cells to release endogenous mediators such as pro-inflammatory cytokines, for example tumour necrosis factor alpha (TNFα), interleukin-1 beta (IL-1β) and interleukin-6 (IL-6). These cytokines have a role in fever pathogenesis. Consequently, the MAT will detect the presence of pyrogens in the test sample. The MAT is suitable, after a product-specific validation, as a replacement for the rabbit pyrogen test.

Pharmaceutical products that contain non-endotoxin pyrogenic or pro-inflammatory contaminants often show very steep or non-linear dose-response curves in comparison with endotoxin dose-response curves. Preparations that contain or may contain non-endotoxin contaminants have to be tested at a range of dilutions that includes minimum dilution.

The following 3 methods are described in the present chapter.

Method A. Quantitative test

Method B. Semi-quantitative test

Method C. Reference lot comparison test

In addition, further useful information on practical aspects of the tests can be found in the 'Guidance notes' section at the end of this general chapter.

The test is carried out in a manner that avoids pyrogen contamination.

2 DEFINITIONS

The maximum valid dilution (MVD) is the maximum allowable dilution of a sample at which the contaminant limit can be determined. The calculation of the MVD is based on the endotoxin reference standard. Determine the MVD using the following expression:

$$\frac{CLC \times C}{LOD}$$

CLC = contaminant limit concentration;
C = concentration of test solution;
LOD = limit of detection.

As the LOD is not always available in advance, an estimated LOD based on historical data can be used to calculate the MVD.

The acceptance criterion for a pass/fail decision is the contaminant limit concentration (CLC), which is expressed in endotoxin equivalents per milligram or millilitre or per units of biological activity of the preparation being examined.

The CLC is calculated using the following expression:

$$\frac{K}{M}$$

K = threshold pyrogenic dose per kilogram of body mass;
M = maximum recommended bolus dose of product per kilogram of body mass.

When the product is to be injected at frequent intervals or infused continuously, *M* is the maximum total dose administered in a single hour period.

Where an endotoxin limit concentration (ELC) has been specified for a product, the CLC is the same as the ELC, unless otherwise prescribed. In this case, the concentration of test solution is expressed in mg/mL if the endotoxin limit is specified by mass (IU/mg), in Units/mL if the endotoxin limit is specified by unit of biological activity (IU/Unit), in mL/mL if the endotoxin limit is specified by volume (IU/mL).

Endotoxin equivalents are values for the contaminant concentration read off the standard endotoxin dose-response curve (Method A) or estimated by comparison with responses to standard endotoxin solutions (Method B). The standard endotoxin stock solution is prepared from an endotoxin reference standard that has been calibrated against the International Standard, for example *endotoxin standard BRP*.

The LOD is determined using the endotoxin standard curve. It is the concentration of endotoxin corresponding to the cut-off value. For the purpose of the test, the LOD is expressed as endotoxin equivalents per millilitre. The cut-off value is expressed in units appropriate to the read-out (e.g. for an enzyme-linked immunosorbent assay (ELISA), use the optical density).

The cut-off value may be calculated using the following expression:

$$\bar{x} + 3s$$

$\bar{x}$ = mean of the 4 replicates for the responses to the blank (R_0);

s = standard deviation of the 4 replicates of the responses to the blank (R_0).

3 GENERAL PROCEDURE

A solution of the preparation being examined is incubated with a source of human monocytes or human monocytic cells, e.g. from human heparinised peripheral blood that is preferably not more than 4 h old, or a monocyte-containing fraction of that blood, such as human peripheral blood mononuclear cells (PBMC) isolated, e.g. by density-gradient centrifugation, or a human monocytic cell line. Human heparinised peripheral blood is usually diluted with culture medium or saline e.g. to 2-50 per cent *V/V* (final concentration). PBMC or monocytic cell lines, in culture medium and with either the donor's own plasma or AB serum, are typically used at a final cell density of 0.1-1.0 × 10^6 cells per well, tube or other receptacle. For monocytic cell lines, heat-inactivated foetal bovine serum may be substituted for AB serum. The cell culture is carried out at 37 ± 1 °C, in an atmosphere appropriate for the culture medium, e.g. 5 per cent CO_2 in humidified air. The duration of the culture is sufficient to allow accumulation of the chosen read-out. The responses of the chosen read-out, e.g. a pro-inflammatory or pyrogenic cytokine, to a solution of the preparation being examined are compared with responses to standard endotoxin or to a reference lot of the preparation being examined.

4 APPARATUS

Depyrogenate all glassware and other heat-stable apparatus in a hot-air oven using a validated process. A commonly used minimum time and temperature is 30 min at 250 °C. If employing plastic apparatus, such as microtitre plates and pipette tips for automatic pipetters, use apparatus shown to be free of detectable pyrogens and which do not interfere with the test.

5 CELL SOURCES AND QUALIFICATION

5-1 WHOLE BLOOD

Whole blood is obtained from single donors or from pooled whole blood which are qualified according to the requirements described under sections 5-3, 5-4, 5-5 and where applicable, section 6-3.

5-2 PERIPHERAL BLOOD MONONUCLEAR CELLS (PBMC)

PBMC are isolated from blood obtained from single donors or from pooled whole blood which are qualified according to the requirements described under sections 5-3, 5-4, 5-5 and where applicable, section 6-3.

5-3 QUALIFICATION OF BLOOD DONORS

Blood donors are to satisfy the following qualification criteria, together with other requirements in force that relate to consent, health and safety and ethical considerations. Blood donors are to describe themselves as being in good health, as not to be suffering from any bacterial or viral infections and to have been free from the symptoms of any such infection for a period of at least 1 week prior to the donation of blood. Blood donors are not to have taken non-steroidal anti-inflammatory drugs during the 48 h prior to donating blood and steroidal anti-inflammatory drugs during the 7 days prior to donating blood. Individuals who have been prescribed immunosuppressant or other drugs known to influence the production of the chosen readout are not to serve as blood donors. Blood donations are to be tested for infection markers according to national requirements for transfusion medicine.

5-4 QUALIFICATION OF CELLS POOLED FROM A NUMBER OF DONORS

Pools (of whole blood or blood fractions, e.g. PBMC), must consist of donations from a minimum of 4 individual donors

but preferably 8 or more donors, where practicable, taking from each donation an approximately equal volume of blood, or cells from an approximately equal volume of blood. For the qualification of pooled cells proceed as follows: within 4 h of collection of blood, generate dose-response curves from the pool using standard endotoxin with at least 4 geometrically diluted endotoxin concentrations, e.g. in the range of 0.01 IU/mL to 4 IU/mL. The dose-response curves are to meet the 2 criteria for the endotoxin standard curve described under section 6-1. If the pool is to be used for the detection of non-endotoxin contaminants, pools are to be qualified as described in section 6-3. Where cells are pooled, the averaging effect is to be considered when setting the pass/fail specification for a given product.

5-5 QUALIFICATION OF CRYO-PRESERVED CELLS

The cell source intended for use in a MAT, e.g. human whole blood, blood fractions, such as PBMC or monocytic cell lines, may be cryo-preserved. Pools of cryo-preserved cells are obtained by pooling before freezing, or by pooling single cryo-preserved donations immediately after thawing. Qualification of cryo-preserved blood or cells is performed immediately after thawing (and pooling if necessary). Dose-response curves for the cryo-preserved blood or cells are to comply with the 2 criteria for the endotoxin standard curve described under section 6-1. Qualification of the cryo-preserved blood or cells is to be performed according to section 6-3 if the intended use is for the detection of non-endotoxin contaminants.

5-6 MONOCYTIC CONTINUOUS CELL LINES

Monocytic cell lines are appropriate for the detection of bacterial endotoxins, but have limited use for the detection of non-endotoxin pyrogens.

A human monocytic cell line is cultured in order to ensure a sufficient supply for the MAT. To optimise the method, clones derived from the cell line can be used.

Cells must be maintained under aseptic conditions and regularly tested for the presence of mycoplasma contamination. Additionally, cells must be regularly checked for identity (e.g. doubling time, morphology, and function) and stability. The functional stability of a cell line is assessed by monitoring its performance in relation to the number of passages during routine testing. Criteria for functional stability are to be established and may include growth criteria, maximum signal obtained in the test, background noise and receptor expression. The receptor expression may be tested with specific ligands e.g. lipopolysaccharide (LPS) for toll-like receptor 4 (TLR4), lipoteichoic acid (LTA) for toll-like receptor 2 (TLR2), synthetic bacterial lipoprotein for TLR2-TLR1, synthetic bacterial lipoprotein for TLR2-TLR6 or flagellin.

The dose-response curves are to meet the 2 criteria for the endotoxin standard curve described under section 6-1. If the cells are to be used for the detection of non-endotoxin contaminants, they are to be qualified as described in section 6-3.

6 PREPARATORY TESTING

To ensure both the precision and validity of the test, preparatory tests are conducted, to assure that the criteria for the endotoxin standard curve are satisfied, that the solution does not interfere with the test, that the test detects endotoxins and non-endotoxins contaminants and that the solution does not interfere in the detection system.

A test for interfering factors is required when any changes are made to the experimental conditions that are likely to influence the result of the test.

6-1 ASSURANCE OF CRITERIA FOR THE ENDOTOXIN STANDARD CURVE

Using the standard endotoxin solution, prepare at least 4 endotoxin concentrations to generate the standard curve. Perform the test using at least 4 replicates of each concentration of standard endotoxin.

The basal release of the chosen read-out (blank) in the absence of added standard endotoxin is optimised to be as low as possible (e.g. an optical density below 0.1 when using an ELISA).

There are 2 acceptance criteria for the standard curve:

— the regression of responses (appropriately transformed if necessary) on $\log_{10}$ dose shall be statistically significant ($p < 0.01$);

— the regression of responses on $\log_{10}$ dose must not deviate significantly from linearity ($p > 0.05$) (see chapter *5.3. Statistical analysis*).

6-2 TEST FOR INTERFERING FACTORS

To assure the validity of the test, preparatory tests are conducted to ensure that the preparation being examined does not interfere with the test. Using an appropriate diluent, dilute the preparation being examined in geometric steps, with all dilutions not exceeding the MVD. Make the same dilutions of the preparation being examined and add endotoxin at a justified concentration. Alternatively, use a diluent containing added endotoxin at a justified concentration. In both cases, this concentration is usually equal to or near the estimated middle of the endotoxin standard curve (Method A) or twice the estimated LOD (Method B). Test these dilution series in parallel in the same experiment. Use the endotoxin standard curve to calculate the concentration of endotoxin-equivalents in each solution. Calculate the mean recovery of the added endotoxin by subtracting the mean concentration of endotoxin equivalents in the solution (if any) from that in the solution containing the added endotoxin. The test solution is considered free of interfering factors if, under the conditions of the test, the measured endotoxin equivalents in the test solution to which endotoxin is added is within 50-200 per cent of the added concentration, after subtraction of any endotoxin equivalents detected in the solution without added endotoxin. When this criterion is not met, Method C is to be preferred over Methods A and B.

In Method C, the dilutions of the test and reference lots depend on the type of analysis used to make the comparison between the two. The type of analysis is to be justified and validated for each product, and is to include assay validity criteria. In an example, a solution of the preparation being examined is tested at 3 dilutions: the highest concentration (lowest dilution) that stimulates the greatest release of the chosen read-out and the 2-fold dilutions immediately below and above the chosen dilution. Since the concentration that stimulates the greatest release of the chosen read-out may be donor-dependent as well as batch-dependent, the product-specific validation is to be performed in at least 3 independent tests, each using cells from different donors. The highest concentration (lowest dilution) that stimulates the greatest release of the chosen read-out in the majority of donors, and the 2-fold dilutions immediately below and above that dilution are deemed to be validated for further testing. If undiluted test solution stimulates the greatest release of the chosen read-out, subsequent testing is to be performed using undiluted test solution and also test solution diluted in the ratios 1:2 and 1:4 before its addition to the monocytic cells. The dilution factors for these 3 solutions are designated f_1, f_2 and f_3.

If the pyrogen content of the product is inherently high, it may be more appropriate to carry out, for example, a parallel-line analysis on the dose-response curves for the test and reference lots. In this situation, solutions of the preparations are tested at 3 or more geometric dilutions which cover the range of the dose-response curve used for the validated analysis (see chapter *5.3. Statistical analysis*).

6-3 METHOD VALIDATION FOR NON-ENDOTOXIN MONOCYTE-ACTIVATING CONTAMINANTS

The preparatory testing is also to show that the chosen test system detects, in addition to bacterial endotoxins, non-endotoxin pro-inflammatory or pyrogenic contaminants. The suitability of the method for the particular product has to be verified. This can be achieved using historic batches found to be contaminated with non-endotoxin contaminants that caused positive responses in the rabbit pyrogens test or adverse drug reactions in man. Where such batches are not available, the preparatory testing is to include validation of the test system using at least 2 non-endotoxin ligands for toll-like receptors, e.g. peptidoglycans, lipoteichoic acids, synthetic bacterial lipoproteins, flagellin and crude bacterial whole cell extract, at least 1 of which is to be spiked into the preparation being examined. The choice of non-endotoxin pyrogens used should reflect the most likely contaminant(s) of the preparation being examined.

6-4 INTERFERENCE IN THE DETECTION SYSTEM

Once the optimum dilution of the solution of the preparation being examined for further testing has been identified, this dilution is tested for interference in the detection system (e.g. ELISA) for the chosen read-out. The agreement between a dilution series of the standard for the chosen read-out, in the presence and absence of the preparation being examined, is to be within, for example ± 20 per cent of the optical density.

7 METHODS

7-1 METHOD A: QUANTITATIVE TEST

Method A involves a comparison of the preparation being examined with a standard endotoxin dose-response curve. The contaminant concentration of the preparation being examined is to be less than the CLC to pass the test.

7-1-1 Test procedure

Using the validated test method, prepare the solutions shown in Table 2.6.30.-1 and culture 4 replicates of each solution with the qualified cells.

Solution A = solution of the preparation being examined at the dilution, here designated *f*, at which the test for interfering factors was carried out, i.e. the highest concentration (lowest dilution) for which the endotoxin recovery is within 50-200 per cent.

Solution B = 2-fold dilution of solution A, not exceeding the MVD.

Solution C = 2-fold dilution of solution B, not exceeding the MVD.

Solution AS = solution A spiked with standard endotoxin at a concentration equal to the middle dose from the endotoxin standard curve (R_3).

Solution BS = solution B spiked with standard endotoxin at a concentration equal to the middle dose from the endotoxin standard curve (R_3).

Solution CS = solution C spiked with standard endotoxin at a concentration equal to the middle dose from the endotoxin standard curve (R_3).

Solution R_0 = negative control.

Table 2.6.30.-1

Solution	Solution	Added endotoxin (IU/mL)	Number of replicates
A	Test solution/*f*	None	4
B	Test solution/2 × *f*	None	4
C	Test solution/4 × *f*	None	4
AS	Test solution/*f*	Middle dose from endotoxin standard curve (R_3)	4
BS	Test solution/2 × *f*	Middle dose from endotoxin standard curve (R_3)	4
CS	Test solution/4 × *f*	Middle dose from endotoxin standard curve (R_3)	4
R_0	Pyrogen-free saline or test diluent	None (negative control)	4
R_1-R_4	Pyrogen-free saline or test diluent	4 concentrations of standard endotoxin	4 of each concentration

Solutions R_1-R_4 = solutions of standard endotoxin at the concentrations used in the test for interfering factors.

7-1-2 Calculation and interpretation

All data to be included in the data analysis are to relate to cells for which the 2 criteria for the endotoxin standard curve are satisfied. For each different cell source, e.g. individual donation, donor pool, or cell line, use the endotoxin standard curve R_1-R_4 to calculate the concentration of endotoxin equivalents in each of the replicates of solutions A, B and C and solutions AS, BS and CS. The recovery of endotoxin equivalents calculated from the endotoxin equivalents concentration found in solutions AS, BS and CS after subtracting the endotoxin equivalents concentration found in solutions A, B and C is within the range of 50-200 per cent. Dilutions not fulfilling the spike recovery argument are not valid and are therefore excluded from further evaluation. The preparation being examined complies with the requirements of the test for a given cell source if the mean concentrations of endotoxin equivalents measured in the replicates of solutions A, B and C, after correction for dilution and concentration, are all less than the CLC specified for the preparation being examined. One valid dilution is the minimum required for a valid test.

7-1-3 Pass/fail criteria of the preparation

When cells from individual donors are used, the preparation being examined is required to comply with the test with the cells from each of 4 different donors. If the preparation being examined passes the test with cells from 3 of the 4 donors, the test is continued with cells from a further 4 donors, none of whom provided cells for the 1st test, and the preparation being examined is required to pass the test with cells from 7 of the 8 different donors (i.e. a maximum of 1 positive reaction in 8 donors is allowed). When the source of monocytes consists of cells pooled from a number of individual donors, the preparation being examined is required to pass the test with 1 pool of cells. Where a human monocytic cell line is used for the test, the preparation being examined is required to pass the test with 1 qualified passage of cells.

7-2 METHOD B. SEMI-QUANTITATIVE TEST

Method B involves a comparison of the preparation being examined with standard endotoxin. The contaminant concentration of the preparation being examined is to be less than the CLC to pass the test. Solution A must be chosen for the pass decision, unless otherwise justified and authorised.

7-2-1 Test procedure

Using the validated test method, prepare the solutions shown in Table 2.6.30.-2 and culture 4 replicates of each solution with the qualified cells.

Table 2.6.30.-2

Solution	Solution	Added endotoxin (IU/mL)	Number of replicates
A	Test solution/*f*	None	4
B	Test solution/f_1	None	4
C	Test solution/f_2	None	4
AS	Test solution/*f*	Standard endotoxin at 2 × estimated LOD for the test system	4
BS	Test solution/f_1	Standard endotoxin at 2 × estimated LOD for the test system	4
CS	Test solution/f_2	Standard endotoxin at 2 × estimated LOD for the test system	4
R_0	Pyrogen-free saline or test diluent	None (negative control)	4
R_1	Pyrogen-free saline or test diluent	Standard endotoxin at 0.5 × estimated LOD for the test system	4
R_2	Pyrogen-free saline or test diluent	Standard endotoxin at 1 × estimated LOD for the test system	4
R_3	Pyrogen-free saline or test diluent	Standard endotoxin at 2 × estimated LOD for the test system	4
R_4	Pyrogen-free saline or test diluent	Standard endotoxin at 4 × estimated LOD for the test system	4

Solution A = solution of the preparation being examined at the dilution, here designated *f*, at which the test for interfering factors was completed.

Solution B = solution of the preparation being examined at a dilution, here designated f_1, not exceeding the MVD, chosen after a review of data from the product-specific validation, e.g. 1:2 × MVD (i.e. 2 times less diluted than the MVD).

Solution C = solution of the preparation being examined at a dilution, here designated f_2, not exceeding the MVD, chosen after a review of data from the product-specific validation, e.g. MVD.

Solution AS = solution A spiked with standard endotoxin at 2 × estimated LOD for the test system (as determined in preparatory testing).

Solution BS = solution B spiked with standard endotoxin at 2 × estimated LOD for the test system.

Solution CS = solution C spiked with standard endotoxin at 2 × estimated LOD for the test system.

Solution R_0 = negative control.

Solution R_1 = standard endotoxin at 0.5 × estimated LOD for the test system.

Solution R_2 = standard endotoxin at 1 × estimated LOD for the test system.

Solution R_3 = standard endotoxin at 2 × estimated LOD for the test system.

Solution R_4 = standard endotoxin at 4 × estimated LOD for the test system.

7-2-2 Calculation and interpretation

All data to be included in the data analysis are to relate to cells for which mean responses to solutions R_0-R_4 increase progressively. The mean response to R_0 may be equal to the mean response to R_1. For each different cell source, the mean response to solution R_2 is to be greater than a positive cut-off value. Data below this cut-off value are considered negative. If the mean response to R_1 or R_2 exceeds the cut-off value, the response to the solution chosen for the pass/fail decision must be negative (= pass). For each negative solution of the preparation being examined (A, B and C), the mean response to the corresponding spiked solution (AS, BS or CS respectively) is compared with the mean response to R_3 to determine the percentage spike recovery.

The contaminant concentration of the preparation being examined is less than the CLC for a given cell source if the solution of the preparation being examined designated for the pass/fail-decision and the dilutions below all give negative results and the endotoxin spike recovery is within the range of 50-200 per cent.

7-2-3 Pass/fail criteria of the preparation

The criteria are the same as for method A (see 7-1-3).

7-3 METHOD C: REFERENCE LOT COMPARISON TEST

Method C involves a comparison of the preparation being examined with a validated reference lot of that preparation. The type of analysis selected to compare the two is to be justified and validated for each product and is to include assay validity criteria. The reference lot is also selected according to criteria that have been justified and authorised. The test is intended to be performed in cases where a preparation being examined shows marked interference but cannot be diluted within the MVD to overcome the interference or because it contains or is believed to contain non-endotoxin contaminants. Responses to non-endotoxin contaminants may dilute out more rapidly than responses to endotoxin, which makes it necessary to perform the test at a range of dilutions that include minimum dilution. The test procedure is described below and includes an example of a type of analysis used for the comparison of a test lot and reference lot.

7-3-1 Test procedure

Using the validated test method, prepare the solutions shown in Table 2.6.30.-3 and culture 4 replicates of each solution with the qualified cells.

Table 2.6.30.-3

Solution	Solution/dilution factor	Number of replicates
A	Solution of reference lot/f_1	4
B	Solution of reference lot/f_2	4
C	Solution of reference lot/f_3	4
D	Solution of preparation being examined/f_1	4
E	Solution of preparation being examined/f_2	4
F	Solution of preparation being examined/f_3	4
G	Positive control (standard endotoxin)	4
R_0	Diluent (negative control)	4

Solutions A, B and C are solutions of the reference lot diluted by the dilution factors, f_1, f_2 and f_3, determined in the test for interfering factors.

Solutions D, E and F are solutions of the preparation being examined diluted by the dilution factors, f_1, f_2 and f_3, determined for the reference lot in the test for interfering factors.

Solution G is the positive test control for the viability of the cells and is a standard endotoxin concentration that gives a clear positive response.

Solution R_0 is the diluent used to dilute the preparation being examined and serves as the test blank.

7-3-2 Calculation and interpretation

All data to be included in the data analysis are to relate to cells for which solution G and at least one of solutions A, B and C give a response that is greater than the basal release of the read-out (Solution R_0). For each different cell source, e.g. individual donation, donor pool, or cell line, use the standard curve for the read-out (a calibration curve in duplicate with a blank and at least 4 geometrically diluted concentrations of the standard for the chosen read-out) and calculate the mean responses of the replicates of solutions A-F. Sum the mean responses to solutions A, B and C and sum the mean responses to solutions D, E and F. Divide the sum of the mean responses to solutions D, E and F by the sum of the mean responses to solutions A, B and C.
The preparation being examined complies with the test for a given cell source if the resulting value complies with a defined acceptance criterion not exceeding a justified value, e.g. 2.5.

7-3-3 Pass/fail criteria of the preparation

The criteria are the same as for method A (see 7-1-3).

To quantify more closely the level of contamination, Methods A, B and C may be performed using other dilutions of the solution of the preparation being examined not exceeding the MVD.

The following section is published for information only.

GUIDANCE NOTES

1 INTRODUCTION

The monocyte-activation test (MAT) is primarily intended to be used as a replacement for the rabbit pyrogen test.
The MAT detects pyrogenic and pro-inflammatory contaminants, including endotoxins from gram-negative bacteria and 'non-endotoxin' contaminants, including pathogen-associated molecular patterns (PAMPs), derived from gram-positive and gram-negative bacteria, viruses and fungi, and product-related and process-related biological or chemical entities.

Since non-endotoxin contaminants are a physico-chemically diverse class of molecules, and usually the nature of the contaminant in a preparation being examined is unknown, the level of contamination is expressed either in endotoxin-equivalent units, derived by comparison with responses to standard endotoxin, or by comparison with a reference lot of the preparation being examined.

In the MAT, responses to standard endotoxin usually dilute out over approximatively 1 $\log_{10}$ and responses to products contaminated with non-endotoxin contaminants (alone or in combination with endotoxins) often show very steep dose-response curves, usually over only 1 or 2 dilution steps when tested for their capability to stimulate monocytes. Frequently, the largest response to such contaminated products is obtained with undiluted solutions of preparations being examined or small dilutions of the preparations being examined. For this reason test solutions of preparations being examined that contain or may contain non-endotoxin contaminants have to be tested at a range of dilutions that includes minimum dilution.

2 METHODS

2-1 INFORMATION REGARDING THE CHOICE OF METHODS

Methods A, B and C, are not normally applied where a preparation being examined has the intrinsic activity of stimulating the release of the chosen read-out or where the preparation being examined is contaminated with the chosen read-out. In both cases, this fact is to be addressed by modifying and validating the chosen method accordingly. The product-specific validation of the chosen method would be expected to identify the frequency of non-responders to a particular product/contaminant(s) combination and to identify steps to address this, e.g. screening of donors, increasing the number of donors per test, and setting pass/fail criteria of appropriate stringency to maximise the likelihood of detecting contaminated batches. Method A is not appropriate if the results of different dilutions (endotoxin equivalents per millilitre) show that the dose response curve is not parallel to the standard endotoxin curve. Method B is a semi-quantitative test that can be applied when responses to dilutions of a preparation being examined are not parallel to responses to dilutions of standard endotoxin.

Method C, the reference lot comparison test, was developed to address extreme donor variability in responses to certain product/contaminant(s) combinations. In this regard, it should be noted that, while monocytes from most donors respond in a broadly similar manner to bacterial endotoxin, responses of monocytes from different donors to non-endotoxin contaminants can differ markedly, so that it is possible to identify non-responders along with low and high responders to certain product/contaminant(s) combinations.

2-2 CALCULATION OF CONTAMINANT LIMIT CONCENTRATION

The acceptance criterion for a pass/fail decision is the contaminant limit concentration (CLC), which is expressed in endotoxin equivalents per milligram or millilitre or in units of biological activity of the preparation being examined. Where an endotoxin limit concentration (ELC) has been specified for a product, the CLC is the same as the ELC, unless otherwise prescribed. The CLC is expressed in terms of endotoxin equivalents. The CLC is calculated using the following expression:

$$\frac{K}{M}$$

K = threshold pyrogenic dose per kilogram of body mass;
M = maximum recommended bolus dose of product per kilogram of body mass.

When the product is to be injected at frequent intervals or infused continuously, M is the maximum total dose administered in a single hour period.

The CLC depends on the product and its route of administration and is stated in some monographs.

Values for K are suggested in Table 2.6.30.-4.

Table 2.6.30.-4

Route of administration	K
Intravenous	5.0 IU of endotoxin per kilogram of body mass
Intravenous, for radiopharmaceuticals	2.5 IU of endotoxin per kilogram of body mass
Intrathecal	0.2 IU of endotoxin per kilogram of body mass
Parenteral formulations administered per square metre of body surface	100 IU/m^2

2-3 INFORMATION REGARDING CRYO-PROTECTANTS

The influence of cryo-protectants, e.g. dimethyl sulfoxide (DMSO), and their residues in thawed cells, is to be considered: DMSO is toxic to cells in culture and, even when cells have been washed thoroughly, cryo-preservation may have altered cell properties, e.g. cell membrane permeability.

2-4 INTERFERENCE TESTING

Where practicable, interference testing is performed on at least 3 different lots of the preparation being examined. Preparations being examined that show marked batch-to-batch variation, that effectively renders each batch unique for the purposes of interference testing, are to be subjected to interference testing within each individual test, i.e. concomitant validation.

Interference testing is preferably performed on batches of the preparation being examined that are free of endotoxins and other pyrogenic/pro-inflammatory contaminants and, where this is not practicable, none of the batches are to be heavily contaminated. If only 1 batch is available the validation has to be performed on that batch in 3 independent tests. Precision parameters for reproducibility, e.g. ± 50 per cent, are to be fulfilled.

2-5 CROSS-VALIDATION

General chapter *5.1.10. Guidelines for using the test for bacterial endotoxins*, states that an MAT should be performed on products where the presence of non-endotoxin pyrogenic substances cannot be ruled out. It is therefore recommended to perform cross-validation experiments with the MAT together with the validation experiments for the test for bacterial endotoxins (BET) using the same 3 batches. Cross-validation should be repeated on 3 batches if important process parameters have changed, as potential contamination by non-endotoxin pyrogens cannot be ruled out.

The rabbit pyrogen test (see general chapter *2.6.8. Pyrogens*) must only be performed for cross validation if none of the MAT methods (A, B or C) can be validated for a certain product.

3 REPLACEMENT OF THE RABBIT PYROGEN TEST BY THE MONOCYTE ACTIVATION TEST

As noted above, MAT is primarily intended to be used as a replacement for the rabbit pyrogen test. Monographs on pharmaceutical products intended for parenteral administration that may contain pyrogenic contaminants require either a test for bacterial endotoxins or a monocyte activation test.

As a general policy:

— in any individual monograph, when a test is required, only 1 test is included, either that for bacterial endotoxins or the MAT. Before including the MAT in a monograph, evidence is required that 1 of the 3 methods described in the MAT chapter can be applied satisfactorily to the product in question;
— the necessary information is sought from manufacturers. Companies are invited to provide any validation data that they have concerning the applicability of the MAT to the substances and formulations of interest. Such data include details of sample preparation and of any procedures necessary to eliminate interfering factors. In addition, any available parallel data for rabbit pyrogen testing that would contribute to an assurance that the replacement of a rabbit pyrogen test by the MAT is appropriate, is to be provided.

4 VALIDATION OF ALTERNATIVE METHODS

Replacement of a rabbit pyrogen test, or replacement of a method for detecting pro-inflammatory/pyrogenic contaminants by another method, is to be regarded as the use of an alternative method in the replacement of a pharmacopoeial test, as described in the General Notices.

The following procedures are suggested for validating a method for the MAT other than the one indicated in the monograph:

— the procedure and the materials and reagents used in the method should be validated as described for the test concerned;
— the presence of interfering factors (and, if needed, the procedure for removing them) should be tested on samples of at least 3 production batches.

MAT should be applied to all new products intended for parenteral administration that have to be tested for the presence of non-endotoxin pyrogens according to the requirements of the European Pharmacopoeia.

I. Assay of Pancreatin

The free protease, lipase and amylase activities of pancreatin are determined by the following methods.

Standard preparation and units

The Standard Preparation is the appropriate FIP Standard which has been adopted as an official preparation by the European Pharmacopoeia Commission and is available as

pancreas powder (protease) EPBRP or *pancreas powder (amylase and lipase) EPBRP* as appropriate.

The Unit of protease activity is contained in that amount of the Standard Preparation that, under the conditions of the assay, hydrolyses casein at an initial rate such that there is liberated per minute an amount of peptides not precipitated by trichloroacetic acid that gives the same absorbance at 275 nm as one micromole of tyrosine. The Unit of lipase activity is contained in that amount of the Standard Preparation that, under the conditions of the assay, liberates one micro-equivalent of acid per minute at pH 9.0 and 37°. The Unit of amylase activity is contained in that amount of the standard preparation that, under the conditions of the assay, decomposes starch at an initial rate such that one micro-equivalent of glycosidic linkage is hydrolysed per minute.

1. Free protease activity

Method

Solution of the standard preparation Triturate for 5 minutes a quantity of the Standard Preparation containing approximately 100 Units of protease activity with 25 mL of *calcium chloride solution* cooled to 5°. Dilute to 100 mL with the cooled *calcium chloride solution* and then dilute a sufficient quantity of the resulting suspension to 100 mL *with borate buffer pH 7.5*, cooled to 5°, so that 1 mL of the final solution contains 0.065 Units of protease activity.

Solution of the substance being examined Triturate for 5 minutes a quantity of the substance being examined containing approximately 100 Units of protease activity with 25 mL of *calcium chloride solution* cooled to 5°. Dilute to 100 mL with the cooled *calcium chloride solution* and then dilute a sufficient quantity of the resulting suspension to 100 mL with *borate buffer pH 7.5*, cooled to 5°, so that the estimated free protease activity corresponds approximately to the activity of the solution of the Standard Preparation.

Label 16 test tubes with the following identification in duplicate; S_1, S_2, S_3, S_1B, S_2B, S_3B, U and UB. To tubes S_1 and S_1B add 2.0 mL and to tubes S_2, S_2B, U and UB, 1.0 mL of *borate buffer pH 7.5*. Then to tubes S_1 and S_1B add 1.0 mL, to tubes S_2 and S_2B, 2.0 mL and to tubes S_3 and S_3B, 3.0 mL of the solution of the Standard Preparation. Add 2.0 mL of the solution of the substance being examined to tubes U and UB. To each of the control tubes (S_1B, S_2B, S_3B and UB) add 5.0 mL of a 5% w/v solution of *trichloroacetic acid* and mix. Place a stirring rod in each tube and warm to, and maintain at, 35° in a water bath. Add 5.0 mL of *concentrated casein substrate* to each of the control tubes and mix.

At accurately timed intervals add 5.0 mL of *concentrated casein substrate*, previously warmed to 35°, to tubes S_1, S_2, S_3 and U and mix immediately. After exactly 30 minutes, in the same order, stop the reaction in tubes S_1, S_2, S_3 and U by adding 5.0 mL of a 5% w/v solution of *trichloroacetic acid* and mix thoroughly. Remove all the test tubes from the water bath and allow to stand at room temperature for 20 minutes. Filter the contents of the tubes through suitable filter paper[1], collect the filtrates and refilter through the same paper. The filtrates must be free from haze.

Measure the *absorbances* of the filtrates at the maximum at 275 nm, Appendix II B, using in the reference cell a mixture of 6.0 mL of *borate buffer pH 7.5* and 5.0 mL of the 5% w/v solution of *trichloroacetic acid* that has been filtered in the same way. Correct the mean absorbances of the filtrates from tubes S_1, S_2 and S_3 by subtracting the mean absorbances of the filtrates from the corresponding control tubes S_1B, S_2B and S_3B.

Prepare a reference curve by plotting the mean corrected absorbances against the potency of the dilution of the solution of the Standard Preparation used. Calculate the corrected mean absorbance of the substance being examined by subtracting the mean absorbance of the filtrates from tubes UB from that of the filtrates from tubes U. Using the corrected mean absorbance, determine the potency of the solution of the substance being examined from the reference curve and calculate the free protease activity per mg of the substance being examined by taking into account the dilution factors.

The test is not valid unless the corrected absorbances are between 0.15 and 0.60.

2. Lipase activity

Apparatus

Use a reaction vessel of about 50 mL capacity fitted with a device that will maintain a temperature of 36.5° to 37.5°, a magnetic stirrer and a lid with holes for the insertion of electrodes, the tip of a burette, a tube for the admission of nitrogen and the introduction of reagents. An automatic or manual titration apparatus may be used. In the latter case, the burette is graduated in 5-µL divisions and the pH meter is provided with a wide reading scale and glass and calomel electrodes. After each test the reaction vessel is evacuated by suction and washed several times with water, the washings being removed each time by suction.

Method

Carry out the assay under nitrogen. In a small mortar cooled to 0° to 4° triturate carefully an amount of the substance being examined containing approximately 2500 Units of lipase activity with 1 mL of cooled *lipase solvent* until a very fine suspension is obtained (about 10 minutes). Dilute with cooled *lipase solvent*, transfer quantitatively to a graduated flask and dilute to 100.0 mL with the cooled solvent; use immediately.

Transfer 29.5 mL of *olive oil substrate emulsion* to the assembled reaction vessel equilibrated at 36.5° to 37.5° and adjust the pH to 9.2 with 0.1M *sodium hydroxide*. Add about 0.5 mL of the suspension of the preparation being examined and record the time at which the pH reaches 9.0. Add continuously from a micrometer syringe sufficient *0.1M sodium hydroxide VS* to maintain the pH at 9.0. Record the volume of *0.1M sodium hydroxide VS* consumed at 1-minute intervals for 5 minutes. Discounting the first reading, calculate the mean rate of alkali consumption *U*. If necessary, dilute with sufficient *lipase solvent* to produce an average alkali consumption of 0.08 to 0.16 mL of *0.1M sodium hydroxide VS* per minute. Repeat the procedure using the Standard Preparation in place of the substance being examined and calculate the mean rate of alkali consumption, *S*. Calculate the potency (P_L) of the substance being examined in Units per mg from the expression:

$$P_L = \frac{U}{S} \times \frac{w_S}{w} \times R$$

[1] *A suitable filter paper complies with the following test. Filter 5 mL of a 5% w/v solution of trichloroacetic acid through a sample of the paper and measure the absorbance of the filtrate at 275 nm, Appendix II B, using the unfiltered 5% w/v solution of trichloroacetic acid in the reference cell. The absorbance is not more than 0.04.*

Where U = the mean volume in mL of *0.1M sodium hydroxide VS* used per minute in the titration of the substance being examined,
S = the mean volume in mL of *0.1M sodium hydroxide VS* used per minute in the titration of the Standard Preparation,
w = weight in mg of the substance being examined,
w_s = weight in mg of the Standard Preparation,
R = potency of the Standard Preparation in Units per mg.

Calculate the potency of the preparation being examined using the average of three separate titrations for both the substance being examined and the Standard Preparation.

3. Amylase activity

Method

Triturate an amount of the preparation being examined containing approximately 1500 Units of amylase activity with 60 mL of *0.2M mixed phosphate buffer pH 6.8* for 15 minutes and add sufficient *0.2M mixed phosphate buffer pH 6.8* to produce 100 mL. To a stoppered tube (200 mm × 22 mm) add 25.0 mL of *starch substrate*, 10.0 mL of *0.2M mixed phosphate buffer pH 6.8* and 1.0 mL of *0.2M sodium chloride*. Stopper the tube, mix the contents and place in a water bath at 24.9° to 25.1°. When the temperature of the mixture has reached 25° add 1.0 mL of the solution of the substance being examined and record the time of addition.
Mix thoroughly and replace in the water bath. After exactly 10 minutes add 2 mL of 1M *hydrochloric acid* to stop the reaction. Transfer the contents of the tube to a stoppered 300 mL flask. While shaking continuously add 10.0 mL of *0.05M iodine VS* followed immediately by 45 mL of *0.1M sodium hydroxide*. Allow to stand in the dark at a temperature of 15° to 25° for 15 minutes. Add 4 mL of a mixture of 1 volume of *sulfuric acid* and 4 volumes of *water* and titrate with *0.1M sodium thiosulfate VS*. Repeat the procedure but add the 2 mL of 1M *hydrochloric acid* before the addition of the solution of the substance being examined. Prepare a solution of the Standard Preparation in the same manner as described for the solution of the substance being examined and repeat the procedure beginning at the words 'To a stoppered tube ...' but using 1.0 mL of this solution in place of the solution of the substance being examined.

Calculate the potency (P_A) of the preparation being examined in Units per mg from the expression:

$$P_A = \frac{(B - A)w_s}{(B_s - A_s)w} \times Q$$

Where A = volume in mL of *0.1M sodium thiosulfate VS* used in the titration of the substance being examined,
A_s = volume in mL of *0.1M sodium thiosulfate VS* used in the titration of the Standard Preparation
B = volume in mL of *0.1M sodium thiosulfate VS* used in the titration of the substance being examined inactivated by the addition of 1M hydrochloric acid,
B_s = volume in mL of *0.1M sodium thiosulfate VS* used in the titration of the Standard Preparation inactivated by the addition of 1M hydrochloric acid,
Q = potency of the Standard Preparation in Units per mg
w = total weight in mg of the substance being examined in the solution prepared for assay,
w_s = total weight in mg of the Standard Preparation in the solution prepared for assay.

J. Blood and Related Products

Coagulants

A1. Assay of Human Coagulation Factor II

(Ph. Eur. method 2.7.18)

Human coagulation factor II is assayed following specific activation to form factor IIa. Factor IIa is estimated by comparing its activity in cleaving a specific chromogenic peptide substrate with the same activity of the International Standard or of a reference preparation calibrated in International Units.

The International Unit is the factor II activity of a stated amount of the International Standard which consists of a freeze-dried concentrate of human blood coagulation factor II. The equivalence in International Units of the International Standard is stated by the World Health Organization.

The chromogenic assay method consists of 2 steps: snake venom-dependent activation of factor II, followed by enzymatic cleavage of a chromogenic factor IIa substrate to form a chromophore that can be quantified spectrophotometrically. Under appropriate assay conditions, there is a linear relation between factor IIa activity and the cleavage of the chromogenic substrate.

REAGENTS

Viper venom specific factor II activator (ecarin). A protein derived from the venom of the saw-scaled viper (*Echis carinatus*) which specifically activates factor II. Reconstitute according to the manufacturer's instructions. Store the reconstituted preparation at 4 °C and use within 1 month.

Factor IIa chromogenic substrate Specific chromogenic substrate for factor IIa such as: *H*-D-phenylalanyl-L-pipecolyl-L-arginine-4-nitroanilide dihydrochloride, 4-toluenesulfonyl-glycyl-prolyl-L-arginine-4-nitroanilide, *H*-D-cyclohexylglycyl-α-aminobutyryl-L-arginine-4-nitroanilide, D-cyclohexylglycyl-L-alanyl-L-arginine-4-nitroanilide diacetate. Reconstitute according to the manufacturer's instructions.

Dilution buffer Solution containing 6.06 g/L of *tris (hydroxymethyl)aminomethane R*, 17.53 g/L of *sodium chloride R* and 1 g/L of *bovine albumin R* or *human albumin R*. Adjust to pH 8.4 if necessary, using *hydrochloric acid R*.

METHOD

Test solution Dilute the preparation to be examined with dilution buffer to obtain a solution containing 0.015 IU of factor II per millilitre. Prepare at least 3 further dilutions in dilution buffer.

Reference solution Dilute the reference preparation to be examined with dilution buffer to obtain a solution containing 0.015 IU of factor II per millilitre. Prepare at least 3 further dilutions in dilution buffer.

Warm all solutions to 37 °C in a water-bath shortly before the test.

The following working conditions apply to microtitre plates. If the assay is carried out in tubes, the volumes are adjusted while maintaining the proportions in the mixture.

Using a microtitre plate maintained at 37 °C, add 25 µL of each dilution of the test solution or the reference solution to each of a series of wells. To each well add 125 µL of dilution buffer, then 25 µL of ecarin and incubate for exactly 2 min. To each well add 25 µL of factor IIa chromogenic substrate.

Read the rate of change of absorbance (*2.2.25*) at 405 nm continuously over a period of 3 min and obtain the mean

rate of change of absorbance (ΔA/min). If continuous monitoring is not possible, read the absorbance at 405 nm at suitable consecutive intervals, for instance 40 s, plot the absorbances against time on a linear graph and calculate ΔA/min as the slope of the line. From the ΔA/min values of each individual dilution of standard and test preparations, calculate the potency of the preparation to be examined and check the validity of the assay by the usual statistical methods (*5.3*).

A2. Assay of Human Coagulation Factor VII

(Ph. Eur. method 2.7.10)

Human coagulation factor VII is assayed by its biological activity as a factor VIIa-tissue factor complex in the activation of factor X in the presence of calcium ions and phospholipids. The potency of a factor VII preparation is estimated by comparing the quantity necessary to achieve a certain rate of factor Xa formation in a test mixture containing the substances that take part in the activation of factor X, and the quantity of the International Standard, or of a reference preparation calibrated in International Units, required to produce the same rate of factor Xa formation.

The International Unit is the factor VII activity of a stated amount of the International Standard, which consists of freeze-dried plasma. The equivalence in International Units of the International Standard is stated by the World Health Organization.

Human coagulation factor VII concentrate BRP is calibrated in International Units by comparison with the International Standard.

The chromogenic assay method consists of 2 consecutive steps: the factor VII-dependent activation of factor X reagent mixture containing tissue factor, phospholipids and calcium ions, followed by enzymatic cleavage of a chromogenic factor Xa substrate into a chromophore that can be quantified spectrophotometrically. Under appropriate assay conditions, there is a linear relation between the rate of factor Xa formation and the factor VII concentration. The assay is summarised in the following scheme.

Step 1

a) factor VII $\xrightarrow{\text{tissue factor, } Ca^{2+}}$ factor VIIa

b) factor X $\xrightarrow[\text{tissue factor/phospholipid}]{\text{factor VIIa, } Ca^{2+}}$ factor Xa

Step 2

chromogenic substrate $\xrightarrow{\text{factor Xa}}$ peptide + chromophore

Both steps employ reagents that may be obtained commercially from a variety of sources. Although the composition of individual reagents may be subject to some variation, their essential features are described in the following specification.

REAGENTS

The coagulation factor reagent comprises purified proteins derived from human or bovine sources. These include factor X and thromboplastin tissue factor/phospholipid as factor VII activator. These proteins are partly purified and do not contain impurities that interfere with the activation of factor VII or factor X. Factor X is present in amounts giving a final concentration during the first step of the assay of 10-350 nmol/L, preferably 14-70 nmol/L. Thromboplastin from natural sources (bovine or rabbit brain) or synthetic preparations may be used as the tissue factor/phospholipid component. Thromboplastin suitable for use in prothrombin time determination is diluted 1:5 to 1:50 in buffer such that the final concentration of Ca^{2+} is 15-25 mmol/L. The final factor Xa generation is performed in a solution containing human or bovine albumin at a concentration such that adsorption losses do not occur and which is appropriately buffered at pH 7.3-8.0. In the final incubation mixture, factor VII must be the only rate-limiting component and each reagent component must lack the ability to generate factor Xa on its own.

The second step comprises the quantification of the formed factor Xa employing a chromogenic substrate that is specific for factor Xa. Generally this consists of a short peptide of between three and five amino acids, bound to a chromophore group. On cleavage of this group from the peptide substrate, its absorption maximum shifts to a wavelength allowing its spectrophotometric quantification. The substrate is usually dissolved in *water R* and used at a final concentration of 0.2-2 mmol/L. The substrate may also contain appropriate inhibitors to stop further factor Xa generation (addition of edetate).

ASSAY PROCEDURE

Reconstitute the entire contents of one ampoule of the reference preparation and the preparation to be examined by adding the appropriate quantity of *water R*; use within 1 h. Add sufficient prediluent to the reconstituted preparations to produce solutions containing between 0.5 IU and 2.0 IU of factor VII per millilitre.

Prepare further dilutions of reference and test preparations using an isotonic non-chelating buffer containing 1 per cent of bovine or human albumin, buffered preferably between pH 7.3 and 8.0. Prepare at least three separate, independent dilutions for each material, preferably in duplicate. Prepare the dilutions such that the final factor VII concentration is below 0.005 IU/mL.

Prepare a control solution that includes all components except factor VII.

Prepare all dilutions in plastic tubes and use within 1 h.

Step 1

Mix dilutions of the factor VII reference preparation and the preparation to be examined with an appropriate volume of the prewarmed coagulation factor reagent or a combination of its separate constituents, and incubate the mixture in plastic tubes or microplate wells at 37 °C.

The concentrations of the various components during the factor Xa generation must be as specified above under the description of the reagents.

Allow the activation of factor X to proceed for a suitable time, usually terminating the reaction before the factor Xa concentration has reached its maximal level in order to obtain a satisfactory linear dose-response relationship. The activation time is also chosen to achieve linear production of factor Xa in time. Appropriate activation times are usually between 2 min and 5 min, but deviations are permissible if acceptable linearity of the dose-response relationship is thus obtained.

Step 2

Terminate the activation by the addition of a prewarmed reagent containing a chromogenic substrate. Quantify the rate of substrate cleavage, which must be linear with the

concentration of factor Xa formed, by measuring the absorbance change at an appropriate wavelength using a spectrophotometer, either monitoring the absorbance continuously, thus allowing the initial rate of substrate cleavage to be calculated, or terminating the hydrolysis reaction after a suitable interval by lowering the pH by the addition of a suitable reagent, such as acetic acid (500 g/L $C_2H_4O_2$) or a citrate solution (1 mol/L) at pH 3. Adjust the hydrolysis time to achieve a linear development of chromophore with time. Appropriate hydrolysis times are usually between 3 min and 15 min, but deviations are permissible if better linearity of the dose-response relationship is thus obtained.

Check the validity of the assay and calculate the potency of the test preparation by the usual statistical methods (for example, *5.3*).

A3. Assay of Factor VIII Fraction (Human Coagulation Factor VIII)

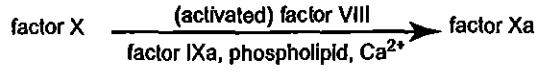
(Ph. Eur. method 2.7.4)

Human coagulation factor VIII is assayed by its biological activity as a cofactor in the activation of factor X by activated factor IX (factor IXa) in the presence of calcium ions and phospholipid. Factor VIII activity may be measured in plasma preparations and therapeutic concentrates (plasma-derived and recombinant). The potency of a factor VIII preparation is estimated by comparing the quantity necessary to achieve a certain rate of factor Xa formation in a test mixture containing the substances that take part in the activation of factor X, and the quantity of the International Standard, or of a reference preparation calibrated in International Units, required to produce the same rate of factor Xa formation.

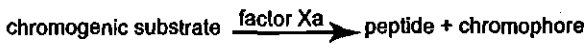
Quantification of factor VIII activity in plasma preparations is expressed in International Units defined by the International Standard for blood coagulation factor VIII in plasma, and *coagulation factors V, VIII, XI and XIII plasma BRP* is suitable for use as a reference preparation. Quantification of factor VIII activity in therapeutic concentrates is expressed in International Units defined by the International Standard for blood coagulation factor VIII concentrate, and *human coagulation factor VIII concentrate BRP* is suitable for use as a reference preparation.

The chromogenic assay method consists of 2 consecutive steps: the factor VIII-dependent activation of factor X in a coagulation-factor reagent composed of purified components, and the enzymatic cleavage of a chromogenic factor Xa substrate to yield a chromophore that can be quantified spectrophotometrically. Under appropriate assay conditions, there is a linear relation between the rate of factor Xa formation and the factor VIII concentration. The assay is summarised by the following scheme.

Step 1



Step 2



Both steps employ reagents that may be obtained commercially from a variety of sources. Although the composition of individual reagents may be subject to some variation, their essential features are described in the following specification. Deviations from this description may be permissible provided that it has been shown, using the appropriate International Standard for blood coagulation factor VIII as the standard, that the results obtained do not differ significantly.

It is important to demonstrate by validation the suitability of the kit used, notably by checking the time course of factor Xa generation in order to determine the time taken to reach 50 per cent of the maximal factor Xa generation.

REAGENTS

The coagulation factor reagent comprises purified proteins derived from human or bovine sources. These include factor X, factor IXa, and a factor VIII activator, usually thrombin. These proteins are partly purified, preferably to at least 50 per cent, and do not contain impurities that interfere with the activation of factor VIII or factor X. Thrombin may be present in its precursor form prothrombin, provided that its activation in the reagent is sufficiently rapid to give almost instantaneous activation of factor VIII in the assay. Phospholipid may be obtained from natural sources or be synthetically prepared, and must, to a substantial extent, consist of the species phosphatidylserine. The components of the complete reagent are usually divided into at least 2 separate reagents, each lacking the ability to generate factor Xa on its own. One of the reagents contains calcium ions. After reconstitution, the reagents may be combined provided that no substantial amounts of factor Xa are generated in the absence of factor VIII. In the final incubation mixture, factor VIII must be the only rate-limiting component.

The 2nd step comprises the quantification of the formed factor Xa, employing a chromogenic substrate that is specific for factor Xa. Generally this consists of a derivatised short peptide of between 3 and 5 amino acids, joined to a chromophore group. On cleavage of this group from the peptide substrate, its chromophoric properties shift to a wavelength allowing its spectrophotometric quantification. The substrate must also contain appropriate inhibitors to stop further factor Xa generation, e.g. chelating agents, and to suppress thrombin activity.

ASSAY PROCEDURE

For the assay of therapeutic concentrates, add sufficient prediluent to the reference and test preparations to produce solutions containing 0.5-2.0 IU/mL. The prediluent consists of haemophilia A plasma, or of an artificially prepared reagent that contains sufficient von Willebrand factor and that gives results that do not differ significantly from those obtained employing haemophilia plasma. The prediluted materials must be stable beyond the time required for the assay. Pre-dilution in haemophilia A plasma is not required for the assay of factor VIII in plasma preparations.

Prepare dilutions of the pre-diluted reference and test concentrate preparations (or the reference and test plasma preparations) using a non-chelating, appropriately buffered solution, for example, tris(hydroxymethyl)aminomethane or imidazole, containing 1 per cent of human or bovine albumin. Prepare at least 2 dilution series of at least 3 further dilutions for each material. Prepare the dilutions such that the final factor VIII concentration in the reaction mixture is preferably below 0.01 IU/mL, during the step of factor Xa generation.

Prepare a control solution that includes all components except factor VIII.

Prepare all dilutions in plastic tubes and use immediately.

Step 1

Mix prewarmed dilutions of the factor VIII reference preparation and of the preparation to be examined with an appropriate volume of the prewarmed coagulation factor reagent or a combination of its separate constituents, and incubate the mixture in plastic tubes or microplate wells at 37 °C. Allow the activation of factor X to proceed for a suitable time, terminating the reaction (step 2) when the factor Xa concentration has reached approximately 50 per cent of the maximal (plateau) level. Appropriate activation times are usually between 2 min and 5 min.

Step 2

Terminate the activation by addition of a prewarmed reagent containing a chromogenic substrate. Quantify the rate of substrate cleavage, which must be linear with the concentration of factor Xa formed, by measuring the absorbance change at an appropriate wavelength using a spectrophotometer, either monitoring the absorbance continuously, thus allowing the initial rate of substrate cleavage to be calculated, or terminating the hydrolysis reaction after a suitable interval by lowering the pH by addition of a suitable reagent, such as a 50 per cent *V/V* solution of acetic acid, or a 1 M pH 3 citrate buffer solution. Adjust the hydrolysis time to achieve a linear development of chromophore over time. Appropriate hydrolysis times are usually between 3 min and 15 min, but deviations are permissible if better linearity of the dose-response relationship is thus obtained.

Calculate the potency of the test preparation by the usual statistical methods (for example, *5.3*).

A4. Assay of Factor IX Fraction (Human Coagulation Factor IX)

(Ph. Eur. method 2.7.11)

The principle of the assay is to measure the ability of a factor IX preparation to reduce the prolonged coagulation time of factor IX-deficient plasma. The reaction is accelerated by addition of a reagent containing phospholipid and a contact activator, e.g. kaolin, silica or ellagic acid. The potency is assessed by comparing the dose-response curve of the preparation to be examined to that of a reference preparation, calibrated in International Units.

The International Unit is the factor IX activity of a stated amount of the International Standard, which consists of a freeze-dried concentrate of human coagulation factor IX. The equivalence in International Units of the International Standard is stated by the World Health Organization.

Human coagulation factor IX concentrate BRP is calibrated in International Units by comparison with the International Standard.

Reconstitute separately the preparation to be examined and the reference preparation as stated on the label and use immediately. Where applicable, determine the amount of heparin present (*2.7.12*) and neutralise the heparin, for example by addition of *protamine sulfate R* (10 µg of protamine sulfate neutralises 1 IU of heparin). Predilute the preparation to be examined and the reference preparation in factor IX-deficient plasma (for example *plasma substrate R2*) to produce solutions containing 0.5-2.0 IU/mL. Prepare at least 3 dilutions for each material, preferably in duplicate, using a suitable buffer solution (for example *imidazole buffer solution pH 7.3 R*) containing 10 g/L of bovine or human albumin. Use these dilutions immediately.

Use an apparatus suitable for measurement of coagulation times or carry out the assay with incubation tubes maintained in a water-bath at 37 °C. Place in each tube 0.1 mL of factor IX-deficient plasma (for example *plasma substrate R2*) and 0.1 mL of one of the dilutions of the reference preparation or of the preparation to be examined. Add to each tube 0.1 mL of a suitable Activated Partial Thromboplastin Time (APTT) reagent containing phospholipid and contact activator and incubate the mixture for a recommended time at 37 °C. To each tube, add 0.1 mL of a 3.7 g/L solution of *calcium chloride R* previously heated to 37 °C. Using a timer, measure the coagulation time, i.e. the interval between the moment of the addition of the calcium chloride and the first indication of the formation of fibrin. The volumes given above may be adapted to the APTT reagent and apparatus used. Calculate the potency using the usual statistical methods (for example, *5.3*).

A5. Assay of Human Coagulation Factor X

(Ph. Eur. method 2.7.19)

Human coagulation factor X is assayed following specific activation to form factor Xa. Factor Xa is estimated by comparing its activity in cleaving a specific chromogenic peptide substrate with the same activity of the International Standard or of a reference preparation calibrated in International Units.

The International Unit is the factor X activity of a stated amount of the International Standard which consists of a freeze-dried concentrate of human coagulation factor X. The equivalence in International Units of the International Standard is stated by the World Health Organization.

The chromogenic assay method consists of 2 steps: snake venom-dependent activation of factor X, followed by enzymatic cleavage of a chromogenic factor Xa substrate to form a chromophore that can be quantified spectrophotometrically. Under appropriate assay conditions, there is a linear relation between factor Xa activity and the cleavage of the chromogenic substrate.

REAGENTS

Russell's viper venom specific factor X activator (RVV). A protein derived from the venom of Russell's viper (*Vipera russelli*) which specifically activates factor X. Reconstitute according to the manufacturer's instructions. Store the reconstituted preparation at 4 °C and use within 1 month.

Factor Xa chromogenic substrate Specific chromogenic substrate for factor Xa such as: *N*-α-benzyloxycarbonyl-D-arginyl-L-glycyl-L-arginine-4-nitroanilide dihydrochloride, *N*-benzoyl-L-isoleucyl-L-glutamyl-glycyl-L-arginine-4-nitroanilide hydrochloride, methanesulfonyl-D-leucyl-glycyl-L-arginine-4-nitroanilide, methoxycarbonyl-D-cyclohexylalanyl-glycyl-L-arginine-4-nitroanilide acetate. Reconstitute according to the manufacturer's instructions.

Dilution buffer Solution containing 3.7 g/L of *tris (hydroxymethyl)aminomethane R*, 18.0 g/L of *sodium chloride R*, 2.1 g/L of *imidazole R*, 0.02 g/L of *hexadimethrine bromide R* and 1 g/L of *bovine albumin R* or *human albumin R*. Adjust to pH 8.4 if necessary using *hydrochloric acid R*.

METHOD

Test solution Dilute the preparation to be examined with dilution buffer to obtain a solution containing 0.18 IU of factor X per millilitre. Prepare at least 3 further dilutions in dilution buffer.

Reference solution Dilute the reference preparation to be examined with dilution buffer to obtain a solution containing 0.18 IU of factor X per millilitre. Prepare at least 3 further dilutions in dilution buffer.

Warm all solutions to 37 °C in a water-bath shortly before the test.

The following working conditions apply to microtitre plates. If the assay is carried out in tubes, the volumes are adjusted while maintaining the proportions in the mixture.

Using a microtitre plate maintained at 37 °C, add 12.5 µL of each dilution of the test solution or the reference solution to each of a series of wells. To each well add 25 µL of RVV and incubate for exactly 90 s. To each well add 150 µL of factor Xa chromogenic substrate, diluted 1 in 6 in dilution buffer.

Read the rate of change of absorbance (*2.2.25*) (at 405 nm continuously over a period of 3 min and obtain the mean rate of change of absorbance (ΔA/min). If continuous monitoring is not possible, read the absorbance at 405 nm at suitable consecutive intervals, for instance 40 s, plot the absorbances against time on a linear graph and calculate ΔA/min as the slope of the line. From the ΔA/min values of each individual dilution of standard and test preparations, calculate the potency of the preparation to be examined and check the validity of the assay by the usual statistical methods (*5.3*).

A6. Assay of Human Coagulation Factor XI

(Ph. Eur. method 2.7.22)

The principle of the assay is to measure the ability of a factor XI preparation to reduce the prolonged coagulation time of factor XI-deficient plasma. The reaction is accelerated by addition of a reagent containing phospholipid and a contact activator, e.g. kaolin, silica or ellagic acid. The potency is assessed by comparing the dose-response curve of the preparation to be examined to that of a reference plasma calibrated against the International Standard for blood coagulation factor XI in plasma.

Reconstitute separately the preparation to be examined and the reference preparation as stated on the label and use immediately. *Coagulation factors V, VIII, XI and XIII plasma BRP* is suitable for use as a reference preparation. Where applicable, determine the amount of heparin present (*2.7.12*) and neutralise the heparin, for example by addition of *protamine sulfate R* (10 µg of protamine sulfate neutralises 1 IU of heparin). Predilute the preparation to be examined and the reference preparation in factor XI-deficient plasma (for example *plasma substrate R3*) to produce solutions containing 0.5-2.0 units/mL. Prepare at least 3 appropriate dilutions for each material, preferably in duplicate, using a suitable buffer solution (for example *imidazole buffer solution pH 7.3 R*) containing 10 g/L of bovine or human albumin. Use these dilutions immediately.

Use an apparatus suitable for measurement of coagulation times or perform the assay with incubation tubes maintained in a water bath at 37 °C. Place in each tube 0.1 mL of factor XI-deficient plasma (for example *plasma substrate R3*) and 0.1 mL of one of the dilutions of the reference preparation or of the preparation to be examined. Add to each tube 0.1 mL of a suitable Activated Partial Thromboplastin Time (APTT) reagent containing phospholipid and contact activator and incubate the mixture for a recommended time at 37 °C. To each tube, add 0.1 mL of a 3.7 g/L solution of *calcium chloride R* previously heated to 37 °C. Using a timer, measure the coagulation time, i.e. the interval between the moment of the addition of the calcium chloride and the first indication of the formation of fibrin. The volumes given above may be adapted to the APTT reagent and apparatus used. Calculate the potency using the usual statistical methods (for example, *5.3*).

A7. Activated Coagulation Factors

(Ph. Eur. method 2.6.22)

Where applicable, determine the amount of heparin present (*2.7.12*) and neutralise the heparin, for example by addition of *protamine sulfate R* (10 µg of protamine sulfate neutralises 1 IU of heparin). Prepare 1 to 10 and 1 to 100 dilutions of the preparation to be examined using *tris(hydroxymethyl) aminomethane buffer solution pH 7.5 R*. Place a series of polystyrene tubes in a water-bath at 37 °C and add to each tube 0.1 mL of *platelet-poor plasma R* and 0.1 mL of a suitable dilution of a phospholipid preparation to act as a platelet substitute. Allow to stand for 60 s. Add to each tube either 0.1 mL of 1 of the dilutions or 0.1 mL of the buffer solution (control tube). To each tube add immediately 0.1 mL of a 3.7 g/L solution of *calcium chloride R* previously heated to 37 °C, and measure, within 30 min of preparing the original dilution, the time that elapses between addition of the calcium chloride solution and the formation of a clot. The test is not valid unless the coagulation time measured for the control tube is 200 s to 350 s.

A8. Assay of Human von Willebrand Factor

(Ph. Eur. method 2.7.21)

The biological functions of human von Willebrand factor are numerous. At present, its ristocetin cofactor activity and its collagen binding activity can be utilised for assays.
The potency of human von Willebrand factor is determined by comparing, in given conditions, its activity with the same activity of a reference preparation calibrated against the International Standard, in International Units where applicable.

The International Unit is the activity of a stated amount of the International Standard, which consists of a freeze-dried human von Willebrand factor concentrate. The equivalence in International Units of the International Standard is stated by the World Health Organization (WHO).

RISTOCETIN COFACTOR ASSAY

The ristocetin cofactor activity of von Willebrand factor is determined by measuring agglutination of a suspension of platelets in the presence of ristocetin A. The assay can be carried out for quantitative determinations by using automated instruments, or for semi-quantitative determinations by visually assessing the endpoint of agglutination in a dilution series. Quantitative assays are preferred.

REAGENTS

Suspension of platelets Use standardised and, for example, formaldehyde- or paraformaldehyde-fixed preparations of freshly isolated and washed human platelets. The suspension may also be freeze-dried. An appropriate amount of ristocetin A is added if necessary. Some platelet reagents may already contain ristocetin A.

Reference preparation The reference preparation for von Willebrand factor is the WHO International Standard for von Willebrand factor concentrate.

METHOD

Semi-quantitative assay Prepare suitable dilutions of the preparation to be examined and of the reference preparation, using as diluent a solution containing 9 g/L of *sodium*

chloride R and 10-50 g/L of *human albumin R*. Add to each dilution an appropriate amount of the suspension of platelets and, if necessary, of ristocetin A. Mix on a glass slide by moving it gently in circles for 1 min. Allow to stand for a further 1 min and read the result against a dark background with side lighting. The last dilution which clearly shows visible agglutination indicates the ristocetin cofactor titre of the sample. Use diluent as a negative control.

Quantitative Assay Reconstitute the entire contents of 1 ampoule of the reference preparation and the preparation to be examined by adding the appropriate quantity of the recommended diluent (for example *water R*); use immediately. Add sufficient prediluent to the reconstituted preparations to produce solutions containing 0.5-2.0 IU/mL. The prediluent consists of an isotonic non-chelating buffer containing, for example, 1-5 per cent of human or bovine albumin, and tris(hydroxymethyl)aminomethane or imidazole, appropriately buffered.

The test is performed in accordance with the manufacturer's instructions with at least 2 dilution series with as many dilutions as are needed to obtain a total of at least 3 different concentrations in the linear range of the assay.

Check the validity of the assay and calculate the potency of the test preparation using the usual statistical methods (for example, *5.3*).

COLLAGEN-BINDING ASSAY

Collagen-binding is determined by an enzyme-linked immunosorbent assay on collagen-coated microtitre plates. The method is based on the specific binding of von Willebrand factor to collagen fibrils and the subsequent binding of polyclonal anti-von Willebrand factor antibody conjugated to an enzyme, which on addition of a chromogenic substrate yields a product that can be quantitated spectrophotometrically. Under appropriate conditions, there is a linear relationship between von Willebrand factor collagen-binding and absorbance.

REAGENTS

Collagen Use native equine or human fibrils of collagen type I or III. For ease of handling, collagen solutions may be used.

Collagen diluent Dissolve 50 g of *glucose R* in *water R*, adjust to pH 2.7-2.9 with *1 M hydrochloric acid* and dilute to 1000 mL with *water R*.

Phosphate-buffered saline (PBS) Dissolve 8.0 g of *sodium chloride R*, 1.05 g of *disodium hydrogen phosphate dihydrate R*, 0.2 g of *sodium dihydrogen phosphate R* and 0.2 g of *potassium chloride R* in *water R*. Adjust to pH 7.2 using *1 M sodium hydroxide* or *1 M hydrochloric acid* and dilute to 1000 mL with *water R*.

Washing buffer PBS containing 1 g/L of *polysorbate 20 R*.

Blocking reagent PBS containing 1 g/L of *polysorbate 20 R* and 10 g/L of *bovine albumin R*.

Dilution buffer PBS containing 1 g/L of *polysorbate 20 R* and 50 g/L of *bovine albumin R*.

Conjugate Rabbit anti-human von Willebrand factor serum horseradish peroxidase conjugate. Use according to the manufacturer's instructions.

Substrate solution Immediately before use, dissolve a tablet of *o*-phenylenediamine dihydrochloride and a tablet of urea hydrogen peroxide in 20 mL of *water R* or use a suitable volume of hydrogen peroxide. Protect from light.

Microtitre plates Flat-bottomed polystyrene plates with surface properties optimised for enzyme immunoassay and high protein-binding capacity.

METHOD

Test solutions Reconstitute the preparation to be examined as stated on the label. Dilute with dilution buffer to produce a solution containing approximately 1 IU of von Willebrand factor. Prepare 2 series of at least 3 further dilutions using dilution buffer.

Reference solutions Reconstitute the reference preparation as directed. Dilute with dilution buffer to produce a solution containing approximately 1 IU of von Willebrand factor. Prepare 2 series of at least 3 further dilutions using dilution buffer.

Allow the solution of collagen to warm to room temperature. Dilute with collagen diluent to obtain a solution containing 30-75 µg/mL of collagen, mix gently to produce a uniform suspension of collagen fibrils. Pipette 100 µL into each well of the microtitre plate. Cover the plate with plastic film and incubate at 37 °C overnight. Empty the wells of the collagen-coated plate by inverting and draining on a paper towel. Add 250 µL of washing buffer. Empty the wells of the plate by inverting and draining on a paper towel. Repeat this operation 3 times. Add 250 µL of blocking reagent to each well, cover the plate with plastic film and incubate at 37 °C for 1 h. Empty the wells of the plate by inverting and draining on a paper towel. Add 250 µL of washing buffer. Empty the wells of the plate by inverting and draining on a paper towel. Repeat this operation 3 times.

Add 100 µL each of the test solutions or reference solutions to the wells. Add 100 µL of dilution buffer to a series of wells to serve as negative control. Cover the plate with plastic film and incubate at 37 °C for 2 h. Empty the wells of the plate by inverting and draining on a paper towel.
Add 250 µL of washing buffer. Empty the wells of the plate by inverting and draining on a paper towel. Repeat this operation 3 times.

Prepare a suitable dilution of the conjugate (for example, a dilution factor of 1 to 4000) with PBS containing 5 g/L of *bovine albumin R* and add 100 µL to each well. Cover the plate with plastic film and incubate at 37 °C for 2 h. Empty the wells of the plate by inverting and draining on a paper towel. Add 250 µL of washing buffer. Empty the wells of the plate by inverting and draining on a paper towel. Repeat this operation 3 times.

Add 100 µL of substrate solution to each of the wells and incubate at room temperature for 20 min in the dark.
Add 100 µL of *1 M hydrochloric acid* to each of the wells.

Measure the absorbance at 492 nm. Use the absorbance values to estimate the potency of the preparation to be examined using the usual statistical methods (*5.3*).

The assay is invalid if the absorbances measured for the negative controls are greater than 0.05.

A9. Test for Prekallikrein Activator

(Ph. Eur. method 2.6.15)

Prekallikrein activator (PKA) activates prekallikrein to kallikrein and may be assayed by its ability to cleave a chromophore from a synthetic peptide substrate so that the rate of cleavage can be measured spectrophotometrically and the concentration of PKA calculated by comparison with a reference preparation calibrated in International Units.

The International Unit is the activity of a stated amount of the International Standard, which consists of freeze-dried prekallikrein activator. The equivalence in International Units

of the International Standard is stated by the World Health Organization.

REAGENTS

Prekallikrein activator in albumin BRP is calibrated in International Units by comparison with the International Standard.

Buffer A Dissolve 6.055 g of *tris(hydroxymethyl) aminomethane R*, 1.17 g of *sodium chloride R*, 50 mg of *hexadimethrine bromide R* and 0.100 g of *sodium azide R* in *water R*. Adjust to pH 8.0 with *2 M hydrochloric acid R* and dilute to 1000 mL with *water R*.

Buffer B Dissolve 6.055 g of *tris(hydroxymethyl) aminomethane R* and 8.77 g of *sodium chloride R* in *water R*. Adjust to pH 8.0 with *2 M hydrochloric acid R* and dilute to 1000 mL with *water R*.

PREPARATION OF PREKALLIKREIN SUBSTRATE

To avoid coagulation activation, blood or plasma used for the preparation of prekallikrein must come into contact only with plastics or silicone-treated glass surfaces.

Draw 9 volumes of human blood into 1 volume of anticoagulant solution (ACD, CPD or a 38 g/L solution of *sodium citrate R*) to which 1 mg/mL of *hexadimethrine bromide R* has been added. Centrifuge the mixture at 3600 *g* for 5 min. Separate the plasma and centrifuge again at 6000 *g* for 20 min to sediment platelets. Separate the platelet-poor plasma and dialyse against 10 volumes of buffer A for 20 h. Apply the dialysed plasma to a chromatography column containing agarose-DEAE for ion-exchange chromatography R that has been equilibrated in buffer A and is equal to twice the volume of the plasma. Elute from the column with buffer A at 20 mL/cm^2/h. Collect the eluate in fractions and record the absorbance at 280 nm (*2.2.25*). Pool the fractions containing the 1st protein peak so that the volume of the pool is about 1.2 times the volume of the platelet-poor plasma.

Test the substrate pool for absence of kallikrein activity by mixing 1 part with 20 parts of the pre-warmed chromogenic substrate solution to be used in the assay and incubate at 37 °C for 2 min. The substrate is suitable if the increase in absorbance is less than 0.001 per minute. Add to the pooled solution 7 g/L of *sodium chloride R* and filter through a membrane filter (nominal pore size 0.45 µm). Freeze the filtrate in portions and store at −25 °C; the substrate may be freeze-dried before storage.

Carry out all procedures from the beginning of the chromatography to freezing in portions during a single working day.

METHOD

The assay may be carried out using an automated enzyme analyser or a suitable microtitre plate system allowing kinetic measurements, with appropriate software for calculation of results. Standards, samples and prekallikrein substrate may be diluted as necessary using buffer B.

Incubate diluted standards or samples with prekallikrein substrate for 10 min such that the volume of the undiluted sample does not exceed 1/10 of the total volume of the incubation mixture to avoid errors caused by variation in ionic strength and pH in the incubation mixture. Incubate the mixture or a part thereof with at least an equal volume of a solution of a suitable synthetic chromogenic substrate, known to be specific for kallikrein (for example, *N-benzoyl-L-prolyl-L-phenylalanyl-L-arginine 4-nitroanilide acetate R* or D-prolyl-L-phenylalanyl-L-arginine 4-nitroanilide dihydrochloride R), dissolved in buffer B. Record the rate of change in absorbance per minute for 2-10 min at the wavelength specific for the substrate used. Prepare a blank for each mixture of sample or standard using buffer B instead of prekallikrein substrate.

Depending on the method used, ΔA/min has to be corrected by subtracting the value obtained for the corresponding blank without the prekallikrein substrate. The results may be calculated using a standard curve, a parallel-line or a slope ratio assay or any other suitable statistical method. Plot a calibration curve using the values thus obtained for the reference preparation and the respective concentrations; use the curve to determine the PKA activity of the preparation to be examined. During method validation, the spiking experiments must show that the sample matrix has no influence on the results. High blank values may impact assay validity and should be appropriately investigated.

A10. Assay of Human Plasmin Inhibitor

(Ph. Eur. method 2.7.25)

Human plasmin inhibitor, also called human α_2-antiplasmin, is a plasma protein that inhibits the plasmin (a serine protease) pathway of fibrinolysis by rapidly forming a complex with free plasmin. Furthermore, upon blood coagulation, human plasmin inhibitor is cross-linked to fibrin strands by factor XIII, and interferes with binding of the proenzyme plasminogen to fibrin.

The potency of human plasmin inhibitor is estimated by comparing the ability of the preparation to be examined to inhibit the cleavage of a specific chromogenic substrate by plasmin with the same ability of a reference standard of human plasmin inhibitor. Plasmin cleavage of the chromogenic substrate yields a chromophore that can be quantified spectrophotometrically.

The individual reagents for the assay may be obtained separately or in commercial kits. Both end-point and kinetic methods are available. Procedures and reagents may vary between different kits and the manufacturer's instructions are followed. The essential features of the procedure are described in the following example of a microtitre-plate kinetic method.

REAGENTS

Dilution buffer pH 7.5 According to the manufacturer's instructions, a suitable buffer is used. Adjust the pH if necessary.

Plasmin A preparation of human plasmin that does not contain significant amounts of other proteases is preferably used. Reconstitute and store according to the manufacturer's instructions.

Plasmin chromogenic substrate A suitable specific chromogenic substrate for plasmin is used: H-D-cyclohexylalanyl-norvalyl-lysyl-*p*-nitroaniline hydrochloride (H-D-CHA-Nva-Lys-*p*NA.HCl) or L-pyroglutamyl-L-phenylalanyl-L-lysyl-*p*-nitroaniline hydrochloride (Glp-Phe-Lys-*p*NA.HCl). Reconstitute in *water R* to give a suitable concentration according to the manufacturer's instructions.

METHOD

Varying quantities of the preparation to be examined are mixed with a given quantity of plasmin and the remaining plasmin activity is determined using a suitable chromogenic substrate.

Reconstitute or thaw the preparation to be examined according to the manufacturer's instructions. Dilute with dilution buffer pH 7.5 and prepare at least 2 independent series of 3 or 4 dilutions for both the preparation to be examined and the reference standard.

Mix 0.020 mL of each dilution with 0.020 mL of dilution buffer pH 7.5 and warm to 37 °C. Add 0.040 mL of a plasmin solution (test concentration in the range of 0.2 nkat/mL to 1.6 nkat/mL) previously heated to 37 °C and leave at 37 °C for 1 min. Add 0.020 mL of the chromogenic substrate solution, previously heated to 37 °C, to each mixture. Immediately start measurement of the change in absorbance at 405 nm (*2.2.25*) using a microtitre plate reader. Calculate the rate of change of absorbance (ΔA/min). Alternatively, an end-point assay might be used by stopping the reaction with acetic acid and measuring the absorbance at 405 nm.

In both cases the duration of the cleavage of the chromogenic substrate should be chosen to produce a linear increase in absorbance at 405 nm, before substrate depletion becomes significant. If the assay is performed in test tubes or cuvettes using a spectrophotometric method, the volumes of reagent solutions are changed proportionally.

Substract the optical density of the blank (prepared with dilution buffer pH 7.5) from the optical density of the preparation to be examined. Check the validity of the assay and calculate the potency of the preparation to be examined by the usual statistical methods (*5.3*).

Anticoagulants

B1. Assay of Heparin in Coagulation Factors

(Ph. Eur. method 2.7.12)

Heparin is assayed as a complex with antithrombin III (AT) via its inhibition of coagulation factor Xa (anti-Xa activity). An excess of AT is maintained in the reaction mixture to ensure a constant concentration of the heparin-AT complex. Factor Xa is neutralised by the heparin-AT complex and the residual factor Xa hydrolyses a specific chromogenic peptide substrate to release a chromophore. The quantity of chromophore is inversely proportional to the activity of the heparin.

Factor Xa chromogenic substrate Specific chromogenic substrate for factor Xa such as: *N*-benzoyl-L-isoleucyl-L-glutamyl-glycyl-L-arginine-4-nitroanilide hydrochloride. Reconstitute according to the manufacturer's instructions.

Dilution buffer 6.05 g/L solution of *tris(hydroxymethyl) aminomethane R*. Adjust to pH 8.4 if necessary using *hydrochloric acid R*.

Test solution Dilute the preparation to be examined with dilution buffer to obtain a solution expected to contain 0.1 IU of heparin per millilitre.

Reference solution Dilute the heparin reference preparation with dilution buffer to obtain a solution containing 0.1 IU of heparin per millilitre.

The following working conditions apply to microtitre plates. If the assay is carried out in tubes, the volumes are adjusted while maintaining the proportions in the mixture.

Warm all solutions to 37 °C in a water-bath shortly before the test.

Distribute in a series of wells, 20 µL of normal human plasma and 20 µL of *antithrombin III solution R1*. Add to the wells a series of volumes (20 µL, 60 µL, 100 µL and 140 µL) of the test solution or the reference solution and make up the volume in each well to 200 µL using dilution buffer (0.02-0.08 IU of heparin per millilitre in the final reaction mixture).

End-point method Transfer 40 µL from each well to a second series of wells, add 20 µL of *bovine factor Xa solution R* and incubate at 37 °C for 30 s. Add 40 µL of a 1 mmol/L solution of factor Xa chromogenic substrate and incubate at 37 °C for 3 min. Terminate the reaction by lowering the pH by the addition of a suitable reagent, such as a 20 per cent *V/V* solution of *glacial acetic acid R* and measure the absorbance at 405 nm (*2.2.25*). Appropriate reaction times are usually between 3 min and 15 min, but deviations are permissible if better linearity of the dose-response relationship is thus obtained.

Kinetic method Transfer 40 µL from each well to a second series of wells, add 20 µL of *bovine factor Xa solution R* and incubate at 37 °C for 30 s. Add 40 µL of a 2 mmol/L solution of factor Xa chromogenic substrate, incubate at 37 °C and measure the rate of substrate cleavage by continuous measurement of the absorbance change at 405 nm (*2.2.25*), thus allowing the initial rate of substrate cleavage to be calculated. This rate must be linear with the concentration of residual factor Xa.

Check the validity of the assay and calculate the heparin activity of the test preparation by the usual statistical methods for a slope-ratio assay (for example, *5.3*).

B2. Assay of Heparin

(Ph. Eur. method 2.7.5)

The anticoagulant activity of heparin is determined *in vitro* by its ability to accelerate the inhibition of thrombin, factor IIa (anti-IIa assay), by antithrombin. The International Unit is the activity contained in a stated amount of the International Standard for unfractionated heparin. *Heparin sodium BRP*, calibrated in International Units by comparison with the International Standard using the 2 assays given below, is used as the reference preparation.

The assay of anti-factor Xa activity is carried out to determine the ratio of anti-factor Xa activity to anti-factor IIa activity.

For anti-IIa and anti-Xa assays, carry out the assay by determining the absorbance (end-point method) or the change of absorbance per minute (kinetic method).

ANTI-FACTOR IIA ACTIVITY

Reference and test solutions

Prepare 4 independent series of 4 dilutions each of the substance to be examined and of *heparin sodium BRP* in *tris (hydroxymethyl) aminomethane-EDTA buffer solution pH 8.4 R1*; a concentration range within 0.005 IU and 0.03 IU per millilitre is suitable. The dilutions chosen must give a linear response when results are plotted as absorbance against log concentration.

Procedure

Label 16 tubes for the dilutions of the substance to be examined and 16 tubes for the dilutions of the reference preparation: T_1, T_2, T_3, T_4 for each of the 4 series of dilutions of the substance to be examined and S_1, S_2, S_3, S_4 for each of the 4 series of dilutions of the reference preparation. To each of the 32 tubes add 100 µL of *antithrombin III solution R5* and 50 µL of the appropriate dilution of the substance to be examined or the reference preparation. After each addition, mix but do not allow bubbles to form. Treating the tubes in 2 subsequent series in the order S_1, S_2, S_3, S_4, T_1, T_2, T_3, T_4, T_1, T_2, T_3, T_4, S_1, S_2, S_3, S_4, allow to equilibrate at 37 °C (water-bath or heating block) for at least 1 min and add to each tube 25 µL of *human thrombin solution R2*. Incubate for exactly 1 min and add 50 µL of a chromogenic substrate specific to factor IIa at a concentration suitable for the assay (for example, D-phenylalanyl-L-pipecolyl-L-arginine-4-nitroanilide

dihydrochloride dissolved in *water R* to give a 1.25 mM solution).

For the kinetic method, transfer the mixtures to semi-micro cuvettes and measure the change in absorbance per minute (*2.2.25*) at 405 nm using a suitable reading device.

For the end-point method, stop the reaction after exactly 4 min by adding 50 µL of a 20 per cent *V/V* solution of *glacial acetic acid R*. Assess whether exactly 4 min of incubation with the chromogenic substrate yields the optimal absorbance reading and, if necessary, adjust the incubation time to give the best dose-response curve. Then, transfer the mixtures to semi-micro cuvettes and measure the absorbance (*2.2.25*) at 405 nm using a suitable reading device.

Determine the blank amidolytic activity at the beginning and at the end of the procedure in a similar manner, using *tris (hydroxymethyl)aminomethane-EDTA buffer solution pH 8.4 R1* instead of the reference and test solutions; the 2 blank values do not differ significantly.

Calculate the regression of the absorbance on log concentrations of the solutions of the substance to be examined and of *heparin sodium BRP*, and calculate the potency of the substance to be examined in International Units per millilitre using the usual statistical methods for parallel-line assays (*5.3*).

ANTI-FACTOR XA ACTIVITY

Reference and test solutions

Prepare 4 independent series of 4 dilutions each of the substance to be examined and of *heparin sodium BRP* in *tris (hydroxymethyl)aminomethane-EDTA buffer solution pH 8.4 R1*; a concentration range within 0.03 IU and 0.375 IU per millilitre is suitable. The dilutions chosen must give a linear response when results are plotted as absorbance against log concentration.

Procedure

Label 16 tubes for the dilutions of the substance to be examined and 16 tubes for the dilutions of the reference preparation: T_1, T_2, T_3, T_4 for each of the 4 series of dilutions of the substance to be examined and S_1, S_2, S_3, S_4 for each of the 4 series of dilutions of the reference preparation. To each of the 32 tubes add 50 µL of *antithrombin III solution R6* and 50 µL of the appropriate dilution of the substance to be examined or the reference preparation. After each addition, mix but do not allow bubbles to form. Treating the tubes in 2 subsequent series in the order S_1, S_2, S_3, S_4, T_1, T_2, T_3, T_4, T_1, T_2, T_3, T_4, S_1, S_2, S_3, S_4, allow to equilibrate at 37 °C (water-bath or heating block) for 1 min and add to each tube 100 µL of *bovine factor Xa solution R2*. Incubate for exactly 2 min and add 100 µL of a chromogenic substrate specific to factor Xa at a concentration suitable for the assay (for example, *N*-α-benzyloxycarbonyl-D-arginyl-L-glycyl-L-arginine-4-nitroanilide dihydrochloride dissolved in *water R* to give a 1 mM solution).

For the kinetic method, transfer the mixtures to semi-micro cuvettes and measure the change in absorbance per minute (*2.2.25*) at 405 nm using a suitable reading device.

For the end-point method, stop the reaction after exactly 4 min by adding 50 µL of a 20 per cent *V/V* solution of *glacial acetic acid R*. Assess whether exactly 4 min of incubation with the chromogenic substrate yields the optimal absorbance reading and, if necessary, adjust the incubation time to give the best dose-response curve. Then, transfer the mixtures to semi-micro cuvettes and measure the absorbance (*2.2.25*) at 405 nm using a suitable reading device.

Determine the blank amidolytic activity at the beginning and at the end of the procedure in a similar manner, using *tris (hydroxymethyl)aminomethane-EDTA buffer solution pH 8.4 R1* instead of the reference and test solutions; the 2 blank values do not differ significantly.

Calculate the regression of the absorbance on log concentrations of the solutions of the substance to be examined and of *heparin sodium BRP*, and calculate the potency of the substance to be examined in International Units per millilitre using the usual statistical methods for parallel-line assays (*5.3*).

B3. Assay of Human Antithrombin III

(Ph. Eur. method 2.7.17)

The antithrombin III content of the preparation to be examined is determined by comparing its ability to inactivate thrombin in the presence of an excess of heparin with the same ability of a reference preparation of human antithrombin III concentrate calibrated in International Units. Varying quantities of the preparation to be examined are mixed with a given quantity of thrombin and the remaining thrombin activity is determined using a suitable chromogenic substrate.

The International Unit is the activity of a stated amount of the International Standard for human antithrombin III concentrate. The equivalence in International Units of the International Standard is stated by the World Health Organization.

Method Prepare 2 independent series of 3 or 4 dilutions in the range 1/75 to 1/200 from 1 IU/mL, for both the preparation to be examined and the reference preparation, using *tris-EDTA BSA buffer solution pH 8.4 R* containing 15 IU of heparin per millilitre.

Warm 200 µL of each dilution at 37 °C for 1-2 min. Add to each dilution 200 µL of a solution of *bovine thrombin R* containing 2 IU/mL in *tris-EDTA BSA buffer solution pH 8.4 R*. Mix and maintain at 37 °C for exactly 1 min. Add 500 µL of a suitable chromogenic substrate (for example, D-phenylalanyl-L-pipecolyl-L-arginine-4-nitroanilide, reconstituted in *water R* to give a solution containing 4 mmol/L and further diluted to a concentration suitable for the assay using *tris-EDTA BSA buffer solution pH 8.4 R* without albumin). Immediately start measurement of the change in absorbance at 405 nm (*2.2.25*), continuing the measurement for at least 30 s. Calculate the rate of change of absorbance (ΔA/min). (Alternatively, an end-point assay may be used by stopping the reaction with acetic acid and measuring the absorbance at 405 nm.)

The rate of change of absorbance (ΔA/min) is inversely proportional to antithrombin III activity.

Check the validity of the assay and calculate the potency of the test preparation by the usual statistical methods (*5.3*).

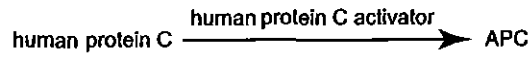
B4. Assay of Human Protein C

(Ph. Eur. method 2.7.30)

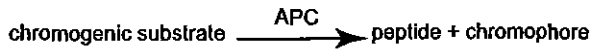
1. CHROMOGENIC ASSAY

Human protein C is a vitamin K-dependent plasma protein that, upon activation to activated protein C (APC), can inhibit blood coagulation through the proteolytic cleavage of factors Va and VIIIa. Human protein C activity is estimated using a two-step method: in the 1st step, human protein C in the preparation is activated by a specific activator from snake venom; in the 2nd step, APC cleaves a specific chromogenic substrate to form a chromophore that can be quantified spectrophotometrically.

Step 1



Step 2



The potency of human protein C is estimated by comparing the ability of the preparation to be examined to cleave a chromogenic substrate with the same ability of a reference standard of human protein C calibrated in International Units. The International Unit is the activity of a stated amount of the International Standard for human protein C. The equivalence in International Units of the International Standard is stated by the World Health Organization.

Individual reagents may be obtained separately or in commercial kits. Both end-point and kinetic methods are available. Procedures and reagents may vary between different kits and the manufacturer's instructions are followed. The essential features of the procedure are described in the following example of a microtitre plate end-point method.

REAGENTS

Dilution buffer pH 8.4 Dissolve 6.055 g of *tris (hydroxymethyl)aminomethane R* and 16.84 g of *caesium chloride R* in *water R* and adjust the pH if necessary. Dilute to 1000.0 mL with *water R*.

Human protein C activator Protein isolated from the venom of the viper *Agkistrodon contortrix contortrix* that specifically activates human protein C. Reconstitute and store according to the manufacturer's instructions. Dilute to 0.25 U/mL with *water R* before use in the assay.

Activated protein C chromogenic substrate Specific chromogenic substrate for APC, for example L-pyroglutamyl-L-prolyl-L-arginyl-*p*-nitroaniline hydrochloride (pyroGlu-Pro-Arg-pNA.HCl). Reconstitute with *water R* to give a concentration of 4.5 mmol/L. Further dilute to 1.1 mmol/L with dilution buffer pH 8.4 before use in the assay.

METHOD

Reconstitute or thaw the preparation to be examined according to the manufacturer's instructions. Dilute with *water R* to produce at least 3 separate dilutions for each preparation in the range 0.050-0.200 IU/mL, preferably in duplicate.

Step 1

Mix 0.025 mL of each dilution with 0.050 mL of the human protein C activator, both previously heated to 37 °C, and leave at 37 °C for exactly 10 min. For each dilution, prepare a blank in the same manner, using *water R* instead of the human protein C activator.

Step 2

Add 0.150 mL of diluted chromogenic substrate, previously heated to 37 °C, to each mixture and leave at 37 °C for exactly 10 min. The incubation time must be adjusted, if necessary, to ensure a linear development of chromophore with time. Terminate the reaction by adding 0.050 mL of a 50 per cent *V/V* solution of *glacial acetic acid R*.

Cleavage of the chromogenic substrate by APC causes release of the chromophore pNA, in proportion to the concentration of human protein C in the preparation. Measure the optical density at a wavelength of 405 nm. Subtract the optical density of the blank from the optical density of the test sample. Check the validity of the assay and calculate the potency of the preparation to be examined using the usual statistical methods (*5.3*).

2. CLOTTING ASSAY

Human protein C activity is estimated following cleavage to APC by a specific activator extracted from the venom of the viper *Agkistrodon contortrix contortrix*. The resulting APC inactivates factors Va and VIIIa, and thus prolongs the APTT (Activated Partial Thromboplastin Time) of a system in which all the coagulation factors are present, constant and in excess, except for human protein C, which is derived from the preparation being tested. Prolongation of the clotting time is proportional to the concentration of human protein C in the preparation.

The potency of human protein C is estimated by comparing the ability of the preparation to be examined to prolong the clotting time with the same ability of a reference standard of human protein C calibrated in International Units.
The International Unit is the activity of a stated amount of the International Standard for human protein C.
The equivalence in International Units of the International Standard is stated by the World Health Organization.

Individual reagents may be obtained separately or in commercial kits. Procedures and reagents may vary between different kits and the manufacturer's instructions are followed. The essential features of the procedure are described in the following example.

REAGENTS

Dilution buffer pH 7.4 Isotonic non-chelating buffer.

Human protein C-deficient plasma Citrated human plasma with no measurable human protein C content. Reconstitute and store according to the manufacturer's instructions.

Human protein C activator Protein isolated from the venom of the viper *Agkistrodon contortrix contortrix* that specifically activates human protein C. Reconstitute and store according to the manufacturer's instructions.

Coagulation activator A suitable APTT reagent containing phospholipids and a contact activator may be used. It may be combined with the human protein C activator.

METHOD

Reconstitute or thaw the preparation to be examined according to the manufacturer's instructions. Dilute with dilution buffer pH 7.4 to produce at least 3 separate dilutions for each preparation in the range 0.010-0.150 IU/mL, preferably in duplicate.

Mix 1 volume of each dilution with 1 volume of human protein C-deficient plasma and 1 volume of the human protein C activator (combined with the APTT reagent where appropriate), all previously heated to 37 °C. Add 1 volume of 0.025 M calcium chloride solution R previously heated to 37 °C, and record the clotting time.

The clotting time is proportional to the concentration of human protein C in each dilution. Check the validity of the assay and calculate the potency of the preparation to be examined using the usual statistical methods (*5.3*).

B5. Assay of Human Protein S

(Ph. Eur. method 2.7.31)

Human protein S is a vitamin K-dependent plasma protein that acts as a cofactor for the anticoagulant functions of activated protein C (APC). Human protein S activity may be determined by the clotting assay described below, which is sensitive to the ability of human protein S to accelerate the inactivation of factor Va by APC. In practice, the assay involves the addition of human protein S to a reagent mixture containing APC, factor Va and human protein S-deficient plasma. Prolongation of the clotting time is proportional to the concentration of human protein S in the preparation. Methods in which APC is added directly as a reagent are preferred to those in which APC is generated during the assay by the addition of a specific human protein C activator purified from snake venom. Activation of coagulation is initiated by the addition of an activating reagent such as thromboplastin or activated factor X, together with phospholipids and calcium chloride. During the assay, factor Va is generated from factor V in the human protein S-deficient plasma following the activation of coagulation. The assay procedure must ensure that human protein S is the only limiting factor.

The potency of human protein S is estimated by comparing the ability of the preparation to be examined to prolong the clotting time with the same ability of a reference standard of human protein S calibrated in International Units.
The International Unit is the activity of a stated amount of the International Standard for human protein S.
The equivalence in International Units of the International Standard is stated by the World Health Organization.

Individual reagents may be obtained separately or in commercial kits. Procedures and reagents may vary between different kits and the manufacturer's instructions are followed. The essential features of the procedure are described in the following example.

REAGENTS

Dilution buffer pH 7.4 Isotonic non-chelating buffer prepared as follows: dissolve 6.08 g of *tris(hydroxymethyl) aminomethane R* and 8.77 g of *sodium chloride R* in *water R* and adjust the pH if necessary; add 10 g of *bovine albumin R* or *human albumin R* and dilute to 1000.0 mL with *water R*.

Human protein S-deficient plasma Citrated human plasma with no measurable human protein S content and, preferably, also free of C4b-binding protein.

Coagulation activator This reagent is used to initiate coagulation in the human protein S-deficient plasma, and thereby also provides a source of activated factor V.
The activator may consist of tissue factor, activated factor X, or an agent capable of directly activating factor X that may be purified from the venom of Russell's viper (*Vipera russelli*).
The reagent also contains APC, phospholipids and *calcium chloride R*, or, alternatively, calcium chloride may be added separately after a timed activation period.

METHOD

Reconstitute or thaw the preparation to be examined according to the manufacturer's instructions. Dilute with dilution buffer pH 7.4 to produce at least 3 separate dilutions for each preparation in the range 0.020-0.100 IU/mL, preferably in duplicate.

Mix 1 volume of each dilution with 1 volume of human protein S-deficient plasma, both previously heated to 37 °C. Add 2 volumes of the coagulation activator, previously heated to 37 °C, and record the clotting time.

Alternative procedures may use a coagulation activator without calcium chloride, and require a precisely timed activation period before the addition of calcium chloride and the measurement of clotting time.

The clotting time is proportional to the concentration of human protein S in each dilution. Check the validity of the assay and calculate the potency of the preparation to be examined using the usual statistical methods (*5.3*).

Immunoglobulins

C1. Test for Fc Function of Immunoglobulin

(Ph. Eur. method 2.7.9)

The test for Fc function of immunoglobulin is carried out using method A or B. Method B is an adaptation of the procedure of method A for the use of microtitre plates for the measurement of complement-mediated haemolysis. Differences in the test procedures between methods A and B are addressed in the test.

REAGENTS

Stabilised human blood Collect group O human blood into ACD anticoagulant solution. Store the stabilised blood at 4 °C for not more than 3 weeks.

Phosphate-buffered saline pH 7.2 Dissolve 1.022 g of *anhydrous disodium hydrogen phosphate R*, 0.336 g of *anhydrous sodium dihydrogen phosphate R* and 8.766 g of *sodium chloride R* in 800 mL of *water R* and dilute to 1000 mL with the same solvent.

Magnesium and calcium stock solution Dissolve 1.103 g of *calcium chloride R* and 5.083 g of *magnesium chloride R* in *water R* and dilute to 25 mL with the same solvent.

Barbital buffer stock solution Dissolve 207.5 g of *sodium chloride R* and 25.48 g of *barbital sodium R* in 4000 mL of *water R* and adjust to pH 7.3 using *1 M hydrochloric acid.* Add 12.5 mL of magnesium and calcium stock solution and dilute to 5000 mL with *water R*. Store at 4 °C in transparent containers.

Albumin barbital buffer solution Dissolve 0.150 g of *bovine albumin R* in 20 mL of barbital buffer stock solution and dilute to 100 mL with *water R*. Prepare immediately before use.

Tannic acid solution Dissolve 10 mg of *tannic acid R* in 100 mL of phosphate-buffered saline pH 7.2. Prepare immediately before use.

Guinea-pig complement Prepare a pool of serum from the blood of not fewer than 10 guinea-pigs. Separate the serum from the clotted blood by centrifugation at about 4 °C. Store the serum in small amounts below −70 °C. Immediately before starting complement-initiated haemolysis, dilute to 125-200 CH_{50} per millilitre with albumin barbital buffer solution and store in an ice-bath during the test.

Rubella antigen Suitable rubella antigen for haemagglutination-inhibition titre (HIT).
Titre > 256 HA units.

Preparation of tanned human red blood cells

Separate human red blood cells by centrifuging an appropriate volume of stabilised human blood, wash the cells at least 3 times with phosphate-buffered saline pH 7.2 and suspend at 2 per cent *V/V* in phosphate-buffered saline pH 7.2. Add 0.2 mL of tannic acid solution to 14.8 mL of phosphate-buffered saline pH 7.2. Mix 1 volume of the freshly prepared dilution with 1 volume of the human red blood cell suspension and incubate at 37 °C for 10 min. Collect the cells by centrifugation (800 *g* for 10 min), discard

the supernatant and wash the cells once with phosphate-buffered saline pH 7.2. Resuspend the tanned cells at 1 per cent *V/V* in phosphate-buffered saline pH 7.2.

Antigen coating of tanned human red blood cells

Take a suitable volume (V_s) of tanned cells, add 0.2 mL of rubella antigen per 1.0 mL of tanned cells and incubate at 37 °C for 30 min. Collect the cells by centrifugation (800 *g* for 10 min) and discard the supernatant. Add a volume of albumin barbital buffer solution equivalent to the discarded supernatant, resuspend and collect the cells as described and repeat the washing procedure. Resuspend with albumin barbital buffer solution using a volume equivalent to 3/4 of V_s, thereby obtaining the initial volume (V_i). Mix 900 µL of albumin barbital buffer solution with 100 µL of V_i, which is thereby reduced to the residual volume (V_r), and determine the initial absorbance at 541 nm (A). Dilute V_r by a factor equal to A using albumin barbital buffer solution, thereby obtaining the final adjusted volume $V_f = V_r \times A$ of sensitised human red blood cells and adjusting A to 1.0 ± 0.1 for a tenfold dilution.

Antibody binding of antigen-coated tanned human red blood cells

Prepare the following solutions in succession and in duplicate, using for each solution a separate half-micro cuvette (for example, disposable type) or test-tube.

(1) *Test solutions.* If necessary, adjust the immunoglobulin to be examined to pH 7.

Where method A is performed, dilute volumes of the preparation to be examined with albumin barbital buffer to obtain 30 mg and 40 mg of immunoglobulin and adjust the volume to 900 µL with albumin barbital buffer.

Where method B is performed, dilute volumes of the preparation to be examined with albumin barbital buffer to obtain 15 mg and 30 mg of immunoglobulin and adjust the volume to 1200 µL with albumin barbital buffer.

(2) *Reference solutions.* Prepare as for the test solutions using *human immunoglobulin for Fc function BRP.*

(3) *Complement control.* Albumin barbital buffer solution.

Where method A is performed, add to each cuvette/test-tube 100 µL of sensitised human red blood cells and mix well. Allow to stand for 15 min, add 1000 µL of albumin barbital buffer solution, collect the cells by centrifugation (1000 *g* for 10 min) of the cuvette/test-tube and remove 1900 µL of the supernatant. Replace the 1900 µL with albumin barbital buffer solution and repeat the whole of the washing procedure, finally leaving a volume of 200 µL. Test samples may be stored in sealed cuvettes/test-tubes at 4 °C for not longer than 24 h.

Where method B is performed, add to each test-tube 300 µL of sensitised human red blood cells and mix well (the final immunoglobulin concentration is in the range of 10-20 mg/mL). Allow to stand for 15 min, add 1500 µL of albumin barbital buffer solution and stir gently until homogeneous. Collect the cells by centrifugation (1000 *g* for 10 min) of the test-tube, remove the supernatant and add approximately 3 mL of albumin barbital buffer solution. Repeat this operation up to 4 times in total, leaving a final volume of 300 µL. Test samples may be stored in sealed test-tubes at 4 °C for not longer than 24 h.

Complement-initiated haemolysis

To measure haemolysis where method A is performed, add 600 µL of albumin barbital buffer solution warmed to 37 °C to the test sample, resuspend the cells carefully by repeated pipetting (not fewer than 5 times) and place the cuvette in the thermostatted cuvette holder of a spectrophotometer. After 2 min, add 200 µL of diluted guinea-pig complement (125-200 CH_{50}/mL), mix thoroughly by pipetting twice and start immediately after the second pipetting the time-dependent recording of absorbance at 541 nm, using albumin barbital buffer solution as the compensation liquid. Stop the measurement if absorbance as a function of time has clearly passed the inflexion point.

To measure haemolysis where method B is performed, add 900 µL of albumin barbital buffer solution warmed to 37 °C to each test-tube and resuspend the cells carefully by repeated pipetting (not fewer than 5 times). The microtitre plate must be prewarmed to 37 °C before starting the test. Transfer 240 µL of each solution into 4 microtitre plate wells then incubate the microplate at 37 °C for 6 min, stirring gently every 10 s. To each microtitre plate well add 60 µL of diluted guinea-pig complement (150 CH_{50}/mL). Mix for 10 s and immediately start recording the absorbance at 541 nm at 37 °C, measuring every 20 s. Stop the measurement if the absorbance as a function of time has clearly passed the inflexion point.

Evaluation

For each cuvette/test-tube/well, determine the slope (S) of the haemolysis curve at the approximate inflexion point by segmenting the steepest section in suitable time intervals (for example, Δt = 1 min), and calculate S between adjacent intersection points, expressed as ΔA per minute. The largest value for S serves as S_{exp}. In addition, determine the absorbance at the start of measurement (A_s) by extrapolating the curve, which is almost linear and parallel to the time axis within the first few minutes. Correct S_{exp} using the expression:

$$S' = \frac{S_{exp}}{A_s}$$

Calculate the arithmetic mean of the values of S' for each preparation (test and reference solution). Calculate the index of Fc function (I_{Fc}) from the expression:

$$I_{Fc} = \frac{100 \times (\overline{S'} - \overline{S'_c})}{\overline{S'_s} - \overline{S'_c}}$$

$\overline{S'}$ = arithmetic mean of the corrected slope for the preparation to be examined;

$\overline{S'_s}$ = arithmetic mean of the corrected slope for the reference preparation;

$\overline{S'_c}$ = arithmetic mean of the corrected slope for the complement control.

Calculate the index of Fc function for the preparation to be examined: the value is not less than that stated in the leaflet accompanying the reference preparation.

C2. Test for Anticomplementary Activity of Immunoglobulin

(Ph. Eur. method 2.6.17)

For the measurement of anticomplementary activity (ACA) of immunoglobulin, a defined amount of test material (10 mg of immunoglobulin) is incubated with a defined amount of guinea-pig complement (20 CH_{50}) and the remaining complement is titrated; the anticomplementary activity is expressed as the percentage consumption of complement relative to the complement control considered as 100 per cent.

The haemolytic unit of complement activity (CH_{50}) is the amount of complement that, in the given reaction conditions, will produce the lysis of 2.5×10^8 out of a total of 5×10^8 optimally sensitised red blood cells.

Magnesium and calcium stock solution Dissolve 1.103 g of *calcium chloride R* and 5.083 g of *magnesium chloride R* in *water R* and dilute to 25 mL with the same solvent.

Barbital buffer stock solution Dissolve 207.5 g of *sodium chloride R* and 25.48 g of *barbital sodium R* in 4000 mL of *water R* and adjust to pH 7.3 using *1 M hydrochloric acid.* Add 12.5 mL of magnesium and calcium stock solution and dilute to 5000 mL with *water R.* Filter through a membrane filter (nominal pore size 0.22 µm). Store at 4 °C in glass containers.

Gelatin solution Dissolve 12.5 g of *gelatin R* in about 800 mL of *water R* and heat to boiling in a water-bath. Cool to 20 °C and dilute to 10 L with *water R.* Filter through a membrane filter (nominal pore size 0.22 µm). Store at 4 °C. Use clear solutions only.

Citrate solution Dissolve 8.0 g of *sodium citrate R,* 4.2 g of *sodium chloride R* and 20.5 g of *glucose R* in 750 mL of *water R.* Adjust to pH 6.1 using a 100 g/L solution of *citric acid monohydrate R* and dilute to 1000 mL with *water R.*

Gelatin barbital buffer solution Add 4 volumes of gelatin solution to 1 volume of barbital buffer stock solution and mix. Adjust to pH 7.3, if necessary, using *1 M sodium hydroxide* or *1 M hydrochloric acid.* Maintain at 4 °C. Prepare fresh solutions daily.

Stabilised sheep blood Collect 1 volume of sheep blood into 1 volume of citrate solution and mix. Store at 4 °C for not less than 7 days and not more than 28 days. (Stabilised sheep blood and sheep red blood cells are available from a number of commercial sources.)

Haemolysin Antiserum against sheep red blood cells prepared in rabbits. (Such antisera are available from a number of commercial sources.)

Guinea-pig complement Prepare a pool of serum from the blood of not fewer than 10 guinea-pigs. Separate the serum from the clotted blood by centrifugation at about 4 °C. Store the serum in small amounts below −70 °C.

METHOD

Preparation of standardised 5 per cent sheep red blood cell suspension

Separate sheep red blood cells by centrifuging an appropriate volume of stabilised sheep blood and wash the cells at least 3 times with gelatin barbital buffer solution and prepare a 5 per cent *V/V* suspension in the same solution. Measure the cell density of the suspension as follows: add 0.2 mL to 2.8 mL of *water R* and centrifuge the lysed solution for 5 min at 1000 *g*; the cell density is suitable if the absorbance (*2.2.25*) of the supernatant at 541 nm is 0.62 ± 0.01. Correct the cell density by adding gelatin barbital buffer solution according to the following equation:

$$V_f = \frac{V_i \times A}{0.62}$$

V_f = final adjusted volume;
V_i = the initial volume;
A = absorbance of the original suspension at 541 nm.

The adjusted suspension contains about 1×10^9 cells/mL.

Haemolysin titration

Prepare haemolysin dilutions as shown in Table 2.6.17.-1.

Table 2.6.17.-1

Required dilution of haemolysin	Prepared using		
	Gelatin barbital buffer solution		Haemolysin
	Volume (mL)	Dilution (1/...)	Volume (mL)
7.5	0.65	undiluted	0.1
10	0.90	undiluted	0.1
75	1.80	7.5	0.2
100	1.80	10	0.2
150	1.00	75	1.0
200	1.00	100	1.0
300	1.00	150	1.0
400	1.00	200	1.0
600	1.00	300	1.0
800	1.00	400	1.0
1200	1.00	600	1.0
1600	1.00	800	1.0
2400	1.00	1200	1.0
3200*	1.00	1600	1.0
4800*	1.00	2400	1.0

* discard 1.0 mL of the mixture.

Add 1.0 mL of 5 per cent sheep red blood cell suspension to each tube of the haemolysin dilution series, starting at the 1/75 dilution, and mix. Incubate at 37 °C for 30 min.

Transfer 0.2 mL of each of these incubated mixtures to new tubes and add 1.10 mL of gelatin barbital buffer solution and 0.2 mL of diluted guinea-pig complement (for example, 1/150). Perform this in duplicate.

As the unhaemolysed cell control, prepare 3 tubes with 1.4 mL of gelatin barbital buffer solution and 0.1 mL of 5 per cent sheep red blood cell suspension.

As the fully haemolysed control, prepare 3 tubes with 1.4 mL of *water R* and 0.1 mL of 5 per cent sheep red cell suspension.

Incubate all tubes at 37 °C for 60 min and centrifuge at 1000 *g* for 5 min. Measure the absorbance (*2.2.25*) of the supernatants at 541 nm and calculate the percentage degree of haemolysis in each tube using the following expression:

$$\frac{A_a - A_1}{A_b - A_1} \times 100$$

A_a = absorbance of tubes with haemolysin dilution;
A_b = mean absorbance of the 3 tubes with full haemolysis;
A_1 = mean absorbance of the 3 tubes with no haemolysis.

Plot the percentage degree of haemolysis as the ordinate against the corresponding reciprocal value of the haemolysin dilution as the abscissa on linear graph paper. Determine the optimal dilution of the haemolysin from the graph by inspection. Select a dilution such that further increase in the amount of haemolysin does not cause appreciable change in the degree of haemolysis. This dilution is defined as 1 minimal haemolytic unit (1 MHU) in 1.0 mL. The optimal haemolytic haemolysin dilution for preparation of sensitised sheep red blood cells contains 2 MHU/mL.

The haemolysin titration is not valid unless the maximum degree of haemolysis is 50 per cent to 70 per cent. If the maximum degree of haemolysis is not in this range, repeat the titration with more or less diluted complement solution.

Preparation of optimised sensitised sheep red blood cells (haemolytic system)
Prepare an appropriate volume of diluted haemolysin containing 2 MHU/mL and an equal volume of standardised 5 per cent sheep red blood cell suspension. Add the haemolysin dilution to the standardised cell suspension and mix. Incubate at 37 °C for 15 min, store at 2 °C to 8 °C and use within 6 h.

Titration of complement
Prepare an appropriate dilution of complement (for example 1/250) with gelatin barbital buffer solution and perform the titration in duplicate as shown in Table 2.6.17.-2.

Table 2.6.17.-2

Tube number	Volume of diluted complement (for example 1/250)(mL)	Volume of gelatin barbital buffer solution (mL)
1	0.1	1.2
2	0.2	1.1
3	0.3	1.0
4	0.4	0.9
5	0.5	0.8
6	0.6	0.7
7	0.7	0.6
8	0.8	0.5
9	0.9	0.4
10	1.0	0.3
11	1.1	0.2
12	1.2	0.1
3 tubes as cell control at 0 per cent haemolysis	–	1.3
3 tubes at 100 per cent haemolysis	–	1.3 mL of water

Add 0.2 mL of sensitised sheep red blood cells to each tube, mix well and incubate at 37 °C for 60 min. Cool the tubes in an ice-bath and centrifuge at 1000 *g* for 5 min. Measure the absorbance of the supernatant at 541 nm and calculate the degree of haemolysis (*Y*) using the following expression:

$$\frac{A_c - A_1}{A_b - A_1}$$

A_c = absorbance of tubes 1 to 12;
A_b = mean absorbance of tubes with 100 per cent haemolysis;
A_1 = mean absorbance of cell controls with 0 per cent haemolysis.

Plot $Y/(1-Y)$ as the abscissa against the amount of diluted complement in millilitres as the ordinate on log–log graph paper. Fit the best line to the points and determine the ordinate for the 50 per cent haemolytic complement dose where $Y/(1-Y) = 1.0$. Calculate the activity in haemolytic units (CH_{50}/mL) using the following expression:

$$\frac{C_d}{C_a \times 5}$$

C_d = reciprocal value of the complement dilution;
C_a = volume of diluted complement resulting in 50 per cent haemolysis, in millilitres;
5 = scaling factor to take account of the number of red blood cells.

The test is not valid unless the plot is a straight line between 15 per cent and 85 per cent haemolysis and the slope is 0.15 to 0.40, and preferably 0.18 to 0.30.

Test for anticomplementary activity
Prepare a complement dilution having 100 CH_{50}/mL by diluting titrated guinea-pig complement with gelatin barbital buffer solution. Depending on the immunoglobulin to be examined and based on validation data, a pH adjustment to 7 may be necessary. Prepare incubation mixtures as follows for an immunoglobulin containing 50 mg/mL:

Table 2.6.17.-3

	Immunoglobulin to be examined	Complement control (in duplicate)
Immunoglobulin (50 mg/mL)	0.2 mL	–
Gelatin barbital buffer	0.6 mL	0.8 mL
Complement	0.2 mL	0.2 mL

Carry out the test on the immunoglobulin to be examined and prepare ACA negative and positive controls using *human immunoglobulin for anticomplementary activity BRP*, as indicated in the leaflet accompanying the reference preparation. Higher or lower volumes of sample and of gelatin barbital buffer solution are added if the immunoglobulin concentration varies from 50 mg/mL; for example, 0.47 mL of gelatin barbital buffer solution is added to 0.33 mL of immunoglobulin containing 30 mg/mL to give 0.8 mL. Close the tubes and incubate at 37 °C for 60 min. Add 0.2 mL of each incubation mixture to 9.8 mL of gelatin barbital buffer solution to dilute the complement. Perform complement titrations on each tube as described above to determine the remaining complement activity (Table 2.6.17.-2). Calculate the anticomplementary activity of the preparation to be examined relative to the complement control considered as 100 per cent, using the following expression:

$$\frac{a - b}{a} \times 100$$

a = mean complement activity (CH_{50}/mL) of complement control;
b = complement activity (CH_{50}/mL) of tested sample.

The test is not valid unless:
— the anticomplementary activities found for ACA negative control and ACA positive control are within the limits stated in the leaflet accompanying the reference preparation;
— the mean complement activity of complement control (a) is in the range 80 CH_{50}/mL to 120 CH_{50}/mL.

C3. Assay of Human Anti-D Immunoglobulin
(Ph. Eur. method 2.7.13)

METHOD A
The potency of human anti-D immunoglobulin is determined by comparing the quantity necessary to produce agglutination of D-positive red blood cells with the quantity of a reference preparation, calibrated in International Units, required to produce the same effect.

The International Unit is the activity contained in a stated amount of the International Reference Preparation.
The equivalence in International Units of the International Reference Preparation is stated by the World Health Organization.

Human anti-D immunoglobulin BRP is calibrated in International Units by comparison with the International Standard and intended for use in the assay of human anti-D immunoglobulin.

Use pooled D-positive red blood cells, collected not more than 7 days earlier and suitably stored, obtained from not

fewer than 4 group O R_1R_1 donors. To a suitable volume of the cells, previously washed 3 times with a 9 g/L solution of *sodium chloride R*, add an equal volume of *bromelains solution R*, allow to stand at 37 °C for 10 min, centrifuge, remove the supernatant and wash 3 times with a 9 g/L solution of *sodium chloride R*. Suspend 20 volumes of the red blood cells in a mixture of 15 volumes of inert serum, 20 volumes of a 300 g/L solution of *bovine albumin R* and 45 volumes of a 9 g/L solution of *sodium chloride R*. Stand the resulting suspension in iced water, stirring continuously.

Using a calibrated automated dilutor, prepare suitable dilutions of the preparation to be examined and of the reference preparation using as diluent a solution containing 5 g/L of *bovine albumin R* and 9 g/L of *sodium chloride R*.

Use a suitable apparatus for automatic continuous analysis. The following protocol is usually suitable: maintain the temperature in the manifold, except for the incubation coils, at 15.0 °C. Pump into the manifold of the apparatus the red blood cell suspension at a rate of 0.1 mL/min and a 3 g/L solution of *methylcellulose 450 R* at a rate of 0.05 mL/min. Introduce the dilutions of the preparation to be examined and the reference preparation at a rate of 0.1 mL/min for 2 min, followed by the diluent solution at a rate of 0.1 mL/min for 4 min before the next dilution is introduced.

Introduce air at a rate of 0.6 mL/min. Incubate at 37 °C for 18 min and then disperse the rouleaux by introducing at a rate of 1.6 mL/min a 9 g/L solution of *sodium chloride R* containing a suitable wetting agent (for example, *polysorbate 20 R* at a final concentration of 0.2 g/L) to prevent disruption of the bubble pattern. Allow the agglutinates to settle and decant twice, first at 0.4 mL/min and then at 0.6 mL/min. Lyse the unagglutinated red blood cells with a solution containing 5 g/L of *octoxinol 10 R*, 0.2 g/L of *potassium ferricyanide R*, 1 g/L of *sodium hydrogen carbonate R* and 0.05 g/L of *potassium cyanide R* at a rate of 2.5 mL/min. A 10-minute delay coil is introduced to allow for conversion of the haemoglobin. Continuously record the absorbance (*2.2.25*) of the haemolysate at a wavelength between 540 nm and 550 nm. Determine the range of antibody concentrations over which there is a linear relationship between the concentration and the resultant change in absorbance (ΔA). From the results, prepare a standard curve and use the linear portion of the curve to determine the activity of the preparation to be examined.

Calculate the potency of the preparation to be examined using the usual statistical methods (*5.3*).

METHOD B

The potency of human anti-D immunoglobulin is determined by competitive enzyme-linked immunoassay on erythrocyte-coated microtitre plates. The method is based on the competitive binding between a polyclonal anti-D immunoglobulin preparation and a biotinylated monoclonal anti-D antibody directed against a D-antigen-specific epitope. The activity of the preparation to be examined is compared with a reference preparation calibrated in International Units.

The International Unit is the activity of a stated amount of International Reference Preparation. The equivalence in International Units of the International Reference Preparation is stated by the World Health Organization.

Human anti-D immunoglobulin BRP is calibrated in International Units by comparison with the International Standard and intended for use in the assay of human anti-D immunoglobulin.

MATERIALS

Reagents not specified are of analytical grade.

PBS (Phosphate-buffered saline) Dissolve 8.0 g of *sodium chloride R*, 0.76 g of *anhydrous disodium hydrogen phosphate R*, 0.2 g of *potassium chloride R*, 0.2 g of *potassium dihydrogen phosphate R* and 0.2 g of *sodium azide R* in *water R* and dilute to 1000 mL with the same solvent.

TBS (Tris-buffered saline) Dissolve 8.0 g of *sodium chloride R* and 0.6 g of *tris(hydroxymethyl)aminomethane R* in *water R*. Adjust to pH 7.2 with *1 M hydrochloric acid* and dilute to 1000 mL with *water R*.

Papain solution Prepare a solution by stirring 1 g of *papain R* at 37 °C for 30 min in 10 mL of *0.067 M phosphate buffer solution pH 5.4 R*, centrifuge at 10 000 *g* for 5 min and filter through a membrane filter (nominal pore size 0.22 µm). To activate, combine 1 mL of the filtrate with 1 mL of a 48.44 g/L solution of *L-cysteine R* and 1 mL of a 3.72 g/L solution of *sodium edetate R* and dilute to 10 mL with *0.067 M phosphate buffer solution pH 5.4 R*. Freeze in aliquots at −20 °C or below.

Red blood cells Use pooled D-positive red blood cells obtained from not fewer than 3 group O R_2R_2 donors. Wash the cells 4 times with PBS. Centrifuge the cells at 1800 *g* for 5 min, mix a suitable volume of prewarmed packed cells with a suitable volume of prewarmed papain solution (2 volumes to 1 volume has been found suitable) and incubate at 37 °C for 10 min. Wash the cells 4 times with PBS. Store at 4 °C in an appropriate stabiliser for up to 1 week.

Biotinylated Brad-5 Use according to instructions.

Alkaline phosphatase-conjugated avidin/streptavidin reagent Preferably modified to combine high specific activity with low non-specific binding. Use according to instructions.

Substrate solution Use *para*-nitrophenyl phosphate according to instructions.

Cell fixation buffer Dissolve 18.02 g of *glucose R*, 4.09 g of *sodium chloride R*, 1.24 g of *boric acid R*, 10.29 g of *sodium citrate R* and 0.74 g of *sodium edetate R* in *water R*. Adjust to pH 7.2-7.3 using *1 M sodium hydroxide* or *1 M hydrochloric acid*, and dilute to 1000 mL with *water R*. Use directly from storage at 4 °C.

Glutaraldehyde solution Immediately before use, add 750 µL of a 250 g/L solution of *glutaraldehyde R* to 50 mL of cold PBS.

Microtitre plates Plates to be coated with red blood cells are flat-bottomed polystyrene plates with surface properties optimised for enzyme immunoassay and high protein-binding capacity. Plates used to prepare immunoglobulin dilutions are U- or V-bottomed polystyrene or poly(vinyl chloride) plates.

METHOD

Prepare a 0.1 per cent *V/V* suspension of papain-treated red blood cells in cold cell-fixation buffer. Pipette 50 µL into each well of the flat-bottomed microtitre plate.

Centrifuge the plate at 350 *g* for 3 min, preferably at 4 °C. Without removing the supernatant, gently add 100 µL of glutaraldehyde solution to each well and leave for 10 min.

Drain the wells by quickly inverting the plate and wash 3 times with 250-300 µL of PBS. This may be done manually or using a suitable automated plate washer. Either carry out the assay as described below, or store the plate at 4 °C after draining off the PBS and adding 100 µL of cell-fixation buffer per well and sealing with plastic film. Plates can be stored at 4 °C for up to 1 month.

Test solutions For freeze-dried preparations, reconstitute as stated on the label. Prepare 4 independent replicates of 5 serial 2-fold dilutions starting with 30 IU/mL in PBS containing 10 g/L of *bovine albumin R*. If necessary, adjust the starting dilution to obtain responses falling in the linear portion of the dose-response curve.

Reference solutions Reconstitute the reference preparation according to instructions. Prepare 4 independent replicates of 5 serial 2-fold dilutions starting with 30 IU/mL in PBS containing 10 g/L of *bovine albumin R*.

Using U- or V-bottomed microtitre plates, add 35 µL of each of the dilutions of the test solution or reference solution to each of a series of wells. To each well add 35 µL of biotinylated Brad-5 at 250 ng/mL.

Empty the wells of the red cell-coated plate by inverting and draining on a paper towel. Add 250 µL of PBS containing 20 g/L of *bovine albumin R* and leave at room temperature for 30 min.

Empty the wells of the red cell-coated plate by inverting and draining on a paper towel and transfer 50 µL from each of the dilutions of the test solution or reference solution containing biotinylated Brad-5 into the wells. Use 50 µL of PBS containing 10 g/L of *bovine albumin R* as negative control. Seal the plate with plastic film and incubate at room temperature for 1 h.

Remove the liquid from the wells of the red cell-coated plate and wash 3 times with 250-300 µL of TBS.

Dilute the alkaline phosphatase-conjugated avidin/streptavidin reagent in TBS containing 10 g/L of *bovine albumin R* and add 50 µL to each well. Incubate for 30 min at room temperature.

Remove the liquid from the wells of the red cell-coated plate and wash 3 times with 250-300 µL of TBS.

Add 100 µL of substrate solution to each of the wells and incubate at room temperature for 10 min in the dark. To stop the reaction, add 50 µL of *3 M sodium hydroxide* to each of the wells.

Measure the absorbances at 405 nm and substract the negative control reading. Use the absorbance values in the linear range of the titration curve to estimate the potency of the preparation to be examined by the usual statistical methods (*5.3*).

METHOD C

The potency of human anti-D immunoglobulin is determined by flow cytometry in a microtitre plate format. The method is based on the specific binding between anti-D immunoglobulin and D-positive red blood cells. The activity of the preparation to be examined is compared with a reference preparation calibrated in International Units.

The International Unit is the activity of a stated amount of International Reference Preparation. The equivalence in International Units of the International Reference preparation is stated by the World Health Organization.

Human anti-D immunoglobulin BRP is calibrated in International Units by comparison with the International Standard and intended for use in the assay of human anti-D immunoglobulin.

MATERIALS

Reagents not specified are of analytical grade.

PBS Dissolve 8.0 g of *sodium chloride R*, 0.76 g of *disodium hydrogen phosphate dodecahydrate R*, 0.2 g of *potassium chloride R* and 0.2 g of *potassium dihydrogen phosphate R* in *water R* and dilute to 1000 mL with the same solvent.

PBS-BSA solution PBS containing 10.0 g/L of *bovine albumin R*.

Red blood cells Use D-positive red blood cells obtained from a group O R_1R_1 donor within 2 weeks of collection. Store if necessary in an appropriate stabiliser at 4 °C. Wash the cells at least twice with PBS-BSA solution and prepare a suspension containing 1×10^4 cells per microlitre but not more than 5×10^4 cells per microlitre in PBS-BSA solution.

Use D-negative red blood cells obtained from a group O rr donor and prepared similarly.

Secondary antibody Use a suitable fluorescent dye-conjugated anti-IgG antibody fragment specific for human IgG or parts of it. Store and use according to the manufacturer's instructions.

Microtitres plates Use flat-bottomed plates without surface treatment for enzyme immunoassays.

METHOD

Test solutions For freeze-dried preparations, reconstitute as stated on the label. Prepare at least 3 independent replicates of at least 3 serial 1.5- or 2-fold dilutions starting with a concentration in the range of 1.2-0.15 IU/mL using PBS/BSA solution as diluent. If necessary, adjust the starting dilution to obtain responses falling in the linear portion of the dose-response curve.

Reference solutions Reconstitute the reference preparation according to instructions. Prepare at least 3 independent replicates of at least 3 serial 1.5- or 2-fold dilutions starting with a concentration in the range of 1.2-0.15 IU/mL using PBS-BSA solution as diluent. If necessary, adjust the starting dilution to obtain responses falling in the linear portion of the dose-response curve.

Distribute 50 µL of the D-positive red blood cells into each well of a microtitre plate. Add 50 µL of each of the dilutions of the test solution or reference solution to each of a series of wells. Use 50 µL of PBS-BSA solution as negative control. Distribute 50 µL of the D-negative red blood cells into 4 wells of the same microtitre plate and add 50 µL of the lowest dilution of the test preparation. To monitor spurious reactions, distribute 50 µL of the D-positive red blood cells into 4 wells of the same microtitre plate and add 50 µL of PBS-BSA solution. Seal with plastic film and incubate at 37 °C for 40 min.

Centrifuge the plates at 50 *g* for 3 min, discard the supernatant and wash the cells with 200-250 µL of PBS-BSA solution. Repeat this at least once.

Centrifuge the plates at 50 *g* for 3 min, discard the supernatant and add 50 µL of the secondary antibody diluted with PBS-BSA solution to a suitable protein concentration. Seal with plastic film and incubate, protected from light, at room temperature for 20 min.

Centrifuge the plates at 50 *g* for 3 min, discard the supernatant and wash the cells with 200-250 µL of PBS-BSA solution. Repeat this at least once.

Centrifuge the plates at 50 *g* for 3 min, resuspend the cells into 200-250 µL of PBS. Transfer the cell suspension into a tube suitable for the flow-cytometry equipment available and further dilute by adding PBS to allow a suitable flow rate.

Proceed immediately with measurement of the median fluorescence intensity in a flow cytometer. Record at least 10 000 events without gating but excluding debris.

Use the median fluorescence intensity in the linear range of the dose-response curve to estimate the potency of the preparation to be examined by the usual statistical methods (*5.3*).

C4. Test for Anti-D Antibodies in Human Immunoglobulin

(Ph. Eur. method 2.6.26)

MATERIALS

Phosphate-buffered saline (PBS) Dissolve 8.0 g of *sodium chloride R*, 0.76 g of *anhydrous disodium hydrogen phosphate R*, 0.2 g of *potassium chloride R* and 0.2 g of *potassium dihydrogen phosphate R* in *water R* and dilute to 1000 mL with the same solvent. If the solution has to be kept for several days, 0.2 g of *sodium azide R* may be added in order to avoid microbial contamination.

PBS-BSA solution PBS containing 2 g/L of *bovine albumin R* (Cohn Fraction V, for ELISA). Store the solution at 2-8 °C but allow it to reach 19-25 °C before use.

Papain solution Use serological-grade papain from a commercial source, the activity of which has been validated.

Red blood cells Use pooled D-positive red blood cells from not fewer than 3 donors, preferably of group OR_2R_2. D-positive red blood cells may also be obtained from OR_1R_1 or OR_1R_2 donors. Mixing phenotypes has not been tested and is therefore not recommended.

Use pooled D-negative red blood cells, preferably from 3 donors of group Orr. When only 1 donor of group Orr is available, D-negative red blood cells from only 1 donor may be used.

Wash the cells 4 times with PBS or until the supernatant is clear. Each wash consists of suspending the cells in a minimum of 2 volumes of PBS, centrifuging the cells at 1800 *g* for 5 min to pack, and discarding the supernatant. Treat the packed cells with papain solution according to the manufacturer's instructions and wash the cells 4 times with PBS.

Red blood cells may be stored for not more than 1 week in a preservative solution at 2-8 °C. A preparation of the following composition is appropriate:

Trisodium citrate	8 g/L
D-glucose	20 g/L
Citric acid	0.5 g/L
Sodium chloride	4.2 g/L
Inosine	0.938 g/L
Adenosine triphosphate (ATP)	0.4 g/L
Chloramphenicol	0.34 g/L
Neomycin sulfate	0.1 g/L

If using stored cells, wash the cells at least twice in PBS or until the supernatant is clear before proceeding.

Microtitre plates Use V-bottomed rigid microtitre plates.

Reference standards *Immunoglobulin (anti-D antibodies test) BRP* and *Immunoglobulin (anti-D antibodies test negative control) BRP* are suitable for use as the positive control and negative control, respectively.

METHOD

The test described in this chapter is performed at room temperature on the positive control solutions, the negative control solutions and the test solutions at the same time and under identical conditions.

Reference solutions Reconstitute the positive control and the negative control according to the instructions. The immunoglobulin G (IgG) concentration is 50 g/L in each of the reconstituted preparations. Make a 2-fold dilution of each reconstituted preparation with PBS-BSA solution to obtain solutions containing IgG at 25 g/L. Prepare 7 further serial 2-fold dilutions of each preparation using PBS-BSA solution to extend the dilution range to 1/256 (0.195 g/L IgG). Add 20 µL of each dilution of each preparation in duplicate to the microtitre plate.

Test solutions Dilute the preparation to be examined with PBS-BSA solution to obtain a starting IgG concentration of 25 g/L. For 50 g/L preparations, this is a 2-fold dilution; adjust the dilution factor accordingly for preparations with an IgG concentration other than 50 g/L to obtain a starting concentration of 25 g/L for testing. This 25 g/L solution is assigned a nominal 2-fold dilution factor for comparison with the reference solutions, even if this does not reflect the true dilution factor used to achieve 25 g/L. Prepare 7 further serial 2-fold dilutions of the preparation using PBS-BSA solution to extend the nominal dilution range to 1/256 (0.195 g/L IgG) for comparison with the reference preparations over the same IgG concentration range. Add 20 µL of each dilution in duplicate to the microtitre plate.

Prepare 3 per cent *V/V* suspensions of papain-treated D-positive (preferably OR_2R_2, but OR_1R_1 or OR_1R_2 may also be used) and D-negative (Orr) red blood cells in PBS-BSA solution. Add 20 µL of D-positive red blood cells to 1 dilution series of each of the preparation to be examined, the positive control and the negative control, and 20 µL of D-negative red blood cells to the other dilution series. Mix by shaking the plate on a shaker for 10 s (or until the cells are resuspended).

Centrifuge the plate at 80 *g* at room temperature for 1 min to pack the cells. Place the plate at an angle of approximately 70°. Read after 4-5 min or when the negative controls (D-negative red blood cells and negative control solution) have streamed. A cell button at the bottom of the well indicates a positive result. A stream of cells represents a negative result.

Record the endpoint titre as the reciprocal of the highest dilution that gives rise to a positive result.

The positive control has a nominal titre of 8 and the negative controls (D-negative red blood cells and negative control solution) must not show agglutination at the starting dilution of 1 in 2. Users must validate their own test conditions, and investigate their assay conditions and reagents in the event of results being significantly different from those expected. Failure to obtain negative reactions with the negative controls may indicate that, for example, insufficient time has elapsed for the cells to stream, or that reagents have been used directly from cold storage.

The titre of the preparation to be examined must not be greater than the titre of the positive control when both preparations are titrated from 25 g/L.

C5. Anti-A and Anti-B Haemagglutinins

(Ph. Eur. method 2.6.20)

METHOD A: INDIRECT METHOD

Prepare in duplicate serial dilutions of the preparation to be examined in a 9 g/L solution of *sodium chloride R*. To each dilution of 1 series add an equal volume of a 5 per cent *V/V* suspension of group A_1 red blood cells previously washed 3 times with the sodium chloride solution. To each dilution of the other series add an equal volume of a 5 per cent *V/V* suspension of group B red blood cells previously washed 3 times with the sodium chloride solution. Incubate the suspensions at 37 °C for 30 min then wash the cells 3 times with the sodium chloride solution. Leave the cells in contact with a polyvalent anti-human globulin reagent for 30 min. Without centrifuging, examine each suspension for agglutination under a microscope.

METHOD B: DIRECT METHOD

MATERIALS

Phosphate-buffered saline (PBS) Dissolve 8.0 g of *sodium chloride R*, 0.76 g of *anhydrous disodium hydrogen phosphate R*, 0.2 g of *potassium chloride R* and 0.2 g of *potassium dihydrogen phosphate R* in *water R* and dilute to 1000 mL with the same solvent. If the solution has to be kept for several days, 0.2 g of *sodium azide R* may be added in order to avoid microbial contamination.

PBS-BSA solution PBS containing 2 g/L of *bovine albumin R* (Cohn Fraction V, for ELISA). Store the solution at 2-8 °C but allow it to reach 19-25 °C before use.

Papain solution Use serological-grade papain from a commercial source, the activity of which has been validated.

Red blood cells Use pooled D-negative A_1 (A_1rr), D-negative B (Brr) and D-negative O (Orr) red blood cells from preferably 3 donors. When *Immunoglobulin for anti-A and anti-B antibodies limit test BRP* is used, 3 donors are to be used. A_2 red blood cells are not recommended as they give weaker reactions.

Wash the cells 4 times with PBS or until the supernatant is clear. Each wash consists of suspending the cells in a minimum of 2 volumes of PBS, centrifuging the cells at 1800 *g* for 5 min to pack, and discarding the supernatant. Treat the packed cells with papain solution according to the manufacturer's instructions and wash the cells 4 times with PBS.

Red blood cells may be stored for not more than 1 week in a preservative solution at 2-8 °C. A preparation of the following composition is appropriate:

Trisodium citrate	8 g/L
D-glucose	20 g/L
Citric acid	0.5 g/L
Sodium chloride	4.2 g/L
Inosine	0.938 g/L
Adenosine triphosphate (ATP)	0.4 g/L
Chloramphenicol	0.34 g/L
Neomycin sulfate	0.1 g/L

If using stored cells, wash the cells at least twice in PBS or until the supernatant is clear before proceeding.

Microtitre plates Use V-bottomed rigid microtitre plates.

Reference standards *Immunoglobulin (anti-A, anti-B antibodies test positive control) BRP* and *Immunoglobulin (anti-A, anti-B antibodies test negative control) BRP* are suitable for use as the positive control and negative control, respectively, and should be used as guides for operators establishing and performing the direct method for anti-A and anti-B haemagglutinins.

Immunoglobulin for anti-A and anti-B antibodies limit test BRP defines the recommended maximum limits permissible for batches of human immunoglobulin and must be used only for comparison with batches of human immunoglobulin that have higher titres than the positive control.

METHOD

The test described in this chapter is performed at room temperature on the positive control solutions, the negative control solutions and the test solutions at the same time and under identical conditions. Whenever necessary, a further test is performed with *Immunoglobulin for anti-A and anti-B antibodies limit test BRP*.

Reference solutions Reconstitute the positive control and the negative control according to the instructions.
The immunoglobulin G (IgG) concentration is 50 g/L in each of the reconstituted preparations. Make a 2-fold dilution of each reconstituted preparation with PBS-BSA solution to obtain solutions containing IgG at 25 g/L. Prepare 7 further serial 2-fold dilutions of each preparation using PBS-BSA solution to extend the dilution range to 1/256 (0.195 g/L IgG). Add 20 µL of each dilution of each preparation in triplicate to the microtitre plate.

Test solutions Dilute the preparation to be examined with PBS-BSA solution to obtain a starting IgG concentration of 25 g/L. For 50 g/L preparations, this is a 2-fold dilution; adjust the dilution factor accordingly for preparations with an IgG concentration other than 50 g/L to obtain a starting concentration of 25 g/L for testing. This 25 g/L solution is assigned a nominal 2-fold dilution factor for comparison with the reference solutions, even if this does not reflect the true dilution factor used to achieve 25 g/L. Prepare 7 further serial 2-fold dilutions of the preparation using PBS-BSA solution to extend the nominal dilution range to 1/256 (0.195 g/L IgG) for comparison with the reference preparations over the same IgG concentration range. Add 20 µL of each dilution in triplicate to the microtitre plate.

For preparations with an IgG concentration lower than 25 g/L, dilute to a starting concentration corresponding to the nearest lower concentration of the reference solutions. This solution is assigned the same nominal dilution factor as the corresponding reference solution having the same IgG concentration. Proceed with the preparation of the appropriate 2-fold dilution series as described above.

Prepare 3 per cent *V/V* suspensions of papain-treated D-negative A_1, B and O red blood cells in PBS/BSA solution. Add 20 µL of D-negative A_1, B and O red blood cells respectively to the 1st, the 2nd and the 3rd dilution series of each of the preparation to be examined, the positive control and the negative control. Mix by shaking the plate on a shaker for 10 s (or until the cells are resuspended).

Centrifuge the plate at 80 *g* at room temperature for 1 min to pack the cells. Place the plate at an angle of approximately 70°. Read after 4-5 min or when the negative controls (D-negative O red blood cells and negative control solution) have streamed. A cell button at the bottom of the well indicates a positive result. A stream of cells represents a negative result.

Record the endpoint titre as the reciprocal of the highest dilution that gives rise to a positive result.

The positive control has nominal anti-A and anti-B titres of 32 (range 32-64 for anti-A; range 16-32 for anti-B) and the negative controls (D-negative O red blood cells and negative control solution) must not show agglutination at the starting dilution of 1 in 2. Users must validate their own test conditions, and investigate their assay conditions and reagents in the event of results being significantly different from those expected. Failure to obtain negative reactions with the negative controls may indicate that, for example, insufficient time has elapsed for the cells to stream, or that reagents have been used directly from cold storage.

If the nominal anti-A or anti-B titre of the preparation to be examined is greater than the titre of the positive control, the test preparation is to be compared with *Immunoglobulin for anti-A and anti-B antibodies limit test BRP*.

The maximum allowable titre is 64.

Other blood-related components

D1. Assay of Human α-1-proteinase Inhibitor

(Ph. Eur. method 2.7.32)

Human α-1-proteinase inhibitor (also known as α-1-antitrypsin or α-1-antiproteinase) content is determined by comparing the ability of the preparation to be examined to inactivate the serine protease elastase (porcine pancreatic elastase or human neutrophil elastase) with the same ability of a reference standard of human α-1-proteinase inhibitor calibrated in milligrams of active (functional) α-1-proteinase inhibitor. Varying quantities of the preparation to be examined are mixed with a given quantity of elastase and the remaining elastase activity is determined using a suitable chromogenic substrate. The method described below is given as an example.

REAGENTS

Tris-albumin buffer solution Dissolve 24.23 g of *trometamol R* in *water R*, adjust to pH 8.0 ± 0.3 using *hydrochloric acid R1* and dilute to 1000 mL with *water R*. To 100 mL of this solution add 0.5 mL of a 20 per cent solution of *human albumin R* or *bovine albumin R*.

Buffer solution containing human or bovine albumin must be prepared fresh on the day of its use; otherwise, it can be conserved by sterile filtration (0.2 µm) and stored at 2-8 °C for up to 2 weeks.

METHOD

Prepare 2 series of 4 or 5 dilutions in an appropriate human α-1-proteinase inhibitor concentration range, for both the preparation to be examined and the reference standard, using the tris-albumin buffer solution.

Transfer 50 µL of the reference solution dilutions into the wells of a microtitre plate and to each well, add 150 µL of a porcine pancreatic elastase solution diluted to an appropriate concentration with the tris-albumin buffer solution. Incubate for a defined period of time, 3-10 min, at room temperature. Since the activity of the solutions of the different porcine pancreatic elastases may vary, the concentration of elastase can be adjusted by evaluation of blank values containing elastase but no human α-1-proteinase inhibitor, to exhibit a suitable change of absorbance at 405 nm under the actual assay conditions.

Add to each well 100 µL of a solution of chromogenic substrate *N*-succinyl-tri-L-alanyl 4-p-nitroanilide (Suc-Ala-Ala-Ala-pNA), reconstituted in *dimethyl sulfoxide R* to give a solution containing 4.5 mg/mL, then further diluted with the tris-albumin buffer solution to a concentration of 0.45 mg/mL. Immediately start measurement of the change in absorbance (*2.2.25*) at 405 nm using a microtitre plate reader, continuing the measurement for at least 5 min. Calculate the rate of change of absorbance (ΔA/min). Alternatively, an end-point assay may be used by stopping the reaction with acetic acid and measuring the absorbance at 405 nm. If the assay is performed in test tubes using spectrophotometers for monitoring the change in absorbance at 405 nm, the volumes of reagent solutions are changed proportionally.

The rate of change of absorbance (ΔA/min) is inversely proportional to human α-1-proteinase inhibitor activity.

Check the validity of the assay and calculate the potency of the test preparation by the usual statistical methods (*5.3*).

D2. Assay of Human C1-Esterase Inhibitor

(Ph. Eur. method 2.7.34)

The human C1-esterase inhibitor content of the preparation to be examined is determined by comparing its ability to inhibit C1-esterase with that of a reference preparation calibrated in International Units. The International Unit is the activity of a stated amount of the International Standard for human plasma-derived C1-esterase inhibitor concentrate. The equivalence in International Units of the International Standard is stated by the World Health Organization. Varying quantities of the preparation to be examined are mixed with an excess of C1-esterase and the remaining C1-esterase activity is determined using a suitable chromogenic substrate.

Individual reagents may be obtained separately or in commercial kits. Procedures and reagents may vary between different kits and the manufacturer's instructions are followed. The essential features of the procedure are described in the following example of a microtitre-plate kinetic method.

Reconstitute the preparation as stated on the label. Prepare an appropriate series of at least 3 dilutions, from 1 IU/mL of C1-esterase inhibitor, for both the preparation to be examined and the reference preparation, using a suitable pH 7.4 buffer solution containing 9 g/L of *sodium chloride R* and either 10 g/L of *human albumin R* or 10 g/L of *bovine albumin R*. Warm all solutions to 37 °C. Place a suitable quantity of 1 of the dilutions of the reference preparation or of the preparation to be examined in microtitre plate wells and incubate at 37 °C. To each well add a suitable quantity of C1-esterase solution and incubate at 37 °C for 5 min. Add an appropriate quantity of a suitable specific chromogenic substrate such as methoxycarbonyl-L-lysyl(ε-benzyloxycarbonyl)-glycyl-L-arginine-4-nitroanilide. Read the rate of increase of absorbance (Δ*A*/min) at 405 nm. A positive control, using the pH 7.4 buffer solution instead of the C1-esterase inhibitor, is included. For preparations that may exhibit proteolytic activity, a test is carried out on a suitable blank composed of the preparation to be examined, the pH 7.4 buffer solution and the chromogenic substrate.

Calculate the C1-esterase inhibitor content using the usual statistical methods, for example slope ratio (*5.3*).

K. Immunological Products

1. Assay of Diphtheria Vaccine (Adsorbed)

(Ph. Eur. method 2.7.6. An alternative in vivo method (Method B) in which the potency is determined by comparing the dose necessary to protect guinea-pigs against the lethal effect of a subcutaneous injection of diphtheria toxin with the dose of a reference preparation calibrated in International Units necessary to give the same protection is also described in the European Pharmacopoeia.)

The potency of diphtheria vaccine is determined by administration of the vaccine to guinea-pigs followed either by challenge with diphtheria toxin (method A or B) or by determination of the titre of antibodies against diphtheria toxin or toxoid in the serum of guinea-pigs (method C). In both cases, the potency of the vaccine is calculated by comparison with a reference preparation, calibrated in International Units.

The International Unit is the activity contained in a stated amount of the International Standard, which consists of a

quantity of diphtheria toxoid adsorbed on aluminium hydroxide. The equivalence in International Units of the International Standard is stated by the World Health Organization (WHO).

Diphtheria vaccine (adsorbed) BRP is suitable for use as a reference preparation.

The method chosen for the assay of diphtheria vaccine (adsorbed) depends on the intended purpose. Method A or B is used:

1. during development of a vaccine, to assay batches produced to validate the production;

2. wherever revalidation is needed following a significant change in the manufacturing process.

Method A or B may also be used for the routine assay of batches of vaccine, but in the interests of animal welfare, method C is used wherever possible.

Method C may be used, except as specified under 1 and 2 above, after verification of the suitability of the method for the product. For this purpose, a suitable number of batches (usually 3) are assayed by method C and method A or B. Where different vaccines (monovalent or combinations) are prepared from diphtheria toxoid of the same origin, and with comparable levels (expressed in Lf/mL) of the same diphtheria toxoid, suitability demonstrated for the combination with the highest number of components can be assumed to be valid for combinations with fewer components and for monovalent vaccines. Any combinations containing a whole-cell pertussis component or containing haemophilus type b conjugate vaccine with diphtheria toxoid or CRM 197 diphtheria protein as carrier in the same vial must always be assessed separately.

For combinations containing diphtheria and tetanus components, the serological assay (method C) can be performed with the same group of animals used for the serological assay of the tetanus vaccine (adsorbed) (*2.7.8*) when the common immunisation conditions for the diphtheria and the tetanus components (for example, doses, duration) have been demonstrated to be valid for the combined vaccine.

The design of the assays described below uses multiple dilutions for the test and reference preparations. Once the analyst has sufficient experience with this method for a given vaccine, it is possible to apply a simplified model such as a single dilution for both test and reference preparations. Such a model enables the analyst to determine whether the potency of the test preparation is significantly higher than the minimum required, but does not give information on linearity, parallelism and the dose-response curve.
The simplified model allows for a considerable reduction in the number of animals required and must be considered by each analyst in accordance with the provisions of the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes.

Where a single-dilution assay is used, production and test consistency over time are monitored via suitable indicators and by carrying out a full multiple-dilution assay periodically, for example every 2 years. For serological assays, suitable indicators to monitor test consistency are:

- the mean and standard deviation of relative antitoxin titres or scores of the serum samples obtained after administration of a fixed dose of the vaccine reference preparation;
- the antitoxin titres or scores of run controls (positive and negative serum samples);
- the ratio of antitoxin titres or scores for the positive serum control to the serum samples corresponding to the reference vaccine.

METHOD A: INTRADERMAL CHALLENGE TEST IN GUINEA-PIGS

SELECTION AND DISTRIBUTION OF THE TEST ANIMALS

Use in the test healthy, white guinea-pigs from the same stock and of a size suitable for the prescribed number of challenge sites, the difference in body mass between the heaviest and the lightest animal being not greater than 100 g. Use guinea-pigs of the same sex or with males and females equally distributed between the groups. Distribute the guinea-pigs in not fewer than 6 equal groups; use groups containing a number of animals sufficient to obtain results that fulfil the requirements for a valid assay prescribed below. If the challenge toxin to be used has not been shown to be stable or has not been adequately standardised, include 5 guinea-pigs as unvaccinated controls.

SELECTION OF THE CHALLENGE TOXIN

Select a preparation of diphtheria toxin containing 67 to 133 lf/100 in 1 Lf and 25 000 to 50 000 minimal reacting doses for guinea-pig skin in 1 Lf. If the challenge toxin preparation has been shown to be stable, it is not necessary to verify the activity for every assay.

PREPARATION OF THE CHALLENGE TOXIN SOLUTION

Immediately before use, dilute the challenge toxin with a suitable diluent to obtain a challenge toxin solution containing about 0.0512 Lf in 0.2 mL. Prepare from this a further series of 5 four-fold dilutions containing about 0.0128, 0.0032, 0.0008, 0.0002 and 0.00005 Lf in 0.2 mL.

DILUTION OF THE TEST AND REFERENCE PREPARATIONS

Using a 9 g/L solution of *sodium chloride R*, prepare dilutions of the vaccine to be examined and of the reference preparation, such that for each, the dilutions form a series differing by not more than 2.5-fold steps and in which the intermediate dilutions, when injected subcutaneously at a dose of 1.0 mL per guinea-pig, will result in an intradermal score of approximately 3 when the animals are challenged.

IMMUNISATION AND CHALLENGE

Allocate the dilutions, 1 to each of the groups of guinea-pigs, and inject subcutaneously 1.0 mL of each dilution into each guinea-pig in the group to which that dilution is allocated. After 28 days, shave both flanks of each guinea-pig and inject 0.2 mL of each of the 6 toxin dilutions intradermally into 6 separate sites on each of the vaccinated guinea-pigs in such a way as to minimise interference between adjacent sites.

DETERMINATION OF THE ACTIVITY OF THE CHALLENGE TOXIN

If necessary, inject the unvaccinated control animals with dilutions containing 80, 40, 20, 10 and 5 × 10^{-6} Lf of the challenge toxin.

READING AND INTERPRETATION OF RESULTS

Examine all injection sites 48 h after injection of the challenge toxin and record the incidence of specific diphtheria erythema. Record also the number of sites free from such reactions as the intra-dermal challenge score. Tabulate the intradermal challenge scores for all the animals receiving the same dilution of vaccine and use those data with a suitable transformation, such as $(score)^2$ or $\arcsin((score/6)^2)$, to obtain an estimate of the relative

potency for each of the test preparations by parallel-line quantitative analysis.

REQUIREMENTS FOR A VALID ASSAY

The test is not valid unless:

— for both the vaccine to be examined and the reference preparation, the mean score obtained at the lowest dose level is less than 3 and the mean score at the highest dose level is more than 3;

— where applicable, the toxin dilution that contains 40×10^{-6} Lf gives a positive erythema in at least 80 per cent of the control guinea-pigs and the dilution containing 20×10^{-6} Lf gives a positive erythema in less than 80 per cent of the guinea-pigs (if these criteria are not met a different toxin has to be selected);

— the confidence limits ($P = 0.95$) are not less than 50 per cent and not more than 200 per cent of the estimated potency;

— the statistical analysis shows no deviation from linearity and parallelism.

The test may be repeated but when more than 1 test is performed the results of all valid tests must be combined in the estimate of potency.

METHOD C. DETERMINATION OF ANTIBODIES IN GUINEA-PIGS

SELECTION AND DISTRIBUTION OF THE TEST ANIMALS

Use in the test healthy guinea-pigs from the same stock, each weighing 250-350 g. Use guinea-pigs of the same sex or with males and females equally distributed between the groups. Distribute the guinea-pigs in not fewer than 6 equal groups; use groups containing a number of animals sufficient to obtain results that fulfil the requirements for a valid assay prescribed below. Use a further group of non-vaccinated guinea-pigs of the same origin to provide a negative serum control. If test consistency has been demonstrated, a reference negative serum control may be used.

REFERENCE PREPARATION

Use a suitable reference preparation such as *diphtheria vaccine (adsorbed) BRP* or a batch of vaccine shown to be effective in clinical studies, or a batch representative thereof, and which has been calibrated in International Units with reference to *diphtheria vaccine (adsorbed) BRP* or the International Standard for diphtheria toxoid (adsorbed).

DILUTION OF THE TEST AND REFERENCE PREPARATIONS

Using a 9 g/L solution of *sodium chloride R* as diluent, prepare serial dilutions of the vaccine to be examined and the reference preparation; series differing by 2.5- to 5-fold steps have been found to be suitable. Use not fewer than 3 dilutions within the range of, for example, 0.5-16 IU/mL for the reference vaccine and within the range of, for example, 1:2 to 1:125 for the vaccine to be examined. Use the dilutions for immunisation preferably within 1 h of preparation. Allocate 1 dilution to each group of guinea-pigs.

IMMUNISATION

Inject subcutaneously to each guinea-pig 1.0 mL of the dilution allocated to its group.

BLOOD SAMPLING

35-42 days after immunisation, take a blood sample from each vaccinated and control guinea-pig using a suitable method.

PREPARATION OF SERUM SAMPLES

Avoid frequent freezing and thawing of serum samples. To avoid microbial contamination, it is preferable to carry out manipulations in a laminar-flow cabinet.

DETERMINATION OF ANTIBODY TITRE

Determine the relative antibody titre or score of each serum sample by a suitable immunochemical method (*2.7.1*). The methods shown below (enzyme-linked immunosorbent assay (ELISA) and Vero cell assay) have been found to be suitable.

CALCULATION OF POTENCY

Calculate the potency of the vaccine to be examined in International Units relative to the reference preparation, using the usual statistical methods (for example, *5.3*).

REQUIREMENTS FOR A VALID ASSAY

The test is not valid unless:

— the confidence limits ($P = 0.95$) are not less than 50 per cent and not more than 200 per cent of the estimated potency;

— the statistical analysis shows a significant slope and no deviation from linearity and parallelism of the dose-response curves (chapter *5.3* describes possible alternatives if significant deviations are observed).

The test may be repeated but when more than 1 test is performed the results of all valid tests must be combined in the estimate of potency.

The following section is published for information.

ASSAY OF DIPHTHERIA VACCINE (ADSORBED): GUIDELINES

METHOD C. DETERMINATION OF ANTIBODIES IN GUINEA-PIGS

PREPARATION OF SERUM SAMPLES

For the preparation of serum samples, the following technique has been found to be suitable. Invert the tubes containing blood samples 6 times and allow to stand at 37 °C for 2 h, then at 4 °C for 2 h. Centrifuge at room temperature at 800 *g* for 20 min. Transfer the serum to sterile tubes and store at a temperature below −20 °C. At least a 40 per cent yield of serum is obtained by this procedure.

DETERMINATION OF ANTIBODY TITRE

The ELISA and Vero cell assays shown below are given as examples of immunochemical methods that have been found to be suitable for the determination of antibody titre.

Determination of antibody titre in guinea-pig serum by enzyme-linked immunosorbent assay (ELISA)

Dilutions of test and reference sera are made on ELISA plates coated with diphtheria toxoid. A positive guinea-pig serum control and a negative guinea-pig serum control are included on each plate to monitor the assay performance. Peroxidase-conjugated rabbit or goat antibody directed against guinea-pig-IgG is added, followed by a peroxidase substrate. Optical density is measured and the relative antibody titre is calculated using the usual statistical methods (for example, *5.3*).

Reagents and equipment

— *ELISA plates*: 96 wells, columns 1-12, rows A-H.

— *Diphtheria guinea-pig antiserum (for vaccines-human use)* (positive control serum), obtained by immunisation of guinea-pigs using *diphtheria vaccine (adsorbed) BRP*.

— *Peroxidase conjugate*. Peroxidase-conjugated rabbit or goat antibody directed against guinea-pig IgG.

— *Diphtheria toxoid*.

— *Carbonate coating buffer pH 9.6.* Dissolve 1.59 g of *anhydrous sodium carbonate R* and 2.93 g of *sodium hydrogen carbonate R* in 1000 mL of *water R.* Distribute into 150 mL bottles and sterilise by autoclaving at 121 °C for 15 min.
— *Phosphate-buffered saline pH 7.4 (PBS).* Dissolve with stirring 80.0 g of *sodium chloride R,* 2.0 g of *potassium dihydrogen phosphate R,* 14.3 g of *disodium hydrogen phosphate dihydrate R* and 2.0 g of *potassium chloride R* in 1000 mL of *water R.* Store at room temperature to prevent crystallisation. Dilute to 10 times its volume with *water R* before use.
— *Citric acid solution.* Dissolve 10.51 g of *citric acid monohydrate R* in 1000 mL of *water R* and adjust the solution to pH 4.0 with a 400 g/L solution of *sodium hydroxide R.*
— *Washing buffer.* PBS containing 0.5 g/L of *polysorbate 20 R.*
— *Diluent blocking buffer.* PBS containing 0.5 g/L of *polysorbate 20 R* and 25 g/L of dried skimmed milk.
— *Peroxidase substrate.* Shortly before use, dissolve 10 mg of *diammonium 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonate) R* (ABTS) in 20 mL of citric acid solution. Immediately before use add 5 µL of *strong hydrogen peroxide solution R.*

Method

The description below is given as an example of a suitable plate layout but others may be used. Wells 1A-H are for negative control serum and wells 2A-H and 12A-H are for positive control serum for assay monitoring. Wells 3-11A-H are for test samples.

Coat each well of the ELISA plates with 100 µL of diphtheria toxoid solution (0.5 Lf/mL in carbonate coating buffer pH 9.6). Allow to stand overnight at 4 °C in a humid atmosphere. To avoid temperature gradient effects, do not stack more than 4 plates high. On the following day, wash the plates thoroughly with washing buffer. Block the plates by addition of 100 µL of diluent block buffer to each well. Incubate in a humid atmosphere at 37 °C for 1 h. Wash the plates thoroughly with washing buffer. Place 100 µL of diluent block buffer in each well of the plates, except those of row A. Prepare suitable dilutions of negative control serum, positive control serum (from about 0.01 IU/mL) and test sera. Allocate the negative control serum to column 1, positive control serum to columns 2 and 12 and test sera to columns 3-11 and add 100 µL of each serum to the first 2 wells of the column to which it is allocated. Using a multichannel micropipette, make twofold serial dilutions from row B, down the plate to row H, by transferring 100 µL from one well to the next well. Discard 100 µL from the last row so that all wells contain 100 µL. Incubate at 37 °C for 2 h. Wash thoroughly with washing buffer. Prepare a suitable dilution (a 2000-fold dilution has been found to be suitable) of peroxidase conjugate in diluent block buffer and add 100 µL to each well. Incubate at 37 °C in a humid atmosphere for 1 h. Wash the plates thoroughly with washing buffer. Add 100 µL of peroxidase substrate to each well. Allow to stand at room temperature, protected from light, for 30 min. Read the plates at 405 nm in the same order as addition of substrate was made.

Determination of antibody titre in guinea-pig serum by Vero cell assay

The method used relies either on metabolic inhibition (method 1) or on cytotoxicity (method 2) as the end point, and on either microscopic (cell morphology) or visual (colour) inspection of the cells.

The limit of detection is specific for each antitoxin and is usually between 0.015 IU/mL (method 1) and 0.05 IU/mL (method 2).

The endpoint is taken as the highest serum dilution protecting cells from the diphtheria toxin effect.
The antitoxin activity is calculated with respect to guinea-pig or WHO reference standard, and expressed in International Units per millilitre.

Reagents and equipment

— *Flat-bottomed tissue culture plates*: 96 wells, columns 1-12, rows A-H.
— *75 cm^2 tissue culture flasks.*
— *Diphtheria toxin.*
— *Diphtheria guinea-pig antiserum (for vaccines-human use)* (positive control serum), obtained by immunisation of guinea-pigs with *diphtheria vaccine (adsorbed) BRP.*
— *Vero cells* (African Green Monkey kidney cells). Cell passages from P2 to P15 are suitable for use.

Method 1 The diphtheria toxin causes a cytopathogenic effect on Vero cells leading to cellular lysis. Antibodies directed against diphtheria toxin may inhibit this cytopathogenic effect. Consequently, the potency of a diphtheria vaccine may be indirectly determined with the help of this cell culture system if different serum dilutions from immunised animals are cultured with a constant toxin concentration. In the Vero cell assay, yellow colour indicates viable cells, red colour dead cells. When only part of the cells are dead, the colour may be orange.

Reagents and equipment

— *Modified MEM.* Minimum Essential Medium (MEM) with Earle's Salts, without L-glutamine and sodium bicarbonate.
— *Modified medium 199.* Medium 199, with Hanks' Solution and L-glutamine, without sodium bicarbonate.
— *Foetal bovine serum.*
— *Sodium bicarbonate 7.5 per cent solution.*
— *Trypsin solution:* trypsin 2.5 per cent solution.
— *EDTA solution:* EDTA 0.02 per cent (Versene 1:5000) solution.
— *Modified D-PBS.* Dulbecco's phosphate buffered saline (D-PBS), without calcium, or magnesium.
— *L-glutamine 200mM solution.*
— *Penicillin/streptomycin solution.*
— *Primary culture medium.* To 50 mL of modified MEM add 440 mL of *water R,* 5 mL of L-glutamine 200 mM solution, and 10 mL of sodium bicarbonate 7.5 per cent solution. To 25 mL of this medium add 1.25 mL of foetal bovine serum.
— *Maintenance culture medium.* Similar to the primary culture medium except that 0.5 mL instead of 1.25 mL of foetal bovine serum is added to 20 mL of the enriched MEM medium.
— *Medium A.* To 50.0 mL of modified medium 199 add 440.0 mL of *water R,* 5.0 mL of L-glutamine 200 mM solution and 10.0 mL of sodium bicarbonate 7.5 per cent solution.
— *Medium B.* To 150.0 mL of medium A add 3.0 mL of foetal bovine serum and 0.3 mL of penicillin/streptomycin solution.
— *Medium C.* To 22.0 mL of medium A add 0.44 mL of foetal bovine serum and 0.44 mL of penicillin/streptomycin solution.

Vero cells are cultured in tissue culture flasks (for example 75 cm^2/250 mL) in an incubator at 36 ± 1 °C, 5 per cent CO_2 and 90 per cent relative humidity. Vero cells

are first grown in the primary culture medium. After 2-3 days of growth, the primary culture medium is replaced by the maintenance culture medium. When a confluent monolayer is obtained, the culture supernatant is discarded and the cell layer washed gently with modified D-PBS. Add a mixture of 1 volume of trypsin solution and 1 volume of EDTA solution to the flask. Swirl the flask gently and incubate in the CO_2 incubator for about 3 min until the cells start to break from the monolayer. Vigorously tap the side of the flask to make the cells fall. Resuspend the cells in 5-6 mL of fresh medium C to obtain a homogeneous suspension. Prepare a cell suspension in medium C containing approximately 1×10^5 cells/mL.

Place 25 µL of medium B in each well except those of column 1. Place 25 µL of the diphtheria guinea-pig antiserum (for vaccines-human use) (positive control serum, working dilution in medium B of 0.40 IU/mL) in wells A1, A2 and A11. Place 25 µL of guinea-pig serum samples in wells B-G of columns 1, 2 and 11. Place 25 µL of negative control serum in row H of columns 1, 2 and 11. Using a multichannel micropipette, make twofold serial dilutions across the plate (from column 2 up to column 10 for rows A-G and up to column 8 for row H). Discard 25 µL from the wells in column 10 in rows A-G, and from well H8.

Reconstitute the diphtheria toxin with saline solution to give a solution of 50 IU/mL. Prepare a 50-fold dilution of this diphtheria toxin dilution in medium B to obtain a working solution of 1.0 IU/mL. Add 25 µL of this working solution to wells A12 and B12 (toxin control). Make twofold serial dilutions by tranferring 25 µL from one well to the next, from well B12 down to H12. Change the tip between each dilution. Discard 25 µL from well H12. Add 25 µL of medium B to wells B12-H12. Then, place 25 µL of the working dilution of the diphtheria toxin (1.0 IU/mL) in each well of rows A-H, from column 1-10, except in wells H9 and H10 (cells only, without serum and without toxin).

Cover the plates with lids or sealer and shake gently. Incubate the plates for at least 2 h in a humid container in a CO_2 incubator at 37 °C. Add 200 µL of cell suspension containing 1×10^5 cells/mL to all the wells. Cover the plates with sealer. Incubate at 37 °C for 5 days. Check for microbial contamination by microscopic examination.

Yellow wells are recorded as negative and red wells indicate dead cells and are recorded as positive. A colour between yellow and red indicates a mixture of viable and dead cells and is recorded as positive/negative. The results based on the change in colour can be confirmed by reading viable and dead cells under the microscope.

The potency of the guinea-pig antiserum samples is obtained by comparing the last well of the standard preparation showing complete neutralisation of the toxin, with the last well of the sample demonstrating the same effect.
For calculations of potency, it must be remembered that the endpoint may be between a negative well and a positive/negative well.

Method 2 Thiazolyl blue MTT is reduced to a blue/black formazan product by the mitochondrial dehydrogenase of viable cells, and thus serves as a quantitative measure of living cells present, indicating when the toxin has been neutralised by the antitoxin. White or colourless wells indicate absence of viable cells due to insufficient antitoxin to neutralise the toxin.

Reagents and equipment

- *MEM* (Minimal Essential Media).
- *Newborn calf serum.*
- *Antibiotic solution* (containing 10 000 units of penicillin, 10 mg of streptomycin and 25 µg of amphotericin B per millilitre).
- *L-glutamine 200mM solution.*
- *Trypsin-EDTA.*
- *Thiazolyl blue MTT* [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide].
- *1 M HEPES buffer pH 8.1.* Dissolve 18.75 g of HEPES in 82.5 mL of *water R* and 30.0 mL of *2 M sodium hydroxide R.*
- *Glucose solution (10 per cent).*
- *Complete culture medium.* Mix 200 mL of MEM with 10 mL of newborn calf serum, 3.0 mL of 1 M HEPES buffer pH 8.1, 2.0 mL of glucose solution (10 per cent), 2.0 mL of antibiotic solution and 2.0 mL of L-glutamine 200mM solution.
- *Phosphate-buffered saline pH 7.4 (PBS).* Dissolve 10.0 g of *sodium chloride R*, 0.75 g of *potassium chloride R*, 1.44 g of *disodium hydrogen phosphate dodecahydrate R*, and 0.125 g of *potassium dihydrogen phosphate R* in *water R*, and dilute to 1000.0 mL with the same solvent. Adjust the pH if necessary. Autoclave at 120 °C for 15 min.
- *Thiazolyl blue MTT solution.* Dissolve 0.1 g of thiazolyl blue MTT in 20 mL of PBS. Sterilise by filtration (0.2 µm) and store in dark bottle.
- *pH adjuster solution.* Mix 40 mL of *acetic acid R* with 1.25 mL of *1 M hydrochloric acid* and 8.75 mL of *water R.*
- *Extraction buffer pH 4.7.* Dissolve 10 g of *sodium laurilsulfate R* in *water R* and add 50 mL of *dimethylformamide R*, and dilute to 100 mL with *water R.* Adjust the pH with an appropriate volume of pH adjuster solution.

Vero cells are cultured in tissue culture flasks (for example 75 cm^2/250 mL) in an incubator at 36 ± 1 °C, 5 per cent CO_2 and 90 per cent relative humidity. Vero cells are grown in the complete culture medium. After 6-7 days of growth, a confluent monolayer is obtained, the culture supernatant is discarded and the cell layer is washed 3 times with trypsin-EDTA: gently pipette out the medium, add 0.5-1 mL of trypsin-EDTA, swirl the flask and tip the trypsin out. Do this twice, and the 3rd time, place the flask in the incubator for 5 min until the cells start to break from the monolayer. Vigorously tap the side of the flask to make the cells fall. Resuspend the cells in 6-25 mL of fresh complete culture medium to obtain a homogeneous suspension. Prepare a cell suspension in complete culture medium containing approximately 4×10^5 cells/mL.

Place 50 µL of complete culture medium in each well except those of column 1. Place 100 µL of diphtheria guinea pig antiserum (for vaccines-human use) (positive control serum, working dilution in complete culture medium of 0.12 IU/mL) in well A1 and 50 µL in well A11. Place 100 µL of guinea pig test serum samples, diluted if necessary, in wells B1-G1. Add 50 µL of the same sample to wells B11-G11 in the corresponding row. Place 100 µL of negative control serum in well H1 and 50 µL in well H11. Using a multi-channel micropipette, make twofold serial dilutions by transferring 50 µL from one well to the next working across the plate (from column 1-10 for rows A-G and from column 1-8 for row H).

Diphtheria toxin of known activity and Lf content is diluted to a suitable working stock containing at least 4 minimum cytopathic doses in complete culture medium. Add 50 µL of the diluted toxin to each well except H9 and H10 (cell control), A11-H11 (serum control) and A12-H12 (toxin control). Add 100 µL of diluted toxin to well A12 and make

twofold serial dilutions by transferring 50 µL from one well to the next working down the plate (from well A12-H12). Discard 50 µL from well H12. Add 50 µL of complete medium to wells H9 and H10.

Cover the plates with a lid or sealer and leave for 1 h at room temperature to allow toxin neutralisation to occur. 50 µL of cell suspension containing approximately 4×10^5 cells/mL is added to each well. The plates are sealed and incubated at 37 °C for 6 days. Check for microbial contamination by microscopic examination. 10 µL of thyazolyl blue MTT solution is added to each well. The plates are incubated at 37 °C for a further 2-4 h. Then, the medium is removed and 100 µL of extraction buffer pH 4.7 is added to each well. The plates are incubated at 37 °C and left overnight to aid the extraction process. Once extraction and solubilisation is complete, plates are visually examined or read at 570 nm.

Blue/black wells are recorded as negative (all the cells are alive, toxin neutralisation by antitoxin) and white or colourless wells indicate dead cells (no toxin neutralisation) and are recorded as positive.

The potency of the test antitoxin is obtained by comparing the last well of the reference antitoxin preparation showing neutralisation of the toxin, with the last well of the antitoxin preparation demonstrating the same effect. The neutralising antibody titre of the sample being examined can be calculated by multiplication of the dilution factor with total number of International Units per millilitre of the reference preparation at the end point. The test is valid if all the cells in the toxin control are dead and reference antitoxin gives a neutralisation in at least the first 2 dilutions tested.

2. Assay of Pertussis Vaccine (Whole Cell)

(Ph. Eur. method 2.7.7)

The potency of pertussis vaccine (whole cell) is determined by comparing the dose necessary to protect mice against the effects of a lethal dose of *Bordetella pertussis*, administered intracerebrally, with the quantity of a reference preparation, calibrated in International Units, needed to give the same protection.

The International Unit is the activity contained in a stated amount of the International Standard which consists of a quantity of dried pertussis vaccine. The equivalence in International Units of the International Standard is stated by the World Health Organization.

Selection and distribution of the test animals

Use in the test healthy mice less than 5 weeks old of a suitable strain from the same stock, the difference in mass between the heaviest and the lightest being not greater than 5 g. Distribute the mice in 6 groups of not fewer than 16 and 4 groups of 10. The mice must all be of the same sex or the males and females distributed equally between the groups.

Selection of the challenge strain and preparation of the challenge suspension

Select a suitable strain of *B. pertussis* capable of causing the death of mice within 14 days of intracerebral injection. If more than 20 per cent of the mice die within 48 h of the injection the strain is not suitable. Make one subculture from the strain and suspend the harvested *B. pertussis* in a solution containing 10 g/L of *casein R* hydrolysate and 6 g/L of *sodium chloride R* and having a pH of 7.0 to 7.2 or in another suitable solution. Determine the opacity of the suspension. Prepare a series of dilutions in the same solution and allocate each dilution to a group of 10 mice. Inject intracerebrally into each mouse a dose (0.02 mL or 0.03 mL) of the dilution allocated to its group. After 14 days, count the number of mice surviving in each group. From the results, calculate the expected opacity of a suspension containing 100 LD_{50} in each challenge dose. For the test of the vaccine to be examined make a fresh subculture from the same strain of *B. pertussis* and prepare a suspension of the harvested organisms with an opacity corresponding to about 100 LD_{50} in each challenge dose. Prepare 3 dilutions of the challenge suspension.

Determination of potency

Prepare 3 serial dilutions of the vaccine to be examined and 3 similar dilutions of the reference preparation such that in each the intermediate dilution may be expected to protect about 50 per cent of the mice from the lethal effects of the challenge dose of *B. pertussis*. Suggested doses are 1/8, 1/40 and 1/200 of the human dose of the vaccine to be examined and 0.5 IU, 0.1 IU and 0.02 IU of the reference preparation, each dose being contained in a volume not exceeding 0.5 mL. Allocate the 6 dilutions, one to each of the groups of not fewer than 16 mice, and inject intraperitoneally into each mouse one dose of the dilution allocated to its group. After 14 - 17 days inject intracerebrally into each animal in the groups of not fewer than 16, one dose of the challenge suspension. Allocate the challenge suspension and the 3 dilutions made from it, one to each of the groups of 10 mice, and inject intracerebrally one dose of each suspension into each mouse in the group to which that suspension is allocated. Exclude from consideration any mice that die within 48 h of challenge. Count the number of mice surviving in each of the groups after 14 days. Calculate the potency of the vaccine to be examined relative to the potency of the reference preparation on the basis of the numbers of animals surviving in each of the groups of not fewer than 16.

The test is not valid unless:

- for both the vaccine to be examined and the reference preparation, the 50 per cent protective dose lies between the largest and the smallest doses given to the mice;
- the number of animals that die in the 4 groups of 10 injected with the challenge suspension and its dilutions indicates that the challenge dose is approximately 100 LD_{50}; and
- the statistical analysis shows no deviation from linearity or parallelism.

The test may be repeated but when more than one test is performed the results of all valid tests must be combined.

3. Assay of Tetanus Vaccine (Adsorbed)

(Ph. Eur. method 2.7.8)

The potency of tetanus vaccine is determined by administration of the vaccine to animals (guinea-pigs or mice) followed either by challenge with tetanus toxin (method A or B) or by determination of the titre of antibodies against tetanus toxoid in the serum of the guinea-pigs (method C). In both cases, the potency of the vaccine is calculated by comparison with a reference vaccine, calibrated in International Units. For methods A and B, in countries where the paralysis method is not obligatory, the LD_{50} method may be used. For the LD_{50} method, the number of animals and the procedure are identical to those described for the paralysis method, but the end-point is the death of the animal rather than paralysis.

The International Unit is the activity contained in a stated amount of the International Standard for tetanus toxoid (adsorbed). The equivalence in International Units of the International Standard is stated by the World Health Organization.

Tetanus vaccine (adsorbed) BRP is calibrated in International Units with reference to the International Standard.

The method chosen for the assay of tetanus vaccine (adsorbed) depends on the intended purpose. Method A or B is used:

1. during development of a vaccine, to assay batches produced to validate the production;

2. wherever revalidation is needed following a significant change in the manufacturing process.

Method A or B may also be used for the routine assay of batches of vaccine, but in the interests of animal welfare, method C is used wherever possible.

Method C may be used, except as specified under 1 and 2 above, after verification of the suitability of the method for the product. For this purpose, a suitable number of batches (usually 3) are assayed by method C and method A or B. Where different vaccines (monovalent or combinations) are prepared from tetanus toxoid of the same origin and with comparable levels (expressed in Lf/mL) of the same tetanus toxoid, suitability demonstrated for the combination with the highest number of components can be assumed to be valid for combinations with fewer components and for monovalent vaccines. Any combinations containing a whole-cell pertussis component or containing haemophilus type b conjugate vaccine with tetanus toxoid in the same vial must always be assessed separately.

For combinations containing diphtheria and tetanus components, the serological assay (method C) can be performed with the same group of animals used for the serological assay of the diphtheria vaccine (adsorbed) (*2.7.6*) when the common immunisation conditions for the tetanus and the diphtheria components (for example, doses, duration) have been demonstrated to be valid for the combined vaccine.

The design of the assays described below uses multiple dilutions for the test and reference preparations. Based on the potency data obtained in multiple-dilution assays, it may be possible to reduce the number of animals needed to obtain a statistically significant result by applying a simplified model such as a single dilution for both test and reference preparations. Such a model enables the analyst to determine whether the potency of the test preparation is significantly higher than the minimum required, but does not give information on the dose-response curves and their linearity, parallelism and significant slope. The simplified model allows for a considerable reduction in the number of animals required and must be considered by each analyst in accordance with the provisions of the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes.

Where a single-dilution assay is used, production and test consistency over time are monitored via suitable indicators and by carrying out a full multiple-dilution assay periodically, for example every 2 years. For serological assays, suitable indicators to monitor test consistency are:

- the mean and standard deviation of relative antitoxin titres or scores of the serum samples obtained after administration of a fixed dose of the vaccine reference preparation;
- the antitoxin titres or scores of run controls (positive and negative serum samples);
- the ratio of antitoxin titres or scores for the positive serum control to the serum samples corresponding to the reference vaccine.

METHOD A. CHALLENGE TEST IN GUINEA-PIGS

SELECTION AND DISTRIBUTION OF THE TEST ANIMALS

Use in the test healthy guinea-pigs from the same stock, each weighing 250-350 g. Use guinea-pigs of the same sex or with males and females equally distributed between the groups. Distribute the guinea-pigs in not fewer than 6 equal groups; use groups containing a number of animals sufficient to obtain results that fulfil the requirements for a valid assay prescribed below. If the activity of the challenge toxin has to be determined, include 3 further groups of 5 guinea-pigs as unvaccinated controls.

SELECTION OF THE CHALLENGE TOXIN

Select a preparation of tetanus toxin containing not less than 50 times the 50 per cent paralytic dose per millilitre. If the challenge toxin preparation has been shown to be stable, it is not necessary to verify the paralytic dose for every assay.

PREPARATION OF THE CHALLENGE TOXIN SOLUTION

Immediately before use, dilute the challenge toxin with a suitable diluent (for example, peptone buffered saline solution pH 7.4) to obtain a stable challenge toxin solution containing approximately 50 times the 50 per cent paralytic dose per millilitre. If necessary, use portions of the challenge toxin solution diluted 1 to 16, 1 to 50 and 1 to 160 with the same diluent to determine the activity of the toxin.

DILUTION OF THE TEST AND REFERENCE PREPARATIONS

Using a 9 g/L solution of *sodium chloride R*, prepare dilutions of the vaccine to be examined and of the reference preparation, such that for each, the dilutions form a series differing by not more than 2.5-fold steps and in which the intermediate dilutions, when injected subcutaneously at a dose of 1.0 mL per guinea-pig, protect approximately 50 per cent of the animals from the paralytic effects of the subcutaneous injection of the quantity of tetanus toxin prescribed for this test.

IMMUNISATION AND CHALLENGE

Allocate the dilutions, 1 to each of the groups of guinea-pigs, and inject subcutaneously 1.0 mL of each dilution into each guinea-pig in the group to which that dilution is allocated. After 28 days, inject subcutaneously into each animal 1.0 mL of the challenge toxin solution (containing 50 times the 50 per cent paralytic dose).

DETERMINATION OF THE ACTIVITY OF THE CHALLENGE TOXIN

If necessary, allocate the 3 dilutions made from the challenge toxin solution, 1 to each of the 3 groups of 5 guinea-pigs, and inject subcutaneously 1.0 mL of each solution into each guinea-pig in the group to which that solution is allocated. The activity and stability of the challenge toxin are determined by carrying out a suitable number of determinations of the 50 per cent paralytic dose. It is then not necessary to repeat the determination for each assay.

READING AND INTERPRETATION OF RESULTS

Examine the guinea-pigs twice daily. Remove and euthanise all animals showing definite signs of tetanus paralysis. Count the number of guinea-pigs without paralysis 5 days after injection of the challenge toxin. Calculate the potency of the vaccine to be examined relative to the potency of the reference preparation on the basis of the proportion of challenged animals without paralysis in each group of vaccinated guinea-pigs, using the usual statistical methods (for example, *5.3*).

REQUIREMENTS FOR A VALID ASSAY

The test is not valid unless:

- for both the vaccine to be examined and the reference preparation, the 50 per cent protective dose lies between the largest and smallest doses of the preparations given to the guinea-pigs;
- where applicable, the number of paralysed animals in the 3 groups of 5 injected with the dilutions of the challenge toxin solution indicates that the challenge was approximately 50 times the 50 per cent paralytic dose;
- the confidence limits (P = 0.95) are not less than 50 per cent and not more than 200 per cent of the estimated potency;
- the statistical analysis shows a significant slope and no deviation from linearity and parallelism of the dose-response curves (chapter *5.3* describes possible alternatives if significant deviations are observed).

The test may be repeated but when more than 1 test is performed the results of all valid tests must be combined in the estimate of potency.

METHOD B. CHALLENGE TEST IN MICE

SELECTION AND DISTRIBUTION OF THE TEST ANIMALS

Use in the test healthy mice from the same stock, about 5 weeks old and from a strain shown to be suitable. Use mice of the same sex or with males and females equally distributed between the groups. Distribute the mice in not fewer than 6 equal groups; use groups containing a number of animals sufficient to obtain results that fulfil the requirements for a valid assay prescribed below. If the challenge toxin to be used has not been shown to be stable or has not been adequately standardised, include 3 further groups of not fewer than 5 mice to serve as unvaccinated controls.

SELECTION OF THE CHALLENGE TOXIN

Select a preparation of tetanus toxin containing not less than 100 times the 50 per cent paralytic dose per millilitre. If the challenge toxin preparation has been shown to be stable, it is not necessary to verify the paralytic dose for every assay.

PREPARATION OF THE CHALLENGE TOXIN SOLUTION

Immediately before use, dilute the challenge toxin with a suitable diluent (for example, peptone buffered saline solution pH 7.4) to obtain a stable challenge toxin solution containing approximately 50 times the 50 per cent paralytic dose in 0.5 mL. If necessary, use portions of the challenge toxin solution diluted 1 to 16, 1 to 50 and 1 to 160 with the same diluent to determine the activity of the toxin.

DILUTION OF THE TEST AND REFERENCE PREPARATIONS

Using a 9 g/L solution of *sodium chloride R*, prepare dilutions of the vaccine to be examined and of the reference preparation, such that for each, the dilutions form a series differing by not more than 2.5-fold steps and in which the intermediate dilutions, when injected subcutaneously at a dose of 0.5 mL per mouse, protect approximately 50 per cent of the animals from the paralytic effects of the subcutaneous injection of the quantity of tetanus toxin prescribed for this test.

IMMUNISATION AND CHALLENGE

Allocate the dilutions, 1 to each of the groups of mice, and inject subcutaneously 0.5 mL of each dilution into each mouse in the group to which that dilution is allocated. After 28 days, inject subcutaneously into each animal 0.5 mL of the challenge toxin solution (containing 50 times the 50 per cent paralytic dose).

DETERMINATION OF THE ACTIVITY OF THE CHALLENGE TOXIN

If necessary, allocate the 3 dilutions made from the challenge toxin solution, 1 to each of the 3 groups of not fewer than 5 mice, and inject subcutaneously 0.5 mL of each solution into each mouse in the group to which that solution is allocated.

READING AND INTERPRETATION OF RESULTS

Examine the mice twice daily. Remove and euthanise all animals showing definite signs of tetanus paralysis. Count the number of mice without paralysis 4 days after injection of the challenge toxin. Calculate the potency of the vaccine to be examined relative to the potency of the reference preparation on the basis of the proportion of challenged animals without paralysis in each group of vaccinated mice, using the usual statistical methods (for example, *5.3*).

REQUIREMENTS FOR A VALID ASSAY

The test is not valid unless:

- for both the vaccine to be examined and the reference preparation, the 50 per cent protective dose lies between the largest and smallest doses of the preparations given to the mice;
- where applicable, the number of paralysed animals in the 3 groups of not fewer than 5 injected with the dilutions of the challenge toxin solution, indicates that the challenge dose was approximately 50 times the 50 per cent paralytic dose;
- the confidence limits (P = 0.95) are not less than 50 per cent and not more than 200 per cent of the estimated potency;
- the statistical analysis shows a significant slope and no deviation from linearity and parallelism of the dose-response curves (chapter *5.3* describes possible alternatives if significant deviations are observed).

The test may be repeated but when more than 1 test is performed the results of all valid tests must be combined in the estimate of potency.

METHOD C. DETERMINATION OF ANTIBODIES IN GUINEA-PIGS

SELECTION AND DISTRIBUTION OF THE TEST ANIMALS

Use in the test healthy guinea-pigs from the same stock, each weighing 250-350 g. Use guinea-pigs of the same sex or with males and females equally distributed between the groups. Distribute the guinea-pigs in not fewer than 6 equal groups; use groups containing a number of animals sufficient to obtain results that fulfil the requirements for a valid assay prescribed below. Use a further group of non-vaccinated guinea-pigs of the same origin to provide a negative serum control. If test consistency has been demonstrated, a reference negative serum control may be used.

REFERENCE PREPARATION

Use a suitable reference preparation such as *tetanus vaccine (adsorbed) BRP* or a batch of vaccine shown to be effective in clinical studies, or a batch representative thereof, and which has been calibrated in International Units with reference to *tetanus vaccine (adsorbed) BRP* or the International Standard for tetanus toxoid (adsorbed).

DILUTION OF THE TEST AND REFERENCE PREPARATIONS

Using a 9 g/L solution of *sodium chloride R* as diluent, prepare serial dilutions of the vaccine to be examined and the

reference preparation; series differing by 2.5- to 5-fold steps have been found to be suitable. Use not fewer than 3 dilutions within the range of, for example, 0.5-16 IU/mL for each series. Use the dilutions for immunisation preferably within 1 h of preparation. Allocate 1 dilution to each group of guinea-pigs.

IMMUNISATION

Inject subcutaneously to each guinea-pig 1.0 mL of the dilution allocated to its group.

BLOOD SAMPLING

35-42 days after immunisation, take a blood sample from each vaccinated and control guinea-pig using a suitable method.

PREPARATION OF SERUM SAMPLES

Avoid frequent freezing and thawing of serum samples. To avoid microbial contamination, it is preferable to carry out manipulations in a laminar-flow cabinet.

DETERMINATION OF ANTIBODY TITRE

Determine the relative antibody titre or score of each serum sample by a suitable immunochemical method (*2.7.1*). The methods shown below (enzyme-linked immunosorbent assay (ELISA) and toxin-binding inhibition (ToBI)) have been found to be suitable.

CALCULATION OF POTENCY

Calculate the potency of the vaccine to be examined in International Units relative to the reference preparation, using the usual statistical methods (for example, *5.3*).

REQUIREMENTS FOR A VALID ASSAY

The test is not valid unless:

— the confidence limits ($P = 0.95$) are not less than 50 per cent and not more than 200 per cent of the estimated potency;

— the statistical analysis shows a significant slope and no deviation from linearity and parallelism of the dose-response curves (chapter *5.3* describes possible alternatives if significant deviations are observed).

The test may be repeated but when more than 1 test is performed the results of all valid tests must be combined in the estimate of potency.

The following section is published for information.

ASSAY OF TETANUS VACCINE (ADSORBED): GUIDELINES

METHOD A. CHALLENGE TEST IN GUINEA-PIGS

READING AND INTERPRETATION OF RESULTS

In order to minimise suffering in the test animals, it is recommended to note the degree of paralysis on a scale such as that shown below. The scale gives typical signs when subcutaneous injection of the challenge toxin is made mid-ventrally, directly behind the sternum with the needle pointing towards the neck of the guinea-pig. Grade T3 is taken as the end-point, but with experience grade T2 can be used instead. Tetanus toxin produces in at least 1 of the forelimbs paralysis that can be recognised at an early stage. The tetanus grades in guinea-pigs are characterised by the following signs:

— T1: slight stiffness of 1 forelimb, but difficult to observe;

— T2: paresis of 1 forelimb which still can function;

— T3: paralysis of 1 forelimb. The animal moves reluctantly, the body is often slightly banana-shaped owing to scoliosis;

— T4: the forelimb is completely stiff and the toes are immovable. The muscular contraction of the forelimb is very pronounced and usually scoliosis is observed;

— T5: tetanus seizures, continuous tonic spasm of muscles;

— D: death.

METHOD B. CHALLENGE TEST IN MICE

READING AND INTERPRETATION OF RESULTS

In order to minimise suffering in the test animals, it is recommended to note the degree of paralysis on a scale such as that shown below. The scale gives typical signs when injection of the challenge toxin is made in the dorsal region, close to one of the hind legs. Grade T3 is taken as the end-point, but with experience grade T2 can be used instead. Tetanus toxin produces in the toxin-injected hind leg paresis followed by paralysis that can be recognised at an early stage. The tetanus grades in mice are characterised by the following signs:

— T1: slight stiffness of toxin-injected hind leg, only observed when the mouse is lifted by the tail;

— T2: paresis of the toxin-injected hind leg, which still can function for walking;

— T3: paralysis of the toxin-injected hind leg, which does not function for walking;

— T4: the toxin-injected hind leg is completely stiff with immovable toes;

— T5: tetanus seizures, continuous tonic spasm of muscles;

— D: death.

METHOD C. DETERMINATION OF ANTIBODIES IN GUINEA-PIGS

PREPARATION OF SERUM SAMPLES

For the preparation of serum samples, the following technique has been found to be suitable. Invert the tubes containing blood samples 6 times and allow to stand at 37 °C for 2 h, then at 4 °C for 2 h. Centrifuge at room temperature at 800 *g* for 20 min. Transfer the serum to sterile tubes and store at a temperature below −20 °C. At least a 40 per cent yield of serum is obtained by this procedure.

DETERMINATION OF ANTIBODY TITRE

The ELISA and ToBI tests shown below are given as examples of immunochemical methods that have been found to be suitable for the determination of antibody titre.

Determination of antibody titre in guinea-pig serum by enzyme-linked immunosorbent assay (ELISA)

Dilutions of test and reference sera are made on ELISA plates coated with tetanus toxoid. A positive guinea-pig serum control and a negative guinea-pig serum control are included on each plate to monitor the assay performance. Peroxidase-conjugated rabbit or goat antibody directed against guinea-pig-IgG is added, followed by a peroxidase substrate. Optical density is measured and the relative antibody titre is calculated using the usual statistical methods (for example, *5.3*).

Reagents and equipment

— *ELISA plates*: 96 wells, columns 1-12, rows A-H.

— *Clostridium tetani guinea-pig antiserum (for vaccines-human use) BRP* (positive control serum).

— *Peroxidase conjugate.* Peroxidase-conjugated rabbit or goat antibody directed against guinea-pig IgG.

— *Tetanus toxoid.*

— *Carbonate coating buffer pH 9.6.* Dissolve 1.59 g of *anhydrous sodium carbonate R* and 2.93 g of *sodium hydrogen carbonate R* in 1000 mL of *water R*. Distribute into 150 mL bottles and sterilise by autoclaving at 121 °C for 15 min.

— *Phosphate-buffered saline pH 7.4 (PBS).* Dissolve with stirring 80.0 g of *sodium chloride R*, 2.0 g of *potassium dihydrogen phosphate R*, 14.3 g of *disodium hydrogen*

phosphate dihydrate R and 2.0 g of *potassium chloride R* in 1000 mL of *water R*. Store at room temperature to prevent crystallisation. Dilute to 10 times its volume with *water R* before use.

— *Citric acid solution.* Dissolve 10.51 g of *citric acid monohydrate R* in 1000 mL of *water R* and adjust the solution to pH 4.0 with a 400 g/L solution of *sodium hydroxide R*.

— *Washing buffer.* PBS containing 0.5 g/L of *polysorbate 20 R*.

— *Diluent block buffer.* PBS containing 0.5 g/L of *polysorbate 20 R* and 25 g/L of dried skimmed milk.

— *Peroxidase substrate.* Shortly before use, dissolve 10 mg of *diammonium 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonate) R* (ABTS) in 20 mL of citric acid solution. Immediately before use add 5 µL of *strong hydrogen peroxide solution R*.

Method

The description below is given as an example of a suitable plate layout but others may be used. Wells 1A-H are for negative control serum and wells 2A-H and 12A-H are for positive control serum for assay monitoring. Wells 3-11A-H are for test samples.

Coat each well of the ELISA plates with 100 µL of tetanus toxoid solution (0.5 Lf/mL in carbonate coating buffer pH 9.6). Allow to stand overnight at 4 °C in a humid atmosphere. To avoid temperature gradient effects, do not stack more than 4 plates high. On the following day, wash the plates thoroughly with washing buffer. Block the plates by addition of 100 µL of diluent block buffer to each well. Incubate in a humid atmosphere at 37 °C for 1 h. Wash the plates thoroughly with washing buffer. Place 100 µL of diluent block buffer in each well of the plates, except those of row A. Prepare suitable dilutions of negative control serum, positive control serum (from about 0.01 IU/mL) and test sera. Allocate the negative control serum to column 1, positive control serum to columns 2 and 12 and test sera to columns 3-11 and add 100 µL of each serum to the first 2 wells of the column to which it is allocated. Using a multichannel micropipette, make twofold serial dilutions from row B down the plate to row H, by transferring 100 µL from one well to the next. Discard 100 µL from the last row so that all wells contain 100 µL. Incubate at 37 °C for 2 h. Wash thoroughly with washing buffer. Prepare a suitable dilution (a 2000-fold dilution has been found to be suitable) of peroxidase conjugate in diluent block buffer and add 100 µL to each well. Incubate at 37 °C in a humid atmosphere for 1 h. Wash the plates thoroughly with washing buffer. Add 100 µL of peroxidase substrate to each well. Allow to stand at room temperature, protected from light, for 30 min. Read the plates at 405 nm in the same order as addition of substrate was made.

Determination of antibody titre in guinea-pig serum by toxin- or toxoid-binding inhibition (ToBI)

Tetanus toxin or toxoid is added to serial dilutions of test and reference sera; the serum/antigen mixtures are incubated overnight. To determine unbound toxin or toxoid, the mixtures are transferred to an ELISA plate coated with tetanus antitoxin. Peroxidase-conjugated equine anti-tetanus IgG is added followed by a peroxidase substrate. Optical density is measured and the antibody titre is calculated using the usual statistical methods (for example, *5.3*). A positive control serum and a negative control serum are included on each plate to monitor assay performance.

Reagents and equipment

— *Round-bottomed, rigid polystyrene microtitre plates.*

— *Flat-bottomed ELISA plates.*

— *Tetanus toxin or tetanus toxoid.*

— *Clostridium tetani guinea-pig antiserum (for vaccines-human use) BRP* (positive control serum).

— *Equine anti-tetanus IgG.*

— *Peroxidase-conjugated equine anti-tetanus IgG.*

— *Carbonate buffer pH 9.6.* Dissolve 1.5 g of *anhydrous sodium carbonate R*, 2.39 g of *sodium hydrogen carbonate R* and 0.2 g of *sodium azide R* in 1000 mL of *water R*, adjust to pH 9.6 and autoclave at 121 °C for 20 min.

— *Sodium acetate buffer pH 5.5.* Dissolve 90.2 g of *anhydrous sodium acetate R* in 900 mL of *water R*, adjust to pH 5.5 using a saturated solution of *citric acid monohydrate R* and dilute to 1000 mL with *water R*.

— *Phosphate-buffered saline pH 7.2 (PBS).* Dissolve 135.0 g of *sodium chloride R*, 20.55 g of *disodium hydrogen phosphate dihydrate R* and 4.80 g of *sodium dihydrogen phosphate monohydrate R* in *water R* and dilute to 15 L with the same solvent. Autoclave at 100 °C for 60 min.

— *Diluent buffer.* PBS containing 5 g/L of *bovine albumin R* and 0.5 g/L of *polysorbate 80 R*.

— *Block buffer.* PBS containing 5 g/L of *bovine albumin R*.

— *Tetramethylbenzidine solution.* 6 g/L solution of *tetramethylbenzidine R* in *ethanol (96 per cent) R*. The substance dissolves within 30-40 min at room temperature.

— *Peroxidase substrate.* Mix 90 mL of *water R*, 10 mL of sodium acetate buffer pH 5.5, 1.67 mL of tetramethylbenzidine solution and 20 µL of *strong hydrogen peroxide solution R*.

— *Washing solution.* Tap water containing 0.5 g/L of *polysorbate 80 R*.

Method

Block the microtitre plates by placing in each well 150 µL of block buffer. Cover the plates with a lid or sealer. Incubate in a humid atmosphere at 37 °C for 1 h. Wash the plates thoroughly with washing solution. Place 100 µL of PBS in each well. Place 100 µL of reference guinea-pig tetanus antitoxin in the first well of a row. Place 100 µL of undiluted test sera in the first well of the required number of rows. Using a multichannel micropipette, make twofold serial dilutions across the plate (up to column 10), by transferring 100 µL from one well to the next. Discard 100 µL from the last column so that all wells contain 100 µL. Prepare a 0.1 Lf/mL solution of tetanus toxin or toxoid using PBS as diluent. Add 40 µL of this solution to each well except those of column 12. The wells of column 11 are a positive control. Add 40 µL of PBS to the wells of column 12 (negative control). Shake the plates gently and cover them with lids. Coat the ELISA plates: immediately before use make a suitable dilution of equine anti-tetanus IgG in carbonate buffer pH 9.6 and add 100 µL to each well. Incubate the 2 series of plates overnight in a humid atmosphere at 37 °C. To avoid temperature gradient effects, do not stack more than 4 plates high. Cover the plates with lids. On the following day, wash the ELISA plates thoroughly with washing solution. Block the plates by placing in each well 125 µL of block buffer. Incubate at 37 °C in a humid atmosphere for 1 h. Wash the plates thoroughly with washing solution. Transfer 100 µL of the pre-incubation mixture from the polystyrene plates to the corresponding wells of the ELISA plates, starting with column 12 and then continuing from 1 to 11. Cover the plates with a lid. Incubate at 37 °C in a humid atmosphere for 2 h. Wash the ELISA plates

thoroughly with washing solution. Make a suitable dilution (a 4000-fold dilution has been found to be suitable) of the peroxidase-conjugated equine anti-tetanus IgG in diluent buffer. Add 100 µL of the dilution to each well and cover the plates with a lid. Incubate at 37 °C in a humid atmosphere for 1.5 h. Wash the ELISA plates thoroughly with washing solution. Add 100 µL of peroxidase substrate to each well. A blue colour develops. Incubate the plates at room temperature. Stop the reaction at a given time (within 10 min) by the addition of 100 µL of a 2 M solution of sulfuric acid prepared from *sulfuric acid R* to each well in the same order as the addition of substrate. The colour changes from blue to yellow. Measure the absorbance at 450 nm immediately after addition of the sulfuric acid or maintain the plates in the dark until reading.

4. Assay of Hepatitis A Vaccine

(Ph. Eur. method 2.7.14)

The assay of hepatitis A vaccine is carried out either *in vitro*, by an immunochemical determination of the antigen content (method A), or *in vivo*, by comparing under given conditions its capacity to induce specific antibodies in mice with the same capacity of a reference preparation (method B).

METHOD A. *IN VITRO* ASSAY

Carry out an immunochemical determination (*2.7.1*) of the hepatitis A antigen content.

Enzyme-linked immunosorbent assay (ELISA) using monoclonal antibodies specific for the detection of a hepatitis A epitope that induces neutralising antibodies has been shown to be suitable.

Suitable numbers of dilutions of the vaccine to be examined and the reference preparation are used, and the potency of the vaccine to be examined is calculated using the usual statistical methods (*5.3*).

The antigen content is within the limits approved for the particular product.

Hepatitis A vaccine (inactivated, non-adsorbed) BRP is calibrated in International Units and may be used as a reference preparation.

METHOD B. *IN VIVO* ASSAY

The test in mice shown below is given as an example of a method that has been found suitable for a given vaccine; other validated methods may also be used.

Selection and distribution of the test animals

Use in the test healthy mice from the same stock, about 5 weeks old and from a strain shown to be suitable. Use animals of the same sex. Distribute the animals in at least 7 equal groups of a number suitable for the requirements of the assay.

Determination of potency of the vaccine to be examined

Using a 9 g/L solution of *sodium chloride R* containing the aluminium adjuvant used for the vaccine, prepare at least 3 dilutions of the vaccine to be examined and matching dilutions of the reference preparation. Allocate the dilutions one to each of the groups of animals and inject subcutaneously not more than 1.0 mL of each dilution into each animal in the group to which that dilution is allocated. Maintain a group of unvaccinated controls, injected subcutaneously with the same volume of diluent. After 28-32 days, anaesthetise and bleed all animals, keeping the individual sera separate. Assay the individual sera for specific antibodies against hepatitis A virus by a suitable immunochemical method (*2.7.1*).

Calculations

Carry out the calculations by the usual statistical methods for an assay with a quantal response (*5.3*).

From the distribution of reaction levels measured on all the sera in the unvaccinated group, determine the maximum reaction level that can be expected to occur in an unvaccinated animal for that particular assay. Any response in vaccinated animals that exceeds this level is by definition a seroconversion.

Make a suitable transformation of the percentage of animals showing seroconversion in each group (for example, a probit transformation) and analyse the data according to a parallel-line log dose-response model. Determine the potency of the test preparation relative to the reference preparation.

Validity conditions

The test is not valid unless:

- for both the test and the reference vaccine, the ED_{50} lies between the smallest and the largest doses given to the animals;
- the statistical analysis shows no significant deviation from linearity or parallelism;
- the confidence limits ($P = 0.95$) are not less than 33 per cent and not more than 300 per cent of the estimated potency.

Potency requirement

The upper confidence limit ($P = 0.95$) of the estimated relative potency is not less than 1.0.

5. Assay of Hepatitis B Vaccine (rDNA)

(Ph. Eur. method 2.7.15)

The assay of hepatitis B vaccine (rDNA) is carried out either *in vivo*, by comparing in given conditions its capacity to induce specific antibodies against hepatitis B surface antigen (HBsAg) in mice or guinea-pigs with the same capacity of a reference preparation, or *in vitro*, by an immunochemical determination of the antigen content.

IN VIVO ASSAY

Selection and distribution of the test animals

Use in the test healthy mice from the same stock, about 5 weeks old. The strain of mice used for this test must give a significant slope for the dose-response curve to the antigen; mice with haplotype H-2^q or H-2^d are suitable. Healthy guinea-pigs weighing 300 g to 350 g (about 7 weeks old) from the same stock are also suitable. Use animals of the same sex. Distribute the animals in at least 7 equal groups of a number appropriate to the requirements of the assay.

Determination of potency of the vaccine to be examined

Using a 9 g/L solution of *sodium chloride R* containing the aluminium adjuvant used for the vaccine or another appropriate diluent, prepare at least 3 dilutions of the vaccine to be examined and matching dilutions of the reference preparation. Allocate the dilutions, 1 to each of the groups of animals, and inject intraperitoneally not more than 1.0 mL of each dilution into each animal in the group to which that dilution is allocated. One group of animals remains unvaccinated and is injected intraperitoneally with the same volume of diluent. After an appropriate time interval (for example, 4-6 weeks), anaesthetise and bleed the animals, keeping the individual sera separate. Assay the individual sera for specific antibodies against HBsAg by a suitable immunochemical method (*2.7.1*).

Calculations

Calculations are carried out by the usual statistical methods for an assay with a quantal response (*5.3*).

From the distribution of reaction levels measured on all the sera in the unvaccinated group, the maximum reaction level that can be expected to occur in an unvaccinated animal for that particular assay is determined. Any response in vaccinated animals that exceeds this level is by definition a seroconversion.

Make a suitable transformation of the percentage of animals showing seroconversion in each group (for example, a probit transformation) and analyse the data according to a parallel-line log dose-response model. Determine the potency of the test preparation relative to the reference preparation.

Validity conditions

The test is not valid unless:

— for both the test and the reference vaccine, the ED_{50} lies between the smallest and the largest doses given to the animals;

— the statistical analysis shows no significant deviation from linearity or parallelism;

— the confidence limits ($P = 0.95$) are not less than 33 per cent and not more than 300 per cent of the estimated potency.

Potency requirement

The upper confidence limit ($P = 0.95$) of the estimated relative potency is not less than 1.0.

***IN VITRO* ASSAY**

Carry out an immunochemical determination (*2.7.1*) of antigen content with acceptance criteria validated against the *in vivo* test.

Enzyme-linked immunosorbent assay (ELISA) and radio-immunoassay (RIA) using monoclonal antibodies specific for protection-inducing epitopes of HBsAg have been shown to be suitable. Suitable numbers of dilutions of the vaccine to be examined and the reference preparation are used and a parallel-line model is used to analyse the data, which may be suitably transformed. Kits for measuring HBsAg *in vitro* are commercially available and it is possible to adapt their test procedures for use as an *in vitro* potency assay.

The acceptance criteria are approved for a given reference preparation by the competent authority in light of the validation data.

6. Assay of Pertussis Vaccine (Acellular)

(Ph. Eur. method 2.7.16)

The assay of acellular pertussis vaccine measures the capacity of the vaccine to induce the formation of specific antibodies in mice or guinea-pigs. Antibody titres for each antigen are determined using a suitable immunochemical method (*2.7.1*) such as enzyme-linked immunosorbent assay (ELISA).

The assay results can be expressed:

— either as a ratio of the geometric mean titre (GMT) of antibodies produced following administration of the test vaccine to the GMT of antibodies produced following administration of a reference vaccine examined in parallel (relative potency assay);

— or directly as a GMT of antibodies induced by the test vaccine (geometric mean unit assay or GMU assay).

For combinations containing pertussis components together with diphtheria and tetanus components, the serological assay in guinea-pigs can be performed with the same group of animals used for the serological assay of diphtheria vaccine (adsorbed) (*2.7.6*) and of tetanus vaccine (adsorbed) (*2.7.8*) when the common immunisation conditions for all components (for example, doses, duration) have been demonstrated to be valid for the combined vaccine.

The guinea-pig model allows for a further reduction in the number of animals required and must be considered by each analyst in accordance with the provisions of the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes.

Methods A and B described below are developed by testing multiple dilutions of the test vaccine and the reference vaccine or internal control (see Glossary), to determine which dilutions are suitable. Once the suitable dilutions have been confirmed for a given vaccine, it is recommended, in accordance with 3R principles (Replacement, Reduction, Refinement), to apply a simplified model such as a single dilution for both the test vaccine and the reference vaccine or internal control. Such a model enables the analyst to determine whether the immunogenicity of the test vaccine is satisfactory.

Suitable indicators to monitor the performance of the serological assay (single or multiple dilutions) are:

— the geometric mean and geometric standard deviation of antibody titres in the serum samples obtained after administration of a fixed dose of the reference vaccine or internal control;

— the antibody titres of run controls (positive control and negative serum samples).

It may be necessary to reconfirm the suitability of the selected dilution with a multiple-dilution assay, e.g. after major process changes or for investigational purposes.

METHOD A. SEROLOGY IN MICE

The following test model is given as an example of a multiple-dilution assay which may form the basis for the establishment of a single-dilution assay.

Selection and distribution of the test animals

Use healthy mice (for example, CD1 strain from the same stock and more than 5 weeks old). Distribute the animals into not fewer than 6 equal groups; use groups containing a number of animals sufficient to meet the pre-defined criteria for variability of the antibody responses prescribed under Calculations and validity criteria. Use serial dilutions of the test vaccine and the reference vaccine or internal control and allocate each dilution to a group of mice. During validation studies a further group of mice may be used as a negative control by injecting the animals with diluent alone.

Immunisation

Inject intraperitoneally or subcutaneously into each mouse 0.5 mL of the dilution allocated to its group.

Collection of serum samples

4-5 weeks after vaccination, bleed the mice individually under anaesthesia. Store the sera at −20 °C until used for antibody determination. Avoid frequent freezing and thawing of serum samples.

Reference antiserum

A reference antiserum of assigned activity serves as the basis for calculation of the antibody titres in the test sera. *Bordetella pertussis mouse antiserum BRP* is suitable for use as a reference antiserum.

Antibody determination

Assay the individual sera for content of specific antibodies against each acellular pertussis antigen using a validated method such as the ELISA test shown below.

ELISA test

Microtitre plates (poly(vinyl chloride) or polystyrene as appropriate for the specific antigen) are coated with the purified antigen at a concentration of 100 ng per well. After washing, unreacted sites are blocked by incubating the plates

with a solution of bovine serum albumin and then washed. 2-fold dilutions of sera from individual mice immunised with the test vaccine or either the reference vaccine or the internal control are prepared on the plates. Reference antiserum is included on each plate. After incubation at 22-25 °C for 1 h, the plates are washed. A suitable solution of enzyme-conjugated anti-mouse IgG antibody is added to each well and incubated at 22-25 °C for 1 h. After washing, a chromogenic substrate is added from which the bound enzyme conjugate liberates a chromophore that can be quantified by measurement of absorbance (*2.2.25*).

METHOD B. SEROLOGY IN GUINEA-PIGS

The following test model is given as an example of a multiple-dilution assay which may form the basis for the establishment of a single-dilution assay.

Selection and distribution of the test animals

Use healthy guinea-pigs from the same stock, each weighing 250-350 g. Use guinea-pigs of the same sex or with males and females equally distributed between the groups. Distribute the guinea-pigs into not fewer than 6 equal groups; use groups containing a number of animals sufficient to meet the pre-defined criteria for variability of the antibody responses prescribed under Calculations and validity criteria. During validation studies a further group of guinea-pigs may be used as a negative control by injecting the animals with diluent alone.

Dilution of the test and reference preparations

Using a 9 g/L solution of *sodium chloride R* as diluent, prepare serial dilutions of the test vaccine and the reference vaccine or internal control; series differing by 2.5- to 5-fold steps have been found to be suitable. Use not fewer than 3 dilutions within the range found to be suitable for all the components in the test vaccine. Use the dilutions for immunisation preferably within 1 h of preparation. Allocate 1 dilution to each group of guinea-pigs.

Immunisation

Inject subcutaneously into each guinea-pig 1.0 mL of the dilution allocated to its group.

Collection of serum samples

5-6 weeks after immunisation, take a blood sample from each vaccinated and negative control guinea-pig using a suitable method. Store the sera at −20 °C until used for antibody determination. Avoid frequent freezing and thawing of serum samples.

Reference antiserum

An in-house guinea-pig reference antiserum of assigned activity serves as the basis for calculation of the antibody titres in the test sera.

Antibody determination

Assay the individual sera for content of specific antibodies against each acellular pertussis antigen using a validated method such as the ELISA test shown below.

ELISA test

Suitable 96-well microtitre plates are coated with the purified antigens (e.g. pertussis toxin (PT), pertactin (PRN), filamentous haemagglutinin (FHA) and/or fimbrial agglutinogens (Fim 2/3)) representing components in the combined vaccine at a concentration of 200-400 ng per well. After washing, unreacted sites are blocked by incubating the plates with a suitable blocking buffer and then washed. 2-fold dilutions of sera from individual guinea-pigs immunised with the test vaccine or either the reference vaccine or the internal control are prepared on the plates. Reference antiserum is included on each plate. After incubation at 37 °C for 1 h, the plates are washed. A suitable solution of enzyme-conjugated anti-guinea-pig IgG antibody is added to each well and incubated at 37 °C for 1 h. After washing, a chromogenic substrate is added from which the bound enzyme conjugate liberates a chromophore that can be quantified by measurement of absorbance (*2.2.25*).

CALCULATIONS AND VALIDITY CRITERIA

Relative potency assay

The antibody titres in the sera of mice or guinea-pigs immunised with the test and reference vaccines are determined for each acellular pertussis antigen using the reference antiserum. From the values obtained, the GMT ratio of the test vaccine in relation to the reference vaccine is calculated for each antigen.

The relative potency assay is not valid unless:

- the number of responder animals for the test and reference vaccines meets the pre-defined criteria;
- the GMT for the reference vaccine is within the limits of the control chart;
- the variability of the antibody responses meets the pre-defined criteria.

GMU assay

The antibody titres in the sera of mice or guinea-pigs immunised with the internal control and test vaccine are determined for each acellular pertussis antigen using the reference antiserum, and the GMTs are calculated for each antigen.

The GMU assay is not valid unless:

- the number of responder animals for the test vaccine and internal control meets the pre-defined criteria;
- the GMT for the internal control is within the limits of the control chart;
- the variability of the antibody responses meets the pre-defined criteria.

GLOSSARY

Internal control for GMU assay

A batch of vaccine shown to be representative of the current manufacturing process and whose response in mice or guinea pigs has been appropriately measured. The stability of the internal control shall be monitored and documented.

Reference vaccine for relative potency assay

A batch of vaccine shown to be effective in clinical trials or a batch representative thereof. The stability of the reference vaccine shall be monitored and documented.

Responder animals

Immunised animals producing antibodies at a titre greater than a threshold defined during the development and validation of the method.

The following section is published for information.

ASSAY OF PERTUSSIS VACCINE (ACELLULAR): GUIDELINES

METHOD B. DETERMINATION OF ANTIBODIES IN GUINEA-PIGS

The ELISA shown below is given as an example of an immunochemical method that has been found to be suitable.

Determination of antibody titre by ELISA method for pertussis toxin (PT), filamentous haemagglutinin (FHA), fimbrial agglutinogens (Fim 2/3) and pertactin (PRN)

2-fold dilutions of sera from test and reference vaccine or internal control are made on ELISA plates coated with

acellular pertussis antigens (PRN, PT, FHA or Fim 2/3). A guinea-pig reference antiserum and a negative guinea-pig serum are included on each plate. Peroxidase-conjugated rabbit or goat antibody directed against guinea-pig IgG is added, followed by a peroxidase substrate.

Reagents and equipment:

— *ELISA plates*: 96 wells, columns 1-12, rows A-H.
— *Reference antiserum* (guinea-pig).
— *Peroxidase conjugate*. Peroxidase-conjugated rabbit or goat antibody directed against guinea-pig IgG.
— *Bordetella pertussis antigens* (PRN, PT, FHA or Fim 2/3).
— *Carbonate coating buffer pH 9.6*. Dissolve 1.59 g of *anhydrous sodium carbonate R* and 2.93 g of *sodium hydrogen carbonate R* in 1000 mL of *water R*. Distribute into 150 mL bottles and sterilise by autoclaving at 121 °C for 15 min.
— *Phosphate-buffered saline pH 7.4 (PBS)*. Dissolve with stirring 80.0 g of *sodium chloride R*, 2.0 g of *potassium dihydrogen phosphate R*, 14.3 g of *disodium hydrogen phosphate dihydrate R* and 2.0 g of *potassium chloride R* in 1000 mL of *water R*. Store at room temperature to prevent crystallisation. Dilute 10-fold with *water R* before use.
— *Citric acid solution*. Dissolve 10.51 g of *citric acid monohydrate R* in 1000 mL of *water R* and adjust to pH 4.0 with a 400 g/L solution of *sodium hydroxide R*.
— *Washing buffer*. PBS containing 0.5 g/L of *polysorbate 20 R*.
— *Diluent block buffer*. PBS containing 0.5 g/L of *polysorbate 20 R* and 25 g/L of dried skimmed milk.
— *Peroxidase substrate*. Shortly before use, dissolve 10 mg of *diammonium 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonate) R* (ABTS) in 20 mL of the citric acid solution. Immediately before use add 5 µL of *strong hydrogen peroxide solution R*.

Method

The description below is given as an example of a suitable plate layout but others may be used. Wells 1A-H are used for negative control serum. Wells 2-12 A-H are used for guinea-pig reference antiserum (usually in 2 positions) and individual sera from guinea-pigs immunised with the test vaccine, or either the reference vaccine or the internal control.

Coat each well of the ELISA plates with 100 µL of the appropriate antigen solution (PT, FHA and Fim 2/3 at 2 µg/mL and PRT at 4 µg/mL, in carbonate coating buffer pH 9.6). Allow to stand overnight at 4 °C in a humid atmosphere. To avoid temperature gradient effects, do not stack more than 4 plates high. On the following day, wash the plates thoroughly with the washing buffer. Block the plates by addition of 150 µL of the diluent block buffer to each well. Incubate in a humid atmosphere at 37 °C for 1 h. Wash the plates thoroughly with the washing buffer. Place 100 µL of the diluent block buffer in each well of the plates, except those of row A. Prepare suitable dilutions of individual test and reference vaccine or internal control serum samples, reference antiserum and negative control serum samples. Allocate the negative control serum to column 1, the reference antiserum to at least 2 other columns and individual test and reference vaccine or internal control sera to the remaining columns and add 100 µL of each serum to the first 2 wells of the column to which it is allocated. Using a multichannel micropipette, make 2-fold serial dilutions from row B down the plate to row H, by transferring 100 µL from one well to the next. Discard 100 µL from the last row so that all wells contain 100 µL. Incubate at 37 °C for 2 h. Wash thoroughly with the washing buffer. Prepare a suitable dilution of the peroxidase conjugate in the diluent block buffer and add 100 µL to each well. Incubate at 37 °C in a humid atmosphere for 1 h. Wash the plates thoroughly with the washing buffer. Add 100 µL of the peroxidase substrate to each well. Allow to stand at room temperature, protected from light, for 30 min. Read the plates at 405 nm in the same order as the addition of substrate was made.

7. *In vivo* Assay of Poliomyelitis Vaccine (Inactivated)

(Ph. Eur. method 2.7.20)

The capacity of the vaccine to induce the formation of neutralising antibodies is determined *in vivo* by one of the following methods.

TEST IN CHICKS OR GUINEA-PIGS

Prepare a suitable series of not fewer than 3 dilutions of the vaccine to be examined using a suitable buffered saline solution. Distribute either guinea-pigs weighing 250-350 g or 3-week-old chicks into groups of 10, and allocate a group to each dilution of the vaccine. Inject intramuscularly into each animal 0.5 mL of the dilution intended for its group. Bleed the animals after 5-6 days and separate the sera. Examine the sera for the presence of neutralising antibodies, at a dilution of 1 in 4, to each of the human poliovirus types 1, 2 and 3. Mix 100 $CCID_{50}$ of virus with the dilution of serum and incubate at 37 °C for 4.5-6 h. Keep at 5 ± 3 °C for 12-18 h where necessary for consistency of results. Inoculate the mixtures into cell cultures for the detection of unneutralised virus and read the results up to 7 days after inoculation. For each group of animals, note the number of sera that have neutralising antibodies and calculate the dilution of the vaccine that gives an antibody response in 50 per cent of the animals. Carry out in parallel a control test using a suitable reference preparation. The vaccine complies with the test if a dilution of 1 to 100 or more produces an antibody response for each of the 3 types of virus in 50 per cent of the animals.

TEST IN RATS

A suitable *in vivo* assay method consists of intramuscular injection into the hind limb(s) of not fewer than 3 dilutions of the vaccine to be examined and a reference vaccine, using for each dilution a group of 10 specific pathogen-free rats of a suitable strain. Use of 4 dilutions is often necessary to obtain valid results for all 3 serotypes. The number of animals per group must be sufficient to obtain results that meet the validity criteria; groups of 10 rats are usually sufficient, although valid results may be obtained with fewer animals per group. If animals of different sex are used, males and females are evenly distributed between all groups.
A weight range of 175-250 g has been found to be suitable. An inoculum of 0.5 mL per rat is used. The dose range is chosen such that a dose response to all 3 poliovirus types is obtained. Bleed the animals after 20-22 days. Neutralising titres against all 3 poliovirus types are measured separately using 100 $CCID_{50}$ of the Sabin strains as challenge viruses, Vero or Hep2 as indicator cells, and neutralisation conditions of 3 h at 35-37 °C followed by 18 h at 2-8 °C where necessary for consistency of results. Results are read following fixation and staining after 7 days of incubation at 35 °C.
For a valid antibody assay, the titre of each challenge virus must be shown to be within the range 10 $CCID_{50}$ to 1000 $CCID_{50}$ and the neutralising antibody titre of a control serum must be within 2 twofold dilutions of the geometric mean titre of the serum. The potency is calculated by comparison of the proportion of responders for the vaccine to

be examined and the reference vaccine by the probit method or, after validation, using a parallel-line model. For the probit method it is necessary to establish a cut-off neutralising antibody titre for each poliovirus type to define a responder. Due to interlaboratory variation, it is not possible to define cut-off values that could be applied by all laboratories. Rather, the cut-off values are determined for each laboratory based on a minimum series of 3 tests with the reference vaccine. The mid-point on a $\log_2$ scale of the minimum and maximum geometric mean titres of the series of 3 or more tests is used as the cut-off value. For each of the 3 poliovirus types, the potency of the vaccine is not significantly less than that of the reference preparation. The test is not valid unless:
- for both the vaccine to be examined and the reference vaccine, the ED_{50} lies between the smallest and the largest doses given to the animals;
- the statistical analysis shows no significant deviation from linearity or parallelism;
- the confidence limits ($P = 0.95$) are not less than 25 per cent and not more than 400 per cent of the estimated potency.

The following section is published for information.

GUIDELINE ON WAIVING OF THE *IN VIVO* ASSAY OF POLIOMYELITIS VACCINE (INACTIVATED) AND ITS COMBINATIONS

This guideline applies to vaccines derived from wild strains of poliovirus. The validation described should be carried out for each product before waiving of the in vivo assay, and should be repeated wherever there is a substantial change to the manufacturing process that may affect the in vitro or in vivo assays.

The European convention on the protection of vertebrate animals used for experimental and other scientific purposes requires that tests in animals shall not be carried out if a scientifically satisfactory alternative is reasonably and practically available. The aim of this guideline is therefore to promote waiving of the *in vivo* assay wherever it can be shown for a given product that the *in vitro* assay (D-antigen determination) gives sufficient assurance of satisfactory potency for routine batch control.

For the *in vivo* assay, the test in rats is considered to be the method of choice. For vaccines that are assayed using chicks or guinea-pigs and that have an established record of production history, the *in vivo* assay may be waived if the rat assay is also applied to the batches included in the validation study described below. For vaccines not yet approved, the results of the rat assay on all final bulks should be included in all data generated for demonstration of consistency of production before waiving of the *in vivo* assay.

Once the *in vivo* assay has been waived, batches of vaccine will be released on the basis of the *in vitro* assay, and the *in vivo* assay should not be used as an alternative for the release of a batch that fails the *in vitro* assay. Repetition of the *in vitro* assay may be performed according to an authorised procedure.

PROCEDURE

The following conditions should be met before performance of the validation study:
- appropriate experience of the rat assay;
- full validation of the D-antigen assay (linearity, repeatability, intermediate precision, accuracy and limits of quantification);
- establishment of acceptance criteria for the D-antigen assay based on a suitable number of consecutive final lots;
- establishment of production consistency on recent final bulks using the currently approved *in vivo* assay; the final bulks should correspond to the final lots used to establish the acceptance criteria for the D-antigen assay and should represent different inactivated harvests of each of the 3 types of poliovirus.

The validation study should be performed on:
- a final bulk/lot that is representative of the current production method;
- 2 sub-potent batches prepared, for example, by heating normal vaccine or mixing it with heat-treated vaccine; the sub-potent batches should have expected titres of about half that of the representative final bulk/lot.

These batches are assayed using as reference standard a homologous production batch:
- by the currently approved *in vivo* assay for the vaccine;
- by the rat assay where this is not the currently approved *in vivo* assay;
- by the D-antigen assay.

Waiving of the *in vivo* assay is acceptable if the representative final bulk/lot complies with the *in vivo* and *in vitro* assays and the sub-potent batches fail to comply. If a sub-potent batch fails to comply with the D-antigen assay but complies with the *in vivo* assay, the latter may be repeated.

8. Flocculation Value (Lf) of Diphtheria and Tetanus Toxins and Toxoids (Ramon Assay)

(Ph. Eur. method 2.7.27)

The content of toxin or toxoid in a sample can be expressed as a flocculation value (Lf) using the Ramon assay. In this assay, antitoxin is added in increasing concentrations to a series of tubes containing a constant amount of toxin or toxoid. At the equivalence point of toxin/toxoid and antitoxin, flocculation occurs in 1 or more tubes. The first tube in which flocculation occurs is used to determine the Lf value of the sample.

The Lf value of a toxin or toxoid is determined by the number of units of antitoxin that, when mixed with the sample, produces an optimally flocculating mixture (Ramon assay).

Practical experience has shown that the results of the calibration of antitoxins in International Units (IU), for example by comparison to international antitoxin standards, depends on the immunochemical method used. For this reason, antitoxins used for the Ramon assay must be directly calibrated against the *international biological reference reagents for diphtheria or tetanus toxoid for flocculation tests*, using the principles described below. The concentration thus determined may be indicated in Lf-equivalents per millilitre (Lf-eq./mL).

By definition, 1 Lf is the quantity of toxin or toxoid that flocculates in the shortest time with 1 Lf-eq. of specific antitoxin.

A range of volumes of the reference standard of antitoxin adjusted to a concentration of 100 Lf-eq./mL is dispensed into a series of, for example, 7 cm × 1 cm flocculation tubes. A sufficient quantity of a 9 g/L solution of *sodium chloride R* is added to each tube to give a constant total volume of, for example, 1 mL. The test sample is diluted to give an expected concentration of approximately 50 Lf/mL, and, for example, 1 mL aliquots of this dilution are dispensed into each of the tubes containing antitoxin. The tubes are properly mixed by shaking, then placed in a

water-bath at a constant temperature between 30 °C and 52 °C, and observed at regular intervals for the first appearance of floccules. This may require the use of a magnifying lens and strong illumination.

The first and the second mixtures to flocculate are recorded as well as the time taken for the first flocculation to appear. 2 tubes may flocculate simultaneously.

The first tube to flocculate is the one that contains the amount of antitoxin closest in equivalence to the amount of antigen in the sample. The antitoxin content of this tube can be used to calculate the Lf value of the sample. If 2 tubes flocculate at the same time, the mean from the tubes are given as the result.

The time taken for the first tube to flocculate (Kf) is a useful indicator of the quality of the antigen. If at a given temperature and concentration of toxoid and antitoxin the Kf value is increased compared with normal, this indicates that the antigen has been damaged. The Kf value may also change with the quality of the antitoxin used.

Example

Tube	A	B	C	D	E	F
Antitoxin added (Lf-eq.)	40	45	50	55	60	65
Antitoxin added (mL)	0.40	0.45	0.50	0.55	0.60	0.65
Saline added (mL)	0.60	0.55	0.50	0.45	0.40	0.35
Diluted sample added	1.0	1.0	1.0	1.0	1.0	1.0

If in this example the first tube to flocculate is tube C then the Lf value of the diluted sample is 50 Lf/mL. However, if the first tube to flocculate is tube A or tube F this does not indicate equivalence at that level. It would be necessary to perform a repeat test using either a different dilution of test sample or selecting a different range of doses of reference antitoxin.

More precision can be obtained by making allowance for the sequence of flocculation after the first tube. Thus, in the example quoted, if the second tube to flocculate had been tube D, the final value for the diluted sample would be 52, whereas if the second tube to flocculate was tube B, the final value would be 48. The test may be performed in duplicate with slightly different dilutions of the test sample.

If there is no indication of the expected Lf value of the sample available, it is advisable to obtain a rough estimate by use of a wider range of antitoxin content in the tubes before proceeding to the final test.

Example

Tube	A	B	C	D	E	F
Antitoxin content (Lf-eq.)	20	30	45	70	100	150

The level of toxin or toxoid and antitoxin concentration in the test may be varied, but this will markedly affect the flocculation time, so that at very low levels the test will take too long, whilst at a high concentration the onset of flocculation may be so rapid as to make it difficult to distinguish the first and second tubes to flocculate.

Assay of low concentrations by blend flocculation

For very low concentrations, it is preferable to measure toxin or toxoid by the method of blend flocculation. This involves comparison of the Lf value of a known toxin or toxoid and that of a mixture of the sample with that toxin or toxoid.

When a toxin or toxoid with a known Lf value and a toxin or toxoid with an unknown Lf value are flocculated together, the mixture will flocculate as the sum of their values if they are homogeneous. If non-homogenous toxins or toxoids are mixed they will produce an aberrant pattern with 2 flocculation maxima.

9. Residual Pertussis Toxin

(Ph. Eur. method 2.6.33)

The test for residual pertussis toxin is performed *in vitro*, using a Chinese Hamster Ovary (CHO) cell-based assay, and is intended for the assay of non-adsorbed purified pertussis components.

Pertussis toxin BRP is suitable as a reference pertussis toxin preparation.

The CHO cell-clustering assay (CHO assay) is based on the induction of clusters in non-confluent CHO cell cultures by active pertussis toxin. The cultures are then examined under a microscope and any clusters present are counted. The assay may be used as a quantitative test or as a limit test with the sensitivity determined within each assay using dilutions of a reference pertussis toxin preparation.

The sensitivity of the CHO assay to pertussis toxin is verified with *pertussis toxin BRP* or a suitable reference preparation calibrated in International Units using a suitable CHO assay. The assay sensitivity is defined as the lowest concentration in the reference preparation dilution series to produce a positive response, i.e. at least 10 CHO cell clusters. A suitable assay has a sensitivity of at least 5 mIU/mL.

The following method is given as an example.

Reagents and equipment

— *Pertussis toxin BRP.*
— *CHO-K1 cell line* (ATCC No. CCL-61 or ECACC No. 85051005).
— *Kaighn's modified Ham's F-12K medium.*

Media that have a slightly different composition from that in Table 2.6.33.-1 may also be used. Proline is an essential component of the medium. Adjust to pH 7.2 ± 0.2 if necessary.

Table 2.6.33.-1. – *Kaighn's modified Ham's F-12K medium*

Amino acids	
L-Alanine	18.0 mg
L-Arginine hydrochloride	0.4220 g
L-Asparagine monohydrate	30.0 mg
L-Aspartic acid	26.6 mg
L-Cysteine hydrochloride monohydrate	70.0 mg
L-Glutamic acid	29.0 mg
L-Glutamine	0.2920 g
Glycine	15.0 mg
L-Histidine hydrochloride monohydrate	45.8 mg
L-Isoleucine	7.88 mg
L-Leucine	26.2 mg
L-Lysine hydrochloride	73.0 mg

Amino acids	
L-Methionine	8.96 mg
L-Phenylalanine	9.92 mg
L-Proline	69.0 mg
L-Serine	21.0 mg
L-Threonine	23.0 mg
L-Tryptophan	4.1 mg
L-Tyrosine disodium salt dihydrate	13.5 mg
L-Valine	23.0 mg
Vitamins	
Biotin	70 µg
D-Calcium pantothenate	0.5 mg
Choline chloride	14.0 mg
Folic acid	1.3 mg
myo-Inositol	18.0 mg
Nicotinamide	37 µg
Pyridoxine hydrochloride	60 µg
Riboflavin	40 µg
Thiamine hydrochloride	0.3 mg
Vitamin B12	1.4 mg
Inorganic salts	
Calcium chloride, anhydrous	0.1020 g
Cupric sulfate pentahydrate	2 µg
Disodium hydrogen phosphate, anhydrous	0.1155 g
Ferric sulfate heptahydrate	0.8 mg
Magnesium chloride, anhydrous	49.7 mg
Magnesium sulfate, anhydrous	0.1920 g
Potassium chloride	0.2850 g
Sodium bicarbonate	2.5000 g
Sodium chloride	7.5300 g
Zinc sulfate heptahydrate	0.144 mg
Other components	
D-Glucose	1.2600 g
Hypoxanthine sodium salt	4.0 mg
Lipoic acid	0.21 mg
Phenol red	3.0 mg
Putrescine dihydrochloride	0.32 mg
Sodium pyruvate	0.2200 g
Thymidine	0.7 mg
Water	to 1000 mL

— *CHO cell culture medium.* Supplement Kaighn's modified Ham's F-12K medium with foetal bovine serum to obtain a final concentration of 10 per cent *V/V*. Add a sufficient quantity of 0.2 M L-glutamine solution to obtain a final concentration of 1 per cent *V/V*. An antibiotic/antimycotic solution may be added if required. Adjust to pH 7.2 ± 0.2 if necessary. Store at 5 ± 3 °C for a maximum of 3 weeks, protected from light.

— *Trypsin-EDTA solution* (trypsin 0.25 per cent).

— *Phosphate-buffered saline pH 7.4 (PBS)*, without calcium or magnesium. Dissolve 9.0 g of *sodium chloride R*, 0.144 g of *potassium dihydrogen phosphate R* and 0.795 g of *disodium hydrogen phosphate heptahydrate R* in *water R*, and dilute to 1000.0 mL with the same solvent. Adjust the pH if necessary.

— *24-well flat-bottomed assay plates with lid.*

— *75 cm^2 tissue culture flasks.*

— *Low protein binding polypropylene microtubes.*

CHO cell culture

The cells are obtained from a cell bank and used between defined passage levels. CHO cells are cultured in tissue culture flasks containing 20 mL of CHO cell culture medium in a humidified incubator set at 37 °C and 5 per cent CO_2. The cells are passaged at a ratio of 1:5 to 1:20 as they approach confluence.

Before use, allow all reagents to reach room temperature or warm to 37 °C.

To passage the cells, remove the CHO cell culture medium. Wash the cell layer gently by rinsing briefly 2-3 times with PBS. Remove the PBS and add 2 mL of trypsin-EDTA solution to the flask, allowing it to cover the monolayer for 20 s. Remove the trypsin-EDTA solution by aspiration. Immediately observe the culture using phase-contrast microscopy. When the cells begin to contract, tap the side of the culture flask firmly to dislodge the cells. When the majority of cells are free-floating, add 10 mL of CHO cell culture medium to stop the trypsin digestion. Trypsinisation used for cell detachment must be carefully controlled to avoid over-digestion which causes cleavage of either pertussis toxin receptors or cell adherence proteins, or both.

Determine the cell concentration and viability using trypan blue exclusion or another appropriate method. The cell viability must be greater than 95 per cent to continue. Transfer a sufficient volume of the cell suspension to a fresh flask for a 1:5 to 1:20 passage ratio and add CHO cell culture medium to bring the volume to 20 mL. Incubate the cells in a humidified incubator set at 37 °C and 5 per cent CO_2 for at least 48 h prior to use in the CHO assay.

Seeding density for CHO assay

Prepare a suspension of CHO cells using the trypsinisation procedure described above. Count and dilute the cell suspension to between 4×10^4 and 8×10^4 CHO cells/mL in CHO cell culture medium. The cell concentration selected must allow clusters to be identified at the end of the stimulation period. Transfer 250 µL of the cell suspension into each well of the 24-well plates. Store the seeded plates in a humidified incubator set at 37 °C and 5 per cent CO_2 while the reference preparation and test sample are prepared.

Preparation of pertussis toxin reference

Reconstitute *pertussis toxin BRP* as stated in the leaflet accompanying the reference preparation. Prepare 7 two-fold serial dilutions in CHO cell culture medium, in such a way that the assay end-point occurs in the middle of the dilution series. The dilutions are prepared in low protein binding polypropylene microtubes. One dilution series for the reference preparation is included in each assay plate.

Preparation of non-adsorbed purified pertussis component samples

Dilute the test sample in CHO cell culture medium to obtain the highest concentration of the test sample which does not significantly dilute the nutrients of the medium, in order to maximise the sensitivity of the assay. Prepare a dilution series as described for the reference preparation.

Stimulation of CHO cells

Transfer 250 µL from each tube of the reference preparation dilution series into the assigned wells of the assay plates seeded with CHO cells. Similarly, transfer 250 µL of each dilution of the test sample into the assigned well(s). Transfer 250 µL of CHO cell culture medium into the negative control wells. Return the assay plates to a humidified incubator set at 37 °C and 5 per cent CO_2 for 48 h.

Scoring and interpretation of results

Observe the cell cultures using phase contrast microscopy at a magnification of 4× or 10×. In all wells the cell cultures must not be confluent and must have sufficient growth space to allow any clusters present to be counted. Assign a positive score when 10 or more CHO cell cluster formations are clearly evident within a single well. Assign a negative score to wells containing fewer than 10 clusters. The end-point concentration is the lowest concentration at which there is a positive score.

Assay validity

The test is not valid unless:

— the negative control wells show no evidence of clustering.
— wells containing the reference toxin preparation at a concentration greater than or equal to 5 mIU/mL exhibit a positive response (i.e. 10 or more CHO cell clusters).
— a clear end-point dilution is observed for the reference preparation.
— replicate values for the end-point of the reference preparation do not differ by more than one 2-fold dilution within one assay.

Calculation

Calculate the residual pertussis toxin activity in the test sample in International Units per millilitre, relative to the reference preparation, using the following expression:

$$\frac{GM(end-point)_S}{GM(end-point)_R} \times A_R$$

$GM(end\text{-}point)_S$ = geometric mean of the reciprocal of the end-point dilutions (last dilution at which there is a positive score) for the test sample;

$GM(end\text{-}point)_R$ = geometric mean of the reciprocal of the end-point dilutions (last dilution at which there is a positive score) for the reference preparation;

A_R = activity of the reference preparation (stock solution), in International Units per millilitre.

Example

Series of 7 two-fold dilutions were prepared for the reference preparation and test sample. In this example, the reference stock solution has an activity of 1000 IU/mL. The scores observed at each dilution of the reference preparation and test sample, the reciprocal end-point dilutions and the geometric means of reciprocal end-point dilutions are shown in Tables 2.6.33.-2 and 2.6.33.-3.

Table 2.6.33.-2 – *Reference preparation dilutions and scores*

Reference preparation dilution	Score	
	Rep. 1	Rep. 2
1:100 000	+	+
1:200 000	+	+
1:400 000	+	+
1:800 000	+	-
1:1 600 000	-	-
1:3 200 000	-	-
1:6 400 000	-	-
End-point dilutions	1:800 000	1:400 000
Reciprocal of end-point dilutions	800 000	400 000
$GM(end\text{-}point)_R$	565 685	

Table 2.6.33.-3 – *Test sample dilutions and scores*

Test sample dilution	Score	
	Rep. 1	Rep. 2
1:100	+	+
1:200	+	+
1:400	+	+
1:800	+	+
1:1600	+	-
1:3200	-	-
1:6400	-	-
End-point dilutions	1:1600	1:800
Reciprocal of end-point dilutions	1600	800
$GM(end\text{-}point)_S$	1131	

The residual pertussis toxin activity in the test sample can be determined as follows: (1131/565685) × 1000 = 2 IU/mL.

L. Nucleic Acid Amplification Techniques

(Ph. Eur. method 2.6.21)

1. INTRODUCTION

Nucleic acid amplification techniques are based on 2 different approaches:

1. amplification of a target nucleic acid sequence using, for example, polymerase chain reaction (PCR), ligase chain reaction (LCR), or isothermal ribonucleic acid (RNA) amplification;

2. amplification of a hybridisation signal using, for example, for deoxyribonucleic acid (DNA), the branched DNA (bDNA) method; in this case signal amplification is achieved without subjecting the nucleic acid to repetitive cycles of amplification.

In this general chapter, the PCR method is described as the reference technique. Alternative methods may be used, if they comply with the quality requirements described below.

2. SCOPE

This section establishes the requirements for sample preparation, *in vitro* amplification of DNA sequences and detection of the specific PCR product. With the aid of PCR, defined DNA sequences can be detected. RNA sequences can also be detected following reverse transcription of the RNA to complementary DNA (cDNA) and subsequent amplification.

3. PRINCIPLE OF THE METHOD

PCR is a procedure that allows specific *in vitro* amplification of segments of DNA or of RNA after reverse transcription into cDNA.

Following denaturation of double-stranded DNA into single-stranded DNA, 2 synthetic oligonucleotide primers of opposite polarity anneal to their respective complementary sequences in the DNA to be amplified. The short double-stranded regions that form as a result of specific base pairing between the primers and the complementary DNA sequence border the DNA segment to be amplified, and serve as starting positions for *in vitro* DNA synthesis by means of a heat-stable DNA polymerase.

Amplification of the DNA occurs in cycles consisting of:
- heat denaturation of the nucleic acid (target sequence) into 2 single strands;
- specific annealing of the primers to the target sequence under suitable reaction conditions;
- extension of the primers, which are bound to both single strands, by DNA polymerase at a suitable temperature (DNA synthesis).

Repeated cycles of heat denaturation, primer annealing and DNA synthesis results in an exponential amplification of the DNA segment limited by the primers.

The specific PCR product known as an amplicon can be detected by a variety of methods of appropriate specificity and sensitivity.

Multiplex PCR assays use several primer pairs designed for simultaneous amplification of different targets in one reaction.

4. TEST MATERIAL

Because of the high sensitivity of PCR, the samples must be protected against external contamination with target sequences. Sampling, storage and transport of the test material are performed under conditions that minimise degradation of the target sequence. In the case of RNA target sequences, special precautions are necessary since RNA is highly sensitive to degradation by ribonucleases. Care must be taken since some added reagents, such as anticoagulants or preservatives, may interfere with the test procedure.

5. TEST METHOD

5.1. Prevention of contamination

The risk of contamination requires a strict segregation of the areas depending on the material handled and the technology used. Points to consider include movement of personnel, gowning, material flow and air supply and decontamination procedures.

The system should be sub-divided into compartments such as:
- master-mix area (area where exclusively template-free material is handled, e.g. primers, buffers, etc.);
- pre-PCR (area where reagents, samples and controls are handled);
- PCR amplification (amplified material is handled in a closed system);
- post-PCR detection (the only area where the amplified material is handled in an open system).

If closed systems are used, the strict segregation of areas is not required.

5.2. Sample preparation

When preparing samples, the target sequence to be amplified needs to be efficiently extracted or liberated from the test material in a reproducible manner and in such a way that amplification under the selected reaction conditions is possible. A variety of physico-chemical extraction procedures and/or enrichment procedures may be employed.

Additives present in test material may interfere with PCR. The procedures described under 7.3.2. must be used as a control for the presence of inhibitors originating from the test material.

In the case of RNA-templates, care must be taken to avoid ribonuclease activity.

5.3. Amplification

PCR amplification of the target sequence is conducted under defined cycling conditions (temperature profile for denaturation of double-stranded DNA, annealing and extension of primers; incubation times at selected temperatures; ramp rates). These depend on various parameters such as:
- the length and base composition of primer and target sequences;
- the type of DNA polymerase, buffer composition and reaction volume used for the amplification;
- the type of thermocycler used and the thermal conductivity rate between the apparatus, reaction tube and reaction fluid.

5.4. Detection

The amplicon generated by PCR may be identified by size, sequence, chemical modification or a combination of these parameters. Detection and characterisation by size may be achieved by gel electrophoresis (using agarose or polyacrylamide slab gels or capillary electrophoresis) or column chromatography (for example, liquid chromatography). Detection and characterisation by sequence composition may be achieved by the specific hybridisation of probes having a sequence complementary to the target sequence or by cleavage of the amplified material reflecting target-specific restriction-enzyme sites. Detection and characterisation by chemical modification may be achieved by, for example, incorporation of a fluorophore into the amplicons and subsequent detection of fluorescence following excitation.

Detection of amplicons may also be achieved by using probes labelled to permit a subsequent chemiluminescent, radioisotopic or immuno-enzyme-coupled detection.

6. EVALUATION AND INTERPRETATION OF RESULTS

A valid result is obtained within a test only if the positive control(s) is unambiguously positive and the negative control(s) is unambiguously negative. Due to the very high sensitivity of the PCR method and the inherent risk of contamination, it is necessary to confirm positive results by repeating the complete test procedure in duplicate, where possible on a new aliquot of the sample. The sample is considered positive if at least one of the repeat tests gives a positive result. As soon as a measurable target threshold is defined, a quantitative test system is required.

7. QUALITY ASSURANCE

7.1. Validation of the PCR assay system

The validation programme must include validation of instrumentation and the PCR method employed. Reference should be made to the ICH guideline Q2(R1) Validation of Analytical Procedures: Text and Methodology.

Appropriate official working reference preparations or in-house reference preparations calibrated against International Standards for the target sequences are used for validation of a PCR test, when available.

7.1.1. Determination of the positive cut-off point

During validation of qualitative tests, the positive cut-off point must be determined. The positive cut-off point is defined as the minimum number of target sequences per volume sample that can be detected in 95 per cent of test runs. The positive cut-off point depends on interrelated factors such as the volume of the sample extracted and the efficacy of the extraction methodology, the transcription of the target RNA into cDNA, the amplification process and the detection.

To define the detection limit of the assay system, reference must be made to the positive cut-off point for each target sequence and the test performance above and below the positive cut-off point.

7.1.2. Quantitative assay systems

For a quantitative assay, the following parameters are determined during validation: accuracy, precision, specificity, quantitation limit, linearity, range and robustness.

7.2. Quality control of reagents

All reagents crucial for the methodology used have to be controlled prior to use in routine applications. Their acceptance/withdrawal is based on pre-defined quality criteria.

Primers are a crucial component of the PCR assay and as such their design, their purity and the validation of their use in a PCR assay require careful attention. Primers may be modified (for example, by conjugation with a fluorophore or antigen) in order to permit a specific method of detection of the amplicon, provided such modifications do not inhibit accurate and efficient amplification of the target sequence.

7.3. Run controls

7.3.1. External controls

In order to minimise the risk of contamination and to ensure adequate sensitivity, the following external controls are included in each PCR assay:

— positive control: this contains a defined number of target-sequence copies, the number being close to the positive cut-off value, and determined individually for each assay system and indicated as a multiple of the positive cut-off value of the assay system;

— negative control: a sample of a suitable matrix already proven to be free of the target sequences.

7.3.2. Internal control

Internal controls are defined nucleic acid sequences containing, unless otherwise prescribed, the primer binding sites. Internal controls must be amplified with defined efficacy, and the amplicons must be clearly discernible. Internal controls must be of the same type of nucleic acid (DNA/RNA) as the material to be tested. The internal control is preferably added to the test material before isolating the nucleic acid and therefore acts as an overall control (extraction, reverse transcription, amplification, detection).

7.3.3. Threshold control

The threshold control for quantitative assays is a test sample with the analyte at a concentration that is defined as the threshold not to be exceeded. It contains the analyte suitably calibrated in International Units and is analysed in parallel in each run of a quantitative assay.

7.4. External quality assessment

Participation in external quality assessment programmes is an important PCR quality assurance procedure for each laboratory and each operator.

The following sections are published for information.

VALIDATION OF NUCLEIC ACID AMPLIFICATION TECHNIQUES (NAT) FOR THE DETECTION OF HEPATITIS C VIRUS (HCV) RNA IN PLASMA POOLS: GUIDELINES

1. SCOPE

The majority of nucleic acid amplification analytical procedures are qualitative (quantal) tests for the presence of nucleic acid with some quantitative tests (either in-house or commercial) being available. For the detection of HCV RNA contamination of plasma pools, qualitative tests are adequate and may be considered to be a limit test for the control of impurities as described in the *Pharmeuropa* Technical Guide for the elaboration of monographs, December 1999, Chapter III 'Validation of analytical procedures'. These guidelines describe methods to validate only qualitative nucleic acid amplification analytical procedures for assessing HCV RNA contamination of plasma pools. Therefore, the 2 characteristics regarded as the most important for validation of the analytical procedure are the specificity and the detection limit. In addition, the robustness of the analytical procedure should be evaluated.

However, this document may also be used as a basis for the validation of nucleic acid amplification in general.

For the purpose of this document, an analytical procedure is defined as the complete procedure from extraction of nucleic acid to detection of the amplified products.

Where commercial kits are used for part or all of the analytical procedure, documented validation points already covered by the kit manufacturer can substitute for the validation by the user. Nevertheless, the performance of the kit with respect to its intended use has to be demonstrated by the user (e.g. detection limit, robustness, cross-contamination).

2. SPECIFICITY

Specificity is the ability to assess unequivocally nucleic acid in the presence of components that may be expected to be present.

The specificity of nucleic acid amplification analytical procedures is dependent on the choice of primers, the choice of probe (for analysis of the final product) and the stringency of the test conditions (for both the amplification and the detection steps).

When designing primers and probes, the specificity of the primers and probes to detect only HCV RNA should be investigated by comparing the chosen sequences with sequences in published data banks. For HCV, primers (and probes) will normally be chosen from areas of the 5' non-coding region of the HCV genome which are highly conserved for all genotypes.

The amplified product should be unequivocally identified by using one of a number of methods such as amplification with nested primers, restriction enzyme analysis, sequencing, or hybridisation with a specific probe.

In order to validate the specificity of the analytical procedure, at least 100 HCV RNA-negative plasma pools should be tested and shown to be non-reactive. Suitable samples of non-reactive pools are available from the European Directorate for the Quality of Medicines & HealthCare (EDQM).

The ability of the analytical procedure to detect all HCV genotypes will again depend on the choice of primers, probes and method parameters. This ability should be demonstrated using characterised reference panels. However, in view of the difficulty in obtaining samples of some genotypes (e.g. genotype 6), the most prevalent genotypes (e.g. genotypes 1 and 3 in Europe) should be detected at a suitable level.

3. DETECTION LIMIT

The detection limit of an individual analytical procedure is the lowest amount of nucleic acid in a sample that can be detected but not necessarily quantitated as an exact value.

The nucleic acid amplification analytical procedure used for the detection of HCV RNA in plasma pools usually yields qualitative results. The number of possible results is limited to 2: either positive or negative. Although the determination of the detection limit is recommended, for practical purposes, a positive cut-off point should be determined for the nucleic acid amplification analytical procedure. The positive cut-off point (as defined in the general chapter 2.6.21) is the minimum number of target sequences per volume sample that can be detected in 95 per cent of test runs. This positive cut-off point is influenced by the distribution of viral genomes in the individual samples being tested and by factors such as enzyme efficiency, and can result in different 95 per cent cut-off values for individual analytical test runs.

In order to determine the positive cut-off point, a dilution series of a working reagent or of the *hepatitis C virus BRP*, which has been calibrated against the WHO HCV International Standard, should be tested on different days to examine variation between test runs. At least 3 independent dilution series should be tested with a sufficient number of replicates at each dilution to give a total number of 24 test results for each dilution, to enable a statistical analysis of the results.

For example, a laboratory could test 3 dilution series on different days with 8 replicates for each dilution, 4 dilution series on different days with 6 replicates for each dilution, or 6 dilution series on different days with 4 replicates for each dilution. In order to keep the number of dilutions at a manageable level, a preliminary test (using, for example, $\log_{10}$ dilutions of the plasma pool sample) should be carried out in order to obtain a preliminary value for the positive cut-off point (i.e. the highest dilution giving a positive signal). The range of dilutions can then be chosen around the predetermined preliminary cut-off point (using, for example, a dilution factor of 0.5 $\log_{10}$ or less and a negative plasma pool for the dilution matrix). The concentration of HCV RNA that can be detected in 95 per cent of test runs can then be calculated using an appropriate statistical evaluation.

These results may also serve to demonstrate the intra-assay variation and the day-to-day variation of the analytical procedure.

4. ROBUSTNESS

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small but deliberate variations in method parameters and provides an indication of its reliability during normal usage.

The evaluation of robustness should be considered during the development phase. It should show the reliability of the analytical procedure with respect to deliberate variations in method parameters. For NAT, small variations in the method parameters can be crucial. However, the robustness of the method can be demonstrated during its development when small variations in the concentrations of reagents (e.g. $MgCl_2$, primers or dNTP) are tested. To demonstrate robustness, at least 20 HCV RNA negative plasma pools (selected at random) spiked with HCV RNA to a final concentration of 3 times the previously determined 95 per cent cut-off value should be tested and found positive.

Problems with robustness may also arise with methods that use an initial ultracentrifugation step prior to extraction of the viral RNA. Therefore, to test the robustness of such methods, at least 20 plasma pools containing varying levels of HCV RNA, but lacking HCV-specific antibodies, should be tested and found positive.

Cross-contamination prevention should be demonstrated by the accurate detection of a panel of at least 20 samples consisting of alternate samples of negative plasma pools and negative plasma pools spiked with high concentrations of HCV (at least 10^2 times the 95 per cent cut-off value or at least 10^4 IU/mL).

5. QUALITY ASSURANCE

For biological tests such as NAT, specific problems may arise that influence both the validation and the interpretation of results. The test procedures must be described precisely in the form of standard operating procedures (SOPs). These should cover:

- the mode of sampling (type of container, etc.);
- the preparation of mini-pools (where appropriate);
- the conditions of storage before analysis;
- the exact description of the test conditions, including precautions taken to prevent cross-contamination or destruction of the viral RNA, reagents and reference preparations used;
- the exact description of the apparatus used;
- the detailed formulae for calculation of results, including statistical evaluation.

The use of a suitable run control (for example, an appropriate dilution of *hepatitis C virus BRP* or plasma spiked with an HCV sample calibrated against the WHO HCV International Standard) can be considered a satisfactory system-suitability check and ensures that the reliability of the analytical procedure is maintained whenever used.

Technical qualification An appropriate installation and operation qualification programme should be implemented for each critical piece of the equipment used.

For confirmation of analytical procedure performance after a change of critical equipment (e.g. thermocyclers), the change should be documented by conducting a parallel test on 8 samples of a plasma pool that is spiked with HCV RNA to a final concentration of 3 times the previously determined 95 per cent cut-off value. All results should be positive.

Operator qualification An appropriate qualification programme should be implemented for each operator involved in the testing.

VALIDATION OF NUCLEIC ACID AMPLIFICATION TECHNIQUES (NAT) FOR THE QUANTIFICATION OF B19 VIRUS (B19V) DNA IN PLASMA POOLS: GUIDELINES

1. SCOPE

The European Pharmacopoeia requires that plasma pools used for manufacture of certain products are tested for the presence of B19 virus (B19V) DNA with a threshold concentration that must not be exceeded. In order to comply with these requirements, quantitative NAT tests are preferred. The characteristics regarded as the most important for validation of the quantitative NAT procedure are accuracy, precision, specificity, quantitation limit, linearity and range. In addition, the robustness of the analytical procedure should be evaluated.

This guideline describes methods to validate NAT analytical procedures for assessing B19V DNA contamination of plasma pools based on the ICH guidelines. However, this document may also be used as a basis for the validation of quantitative NAT in general.

For the purpose of this document, an analytical procedure is defined as the complete procedure from extraction of nucleic acid to detection of the amplified products.

Where commercial kits are used for part or all of the analytical procedure, documented validation points already covered by the kit manufacturer can substitute for the validation by the user. Nevertheless, the performance of the kit with respect to its intended use has to be demonstrated by the user (e.g. precision, accuracy, range, robustness).

2. ACCURACY

Accuracy expresses the closeness of agreement between the value that is accepted as either a conventional true value or an accepted reference value and the value found.
The accuracy of an assay is dependent on the calibration of the assay and on the variance of the different assay steps. Though it is recommended to establish the accuracy across the specified range of the analytical procedure, the most important assessment of accuracy is in the range of the threshold concentration. In the case of B19V NAT assays for investigation of plasma pools it is recommended to assess the accuracy of the calibrated assay by assaying at least 5 concentrations (dilution factor of 0.5 $\log_{10}$) of *B19 virus DNA for NAT testing BRP* or another material, suitably calibrated in International Units against the actual WHO B19 DNA International Standard, covering the range of the currently recommended threshold concentration of 10.0 IU/µL B19V DNA (e.g. 10^5 IU/mL, $10^{4.5}$ IU/mL, 10^4 IU/mL, $10^{3.5}$ IU/mL and 10^3 IU/mL), with at least 3 replicates for each dilution. Accuracy should be reported for the different concentrations in terms of percentage determined compared with the known amount of B19V DNA. It should reflect the level of technology of the respective assays, which should also be defined, for example in collaborative studies.

3. PRECISION

Precision expresses the closeness of agreement between a series of measurements, obtained from multiple sampling of the same homogenous sample. The precision is defined at 3 levels:

- repeatability expresses the precision under the same operating conditions over a short interval of time (intra-assay precision); it is assessed by using 1 assay and testing 3 replicates of appropriate dilutions of a B19V DNA-positive sample suitably calibrated in International Units and covering the whole quantitative range of the assay; the coefficient of variation for the individual samples is calculated (intra-assay variability);
- intermediate precision expresses the intra-laboratory variations (inter-assay precision); it is established by assaying replicates (as routinely used for the assay) of appropriate dilutions of a B19V DNA-positive sample suitably calibrated in International Units covering the whole quantitative range of the assay under different circumstances (e.g. different days, different analysts, different equipment, different reagents); the coefficient of variation for the individual samples is calculated;
- reproducibility expresses the precision between different laboratories (inter-laboratory precision); it is assessed by participation in quantitative collaborative studies on B19V DNA-NAT assays, e.g. under the Proficiency Testing Scheme (PTS), including the comparative analysis of the obtained quantitative results, where appropriate.

4. SPECIFICITY

Specificity expresses the ability to assess unequivocally nucleic acid in the presence of components that may be expected to be present. The specificity of NAT analytical procedures is dependent on the choice of primers, the choice of probe (for analysis of the final product) and the stringency of the test conditions (for both the amplification and the detection steps).

When designing primers and probes, the specificity of the primers and probes to detect only human B19V DNA should be investigated by comparing the chosen sequences with sequences in published data banks. There should be no major homology found with sequences unrelated to B19V.

The amplified product should be unequivocally identified by using one of a number of methods such as amplification with nested primers, restriction enzyme analysis, sequencing, or hybridisation with a specific probe.

In order to examine the specificity of the analytical procedure, at least 20 B19V DNA-negative plasma pools should be tested and shown to be non-reactive.

Parvovirus B19 genotypes The International Committee on Taxonomy of Viruses (ICTV) has classified representatives of the 3 genotypes as strains of human parvovirus B19. Genotype 1 represents prototype B19V, genotype 2 represents viral sequences like A6, and genotype 3 represents V9-like sequences. By performing sequence alignment with respective B19V genotype sequences available from nucleic acid sequence databases, primers and probes should be designed to detect and quantify consistently the different human parvovirus B19 genotypes. Reference materials should be used to check the approach chosen. Since biological reference preparations reflecting some genotypes might be difficult to obtain, respective plasmid preparations or synthetic nucleic acids may also serve as a characterised target sequence source. However, those cannot be used to validate the extraction procedure.

5. QUANTITATION LIMIT

The quantitation limit is the lowest amount of nucleic acid in a sample that can be determined quantitatively with suitable precision and accuracy. The quantitation limit of the B19V NAT assay is assessed during the repeatability and intermediate-precision studies by limiting dilution analysis. The lowest concentration of target nucleic acids that is quantitated with suitable precision and accuracy is defined.

6. LINEARITY

The linearity of an assay is its ability to obtain test results that are directly proportional to the concentration of the nucleic acid. The linearity of the B19V NAT assay is assessed during the repeatability and intermediate-precision studies by testing replicates of diluted samples with the concentrations covering the whole quantitative range. The interval between the upper and the lower concentration of the target nucleic acid where test results are directly proportional to the concentrations is defined.

7. RANGE

The range of an assay is the interval between the upper and the lower concentration of nucleic acid in the sample for which it has been demonstrated that the procedure has a suitable level of precision, accuracy and linearity. The range of the B19V NAT assay is assessed during the repeatability and intermediate-precision studies by testing replicates of diluted samples. The interval between the upper and the lower concentration that can be expressed with an acceptable degree of accuracy and precision is defined.

8. ROBUSTNESS

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small but deliberate variations in method parameters and provides an indication of its reliability during normal usage. The evaluation of robustness should be considered during the development phase. It should show the reliability of the analytical procedure with respect to deliberate variations in method parameters. For NAT, small variations in the method parameters can be crucial. Nonetheless, the robustness of NAT can be demonstrated during the development of the method when small variations in the concentrations of reagents, for example $MgCl_2$, primers or dNTP, are tested. To demonstrate robustness, at least 20 B19V DNA-negative plasma pool samples spiked with B19V DNA at the threshold concentration should be tested and found to have acceptable quantitative values.

Cross-contamination prevention should be demonstrated by the accurate detection of a panel of at least 20 samples consisting of alternate samples of plasma pools without B19V DNA or with levels below the threshold concentration (10 samples) and plasma pools spiked with high concentrations of B19V DNA (at least 10^2 times the threshold level, 10 samples).

9. QUALITY ASSURANCE

For biological tests such as NAT, specific problems may arise that may influence both the validation and the interpretation of results. The test procedures must be described precisely in the form of standard operating procedures (SOPs). These should cover:

- the mode of sampling (type of container, etc.);
- the preparation of mini-pools by manufacturers (where appropriate);
- the conditions of storage before analysis;
- the exact description of the test conditions including precautions taken to prevent cross-contamination or destruction of the viral nucleic acids, reagents and reference preparations used;
- the exact description of the apparatus used;
- the detailed formulae for calculation of results, including statistical evaluation.

The inclusion of an appropriate threshold control (for example, plasma spiked with a B19V DNA sample suitably calibrated in International Units, such as *B19 virus DNA for NAT testing BRP*) is considered to be a satisfactory system-suitability check and ensures that the reliability of the analytical procedure is maintained whenever used.

Technical qualification An appropriate installation and operation qualification programme should be implemented for each critical piece of the equipment used.
For confirmation of analytical procedure performance after a change of critical equipment (e.g. thermocyclers), the change should be documented by conducting a parallel test on 8 samples of a plasma pool that is spiked with a concentration of B19V DNA around the threshold concentration. All results should be acceptable and reflect the features of the assay as determined during the validation phase.

Operator qualification An appropriate qualification programme should be implemented for each operator involved in the testing.

M. Assay of Interferons

(Ph. Eur. general texts 5.6)

The following chapter is published for information.

1. INTRODUCTION

Monographs on human interferons generally contain a bioassay based on the inhibitory activity of the interferon on the cytopathic action of a virus on a cell line in culture. In most cases, however, the virus, cell line and the assay details are not specified, in order to allow the appropriate flexibility, where the monograph covers more than one sub-class of interferon.

The present text is intended to provide outline information for the analyst on how to design, optimise and validate such an assay once an appropriate combination of cell line and cytopathic virus has been identified. A detailed procedure for a particular cytopathic antiviral assay is described as an example of a suitable method, together with information on other virus-cell line combinations and guidance on how to adapt and validate the procedure for these other combinations.

2. ANTIVIRAL (CYTOPATHIC EFFECT REDUCTION) ASSAYS

The antiviral assay of human interferons is based on the induction of a cellular response in human cells, which prevents or reduces the cytopathic effect of an infectious virus. The potency of interferon is estimated by comparing its protective effect against a viral cytopathic effect with the same effect of the appropriate reference preparation calibrated in International Units.

3. INTERFERON ASSAY USING HEP2C CELLS AND INFECTIOUS ENCEPHALOMYOCARDITIS VIRUS

The antiviral assay of human interferons described is of the cytopathic effect reduction type. It uses human Hep2c cells infected by encephalomyocarditis virus (EMCV) to measure the potencies of different human interferon test preparations. This assay has been used in three World Health Organization (WHO) international collaborative studies of candidate International Standards for human interferon alpha, human interferon beta and human interferon gamma and has repeatedly been demonstrated to be sensitive, reliable and reproducible for potency estimations of the different types of human interferon.

For the culture of mammalian cells, all procedures are carried out using standard operating procedures for the

maintenance of such cell lines in culture. Volumes of reagents are indicated for cell cultures carried out in 75 cm^2 flasks. Other types of containers (flasks or plates) may be used but volumes must be adapted accordingly.

3.1. MAINTENANCE AND PREPARATION OF HEP2C CELLS

Hep2c cells are maintained and passaged in culture medium A.

Cells are stored as frozen stocks using standard operating procedures. Growing cells may be maintained in culture up to a permitted passage number of 30, after which new cultures are established from frozen stocks.

At the beginning of the assay procedure, harvest the cells from the flasks showing 90 per cent confluent monolayers using the trypsin-treatment procedure described below.

— Remove the culture medium from the flasks.
— To each flask, add 5 mL of trypsin solution heated at 37 °C (a trypsin stock solution contains 4 mg/mL of *trypsin R* and 4 mg/mL of *sodium edetate R*; immediately before use, dilute 50 times with phosphate buffered saline). Swirl the capped flask to wash the cell monolayer. Remove the excess of trypsin solution.
— Incubate the flasks for 5 min to 10 min at 37 °C. Microscopically or visually observe the cells for signs of detachment. When viewed microscopically, the cells appear rounded up or detached and free-floating. Shake the flask vigorously to detach all the cells, add approximately 5 mL of culture medium A. Shake vigorously to yield a suspension of single cells.
— To prepare the cell suspensions for the assay procedure, carefully disperse the cells by pipetting up and down to disrupt cell aggregates, count the cells and resuspend at a concentration of 6×10^5 cells/mL.

3.2. PROPAGATION OF ENCEPHALOMYOCARDITIS VIRUS

Encephalomyocarditis virus is propagated in mouse L-929 cells in order to produce a stock of progeny virus. L-929 cells are maintained by trypsin treatment and passage as described for Hep2c cells (*NOTE: it may be necessary to substitute neonatal calf serum with foetal bovine serum if the cells show poor growth*).

Take several flasks containing confluent cultures of L-929 cells. Pour off the medium from the flasks. Inoculate with 2 mL of the EMCV suspension appropriately diluted in culture medium B so that it contains approximately 2.5×10^8 plaque forming units (PFU) per millilitre. Each flask will contain $4\text{-}6 \times 10^7$ L-929 cells and therefore the multiplicity of infection (m.o.i.) will be approximately 10 PFU/cell. Carefully swirl the virus suspension over the entire cell monolayer and return the flasks to the incubator for approximately 1 h. Maintain the medium at pH 7.4 to 7.8.

After adsorption of the EMCV, add approximately 40 mL of culture medium B to each flask and return the flasks to the incubator at 37 °C for about 30 h. Maintain the medium at pH 7.4 to 7.8 to obtain a maximum virus yield. Remove the culture fluid and store at approximately 4 °C.

Place the flasks at −20 °C to freeze the cell monolayer. Then thaw to room temperature. Add approximately 5 mL of culture medium and shake the flask to disrupt the cell walls. Transfer the contents of each flask to the container of culture fluid. Transfer the culture fluid containing the EMCV to 50 mL plastic centrifuge tubes and centrifuge at approximately 500 *g* for about 10 min to remove cell debris. Dispense the clarified culture fluid into glass screw-capped bottles, in quantities of 20 mL, 10 mL, 5 mL, 1 mL, 0.5 mL or 0.2 mL, as appropriate. Store at −70 °C. Larger volumes can be thawed, dispensed into smaller quantities and re-frozen when required. The EMCV stock will retain its original titre if stored permanently at approximately −70 °C, but repeated freeze-thaw cycles or storage at higher temperatures, e.g. at approximately −20 °C, results in progressive loss of titre.

3.3. ASSAY PROCEDURE

3.3.1. Determination of the dose-response range

Preparation of the solutions

Dilute the appropriate standard for interferon (for example a specific WHO sub-type interferon standard) in culture medium A, in 10-fold dose increments, to give doses covering the range of 1000 - 0.001 IU/mL. Carry out the assay procedure in 96-well microtitre plates. To each well add 100 µL of culture medium A. Add approximately 100 µL of each dilution of the reference preparation to each well except for those intended for virus controls. Using a multichannel pipette set at 100 µL, mix the contents of the wells.

Dispensing of the cell suspension

Pour the cell suspension of Hep2c cells, which has been adjusted to contain approximately 6×10^5 cells/mL of culture medium A, into a plastic Petri-dish. Dispense the cell suspension from the Petri-dish into each well of the microtitre plates, using a multichannel pipette set at 100 µL.

Incubate the plates for about 24 h in an incubator set at 37 °C and 5 per cent CO_2.

Viral infection

At this stage, using an inverted microscope, check that the monolayers of Hep2c cells are confluent, that they show a relatively even distribution of cells, that they have correct morphology and that they are healthy.

Remove most of the culture medium from the wells by inverting the plate and shaking it and blotting on a paper towel (proceed in an identical way when discarding fluids from micro-titre plates as described later). Dilute the EMCV stock with fresh culture medium A to a titre of approximately 3×10^7 PFU/mL (*NOTE: each plate requires approximately 20 mL of diluted virus, plus 5 per cent to 10 per cent of extra volume*). Dispense the diluted suspension from a 9 cm sterile Petri-dish using a multichannel pipette set at 200 µL to all wells including virus controls, but excluding cell controls. Add approximately 200 µL of culture medium A without virus to each of the cell control wells.

Return the plates to the incubator set at 37 °C and 5 per cent CO_2 for approximately 24 h.

Staining

Examine the plates microscopically to check that the EMCV has caused a cytopathic effect (c.p.e.) in the virus controls. The time interval for maximum c.p.e. may vary from one assay to the next because of inherent variation of Hep2c cells to virus challenge over a given period of continuous cultivation.

Remove most of the culture medium from the wells by discarding into an appropriate decontaminating solution (sodium hypochlorite is suitable). Dispense *phosphate buffered saline pH 7.4 R* into each well. Discard the *phosphate buffered saline pH 7.4 R* into a decontaminating solution. Dispense into each well 150 µL of staining solution. Stain the cells for approximately 30 min at room temperature. Discard the staining solution into a decontaminating solution. Dispense approximately 150 µL of fixing solution. Fix for 10 min at

room temperature. Discard the fixing solution into a decontaminating solution and wash the cell monolayers by immersing the assay plates in a plastic box containing running water. Discard the water and superficially dry the plates with paper towels. Dry the assay plates at 20 °C to 37 °C until all moisture has evaporated.

Add 150 µL of *0.1 M sodium hydroxide* to each well. Elute the stain by gentle agitation of the plates or by hitting them against the palm of the hand. Make sure that the stain is evenly distributed in all wells before making spectrophotometric readings.

Read the absorbance at 610 nm to 620 nm, using a microtitre plate reader, taking as a blank a well or a column of wells containing no cells and approximately 150 µL of *0.1 M sodium hydroxide*.

Estimate the concentrations of interferon standard that give the maximum and minimum reduction of cytopathic effect. This is the dose response corresponding to the working range of the assay.

3.3.2. Assay procedure

Carry out the assay as described above, using:

— as test solutions, the substance to be examined, diluted in two-fold increments with culture medium A to give nominal concentrations covering the working range of the assay,

— as reference solutions, the appropriate standard for interferon (for example, a specific WHO sub-type interferon) in culture medium A, diluted in two-fold increments to give nominal concentrations covering the working range of the assay.

3.3.3. Data analysis

Results of the cytopathic effect reduction assay generally fit a sigmoidal dose-response curve, when the interferon concentration (the log of the reciprocal of the interferon dilution) is plotted versus stain absorbance.

Plot the interferon concentration (log reciprocal of dilution) versus the stain absorbance for the interferon reference preparation and for the interferon test solutions. Using the linear portion of the curve, calculate the concentration of interferon in the sample by comparing the responses for test and reference solutions, using the usual statistical methods for a parallel line assay.

4. VALIDATION OF OTHER PROCEDURES

4.1. CHOICE OF CELL LINE AND VIRUS

A number of other combinations of cell line and virus have been used in anti-viral assays for interferons. For example, EMCV has been used in combination with the A549 epithelial lung carcinoma cell line, Semliki Forest virus or Sindbis virus have been used with human fibroblasts, and vesicular stomatitis virus has been used with either human diploid fibroblasts, the human amnion WISH cell line or the Madin-Darby bovine kidney cell line. In each case the choice of the cell line/virus combination is usually based on that which gives the most sensitive response to the interferon preparation to be assayed, and gives parallel responses when comparing the test preparation and interferon standard.

4.2. CHOICE OF RESPONSE

The staining procedure described above measures remaining viable cells. A number of other responses have been used, including methyl violet or crystal violet staining, or the thiazolyl blue (MTT) conversion procedure. In each case, the method is selected on the basis of producing a suitably linear and sensitive relationship between response colour and viable cell count.

4.3. STATISTICAL VALIDATION

As with all parallel line bioassays, the assay must satisfy the usual statistical criteria of linearity of response, parallelism and variance.

4.4. VALIDATION OF ASSAY LAYOUT

As with all microtitre plate assay procedures, attention must be given to validating the assay layout. In particular, bias due to non-random pipetting order or plate edge effects must be investigated and eliminated, by randomising the assay layout, or by avoiding the use of edge wells.

REAGENTS AND CULTURE MEDIA

Culture medium A (10 per cent neonatal calf serum)

RPMI 1640 culture medium, supplemented with antibiotics if necessary (penicillin 10 000 IU/mL; streptomycin 10 ng/mL)	450 mL
L-Glutamine, 200 mM, sterile	5 mL
Neonatal calf serum	50 mL

Culture medium B (2 per cent foetal bovine serum)

RPMI 1640 culture medium, supplemented with antibiotics if necessary (penicillin 10 000 IU/mL; streptomycin 10 ng/mL)	490 mL
L-Glutamine, 200 mM, sterile	5 mL
Foetal bovine serum	10 mL

Staining solution

Naphthalene black	0.5 g
Acetic acid, glacial	90 mL
Sodium acetate, anhydrous	8.2 g
Water	to 1000 mL

Fixing solution

Formaldehyde, 40 per cent	100 mL
Acetic acid, glacial	90 mL
Sodium acetate, anhydrous	8.2 g
Water	to 1000 mL

N1. Numeration of CD34/CD45+ Cells in Haematopoietic Products

(Ph. Eur. method 2.7.23)

This chapter describes immunolabelling and analysis by flow cytometry (*2.7.24*) to determine the number of CD34/CD45+ cells contained in haematopoietic products. The determination is carried out by a single platform method using calibrated fluorospheres, after lysis of the sample red blood cells if necessary.

This method applies to all types of preparations and whole blood. However, the characteristics of this method make it particularly suitable for preparations containing very low percentages of CD34/CD45+ cells.

Graft quality assessment by CD34/CD45+ cell enumeration

A variety of studies have established that the 1-3 per cent of cells in the bone marrow that express the CD34 cell surface antigen are capable of reconstituting long-term, multilineage haematopoiesis after myeloablative therapy.

CD34/CD45+ cells are also found in the peripheral circulation of normal individuals but are extremely rare (0.01-0.1 per cent). However, CD34/CD45+ cells may also be mobilised from marrow to the peripheral circulation in greater numbers by haematopoietic cytokines such as granulocyte colony-stimulating factor and/or chemotherapy.

The technique used for enumeration of CD34/CD45+ cells must meet the following requirements:
— high sensitivity, since haematopoietic stem cells are rare events;
— accuracy, to provide clinically relevant results;
— reproducibility, to provide clinically reliable results;
— speed, to provide real-time analysis.

Selection of parameters
The flow cytometry assay uses commercially available, directly conjugated fluorochrome-labelled monoclonal antibodies, routine staining and whole blood lysing procedures, and a gating strategy using light scatter and immunofluorescence analysis using a pan-CD45/CD34 monoclonal antibody combination.

It is possible to determine CD34/CD45+ cell viability by appropriate nucleic acid staining with a stain that does not cross the intact cell membrane (for example, with 7-aminoactinomycin D).

Selection of monoclonal antibodies
CD34 antibodies Use class III CD34 antibodies that detect all glycosylation variants of the molecule (for example, clone 8G12 or 581). To detect rare events, use an antibody conjugated to the brightest fluorochrome excitable using an argon laser-based flow cytometer, for example phycoerythrin (PE).

CD45 antibodies Pan-CD45 antibodies that detect all isoforms and all glycoforms of this structure are required. A CD45 antibody conjugated to fluorescein isothiocyanate (FITC) fluorochrome is generally used (for example, J33, HLe1, 2D1).

Isotypic or isoclonic controls A negative control is analysed to detect any non-specific signal in the PE fluorescence region. If using an isotypic control (a monoclonal antibody to an irrelevant antigen of the same isotype as the CD34 antibody employed), the PE-conjugated isotype is combined with CD45-FITC (or PerCP). If using an isoclonic control, the unconjugated (in excess) and PE-conjugated CD34 identical monoclonal antibody is combined with conjugated CD45. Alternative combinations may be used.

Absolute count of CD34/CD45+
Calibrated fluorospheres Depending on the technique used, the internal standard either consists of calibrated beads in suspension or is directly introduced into the associated tubes by the manufacturer.

The absolute count of the CD34/CD45+ cells per microlitre is calculated using the following expression:

$$\frac{A}{B} \times C$$

A = number of CD34/CD45+ cells counted;
B = number of fluorosphere singlets counted;
C = known fluorosphere concentration.

Gating strategies
The purpose of sequential gating is to select the population of interest and simultaneously minimise interference from debris and mature cells to which antibodies can bind non-specifically. If using a commercial kit, apply the gating recommended by the manufacturer. If using an in-house assay, it is preferable to apply a currently recommended strategy. A gating strategy that uses light scattering parameters and CD34/CD45 fluorescence will aid in the accurate identification and enumeration of CD34/CD45+ cells.

Number of events analysed
A sufficient number of events are analysed to maintain acceptable accuracy and precision, for example not fewer than 100 CD34+ events and not fewer than 60 000 CD45+ events; the total number of cells counted may be greater if the percentage of CD34 is 0.1 per cent or less.

Specimen collection
Acid citrate dextrose (ACD) formula A is the anticoagulant used in apheresis procedures. This anticoagulant allows both an automated leucocyte count and flow cytometry evaluation to be performed on the same specimen. Edetic acid (EDTA) is the anticoagulant of choice for peripheral blood sampling.

Specimen transport
Transport conditions guarantee the physical and thermal safety of samples.

Specimen integrity and storage
Fresh (less than 24 h old) apheresis products, whole blood samples, umbilical cord blood specimens or bone marrow samples can be processed. Old specimens (more than 24 h old) and specimens that have been frozen and thawed are stained with a viability dye. On receipt, the temperature within the package is verified.

TECHNIQUE

Sample preparation
Ensure that the concentration of leucocytes is suitable prior to staining with monoclonal antibodies. If necessary, dilute the sample with medium that is compatible with the product to be tested and the lysing system. Record the dilution factor. It is recommended to perform the test with a negative control.

Flow cytometry analysis
Autostandardisation

For analysis of cells labelled with a commercially available kit, manufacturers have developed some quality tools for setting the flow cytometer. These settings are then automatically transferred on protocol analysis of samples. Specific fluorospheres are used to set the photomultiplier tube (PMT) on target values, compensation is set and the system is checked using a control preparation.

System settings
— *Discriminator/threshold*: the forward angle light scatter threshold is set to exclude debris (low forward scatter) but not small lymphocytes from the light-scatter plot.
— *PMT high voltage settings*: these must be consistent with cell-surface marker analysis and established within each laboratory so that negative and positive cell populations of moderate antigen density can be distinguished; PMT voltages are reviewed and adjusted periodically according to standardised laboratory procedures.
— *Compensation*: this must be acceptable for the colour spectra overlap (for example, FITC/PE) encountered in cell-surface marker analysis; colour compensation is analysed and adjusted according to standardised laboratory procedures.
— *Flow rate*: this must be consistent with routine cell-surface marker analysis.
— *Gating regions*: the gating regions established for the CD34/CD45 samples are maintained unaltered for the analysis of the negative region.

Calculation of absolute number of CD34/CD45+ cells
The absolute number of CD34/CD45+ cells is calculated using the following expression:

$$n \times D \times V$$

n = total number of CD34/CD45+ cells per microlitre;
D = dilution factor;
V = volume of the product to be tested, in microlitres.

Results are reported as both the percentage of CD34/CD45+ cells and the absolute number per microlitre. They may also be reported as the absolute number per kilogram of recipient body mass, where this is possible.

N2. Colony-forming Cell Assay for Human Haematopoietic Progenitor Cells

(Ph. Eur. method 2.7.28)

The haematopoietic system represents a continuum of cells whose phenotype and properties change as they progress from stem cells to differentiated cells.

Haematopoietic progenitor cells (HPCs) are capable of forming colonies or 'cell clusters' in cultures grown in semi-solid media and are said to be 'clonogenic'.

The determination of the number of colony-forming cells (CFCs) in a cellular product is an indicator of the functional capacity of the progenitor cells and is a predictor of haematopoietic reconstitution. The measured number of CFCs correlates with the minimum number of progenitors present in the sample.

CELL-SURFACE MARKERS

The capacity of colony-forming cells to give rise to haematopoietic colonies *in vitro* and/or to reconstitute the haematopoietic system has been correlated with the expression of specific cell-surface antigens. The expression of the membrane antigen CD34 is an accepted marker for most of the haematopoietic progenitors and stem cells.

COLONY ASSAY SPECIFICITY

Colony-forming cells are identified with a nomenclature based on the lineages of mature cells present in the colony (for example, CFU-Mix, CFU-GEMM, CFU-GM, CFU-G, CFU-M, BFU-E, CFU-E, CFU-Meg) and are a population of progenitors able to give rise to colonies containing one or more lineages of haematopoietic cells. No or low capacity for self-renewal has been ascribed to this population of human HPCs compared with the most immature stem cells.

The amount and type of growth factors supplied during the culture modulate the type and size of colonies that will be formed.

Greater specificity on the general class of HPCs and on their relative proliferative potential is provided by the time required to differentiate *in vitro* into mature cells. The time required by post-natal colony-forming cells to give rise to a colony formed of mature cells *in vitro* is 10-14 days.

QUALITY ASSURANCE FOR A CFC ASSAY

It is paramount for the overall quality of the colony-forming cell assay to apply a strictly standardised approach. It is therefore recommended to carry out intra- and inter-laboratory validations. The source of the materials, including reagents, growth factors and disposables, is identified.

The main factors affecting variability in the CFC assay are the number of cells plated and the identification of colonies. Up to 15 per cent intra-laboratory variability may be observed for the same test. If it is necessary to evaluate the number of colony-forming cells in a purified cell population, it is possible to use a limiting dilution approach where the number of wells positive for cell proliferation is measured with an automated system.

The other main source of variability stems from the use of undefined materials (for example, foetal bovine serum or bovine serum albumin) in the CFC assay. These products derive from pools of source materials and provide a non-specific stimulation of cellular proliferation. However, it is not uncommon to have batches with particular characteristics that selectively stimulate the proliferation of specific haematopoietic lineages.

Finally, a low level of endotoxins (less than 0.01 IU/mL or less than 0.01 IU/mg) in all the materials used for the clonogenic assay is advisable, as higher levels result first in a progressive skewing of the haematopoietic lineages expression in the cultures, and afterwards in a more general inhibition of cell proliferation and clonogenesis.

CFC CLONOGENIC ASSAY

The CFC assay is based on the capacity of progenitor cells to form a colony when plated in a semi-solid medium or in a gel in the presence of specific growth factors. Different types of semi-solid media may be used (for example, methylcellulose, collagen, agar and plasma-clot) depending on the desired readout. Commercially available media usually give more reproducible results.

MATERIALS

A validation is performed at least for the following critical materials.

Growth factors

Both multilineage (such as Kit-ligand or stem cell factor (SCF), interleukin-3, granulocyte-macrophage colony-stimulating factor (GM-CSF)) and lineage-specific (erythropoietin, granulocyte colony-stimulating factor (G-CSF)) growth factors are required to obtain the highest number of colonies from a cell suspension containing a mixed population of HPCs.

Other media components

Media may be supplemented by serum (notably by foetal bovine serum) and/or albumin.

CELL CULTURE

Cells

The sample placed in culture must be representative of the cellular product injected. Cell suspensions are required for this assay. In the case of bone marrow aspirates, such suspensions can be obtained by forcing the bone marrow through a sieve or through progressively smaller calibre needles. Repeated passages through a 21-gauge needle are usually sufficient to disperse cell clusters into a cell suspension.

PLATING AND SCORING

The cells diluted in the culture medium are mixed in the semi-solid medium. It is common to plate 1 mL of the mixture in an untreated sterile Petri dish (Ø 35 mm).

Because of the viscosity of the medium, the solution cannot be plated with air displacement pipettes and the use of syringes equipped with large bore (≤ 18-gauge) needles is required.

The number of cells to be plated depends on the HPC concentration in the sample to be tested. So that no colony is derived from 2 different HPCs, the number of cells plated must allow between 40 and 80 colonies per plate (Ø 35 mm) to be counted. The 'target' number of colonies per plate may be obtained either from the percentage of CD34+ (or concentration of CD34+ cells/mL) determined by flow

cytometry (*2.7.24*) or from different dilutions of the cell suspension (usually 2 concentrations are tested).

The plates are incubated in aerobic conditions with a carbon dioxide concentration of 5 per cent, at 37 °C in a humid (saturated) atmosphere for 10-14 days, and the number of colonies is then scored under an inverted microscope. Care must be taken when manipulating the dishes containing the colonies as the methylcellulose-based medium is viscous but not jellified. An inclined plate will result in mixed and 'comet'-shaped colonies making the scoring likely to be incorrect.

IDENTIFICATION OF THE COLONIES

The size and structure of the colonies depend on the type of mature cells that are their constituents. 50 cells per colony is usually considered a minimum. The presence of haemoglobinised cells identifies progenitors of the erythroid lineage. As the amount of mature cells for each lineage largely depends on the growth factors added to the cultures, performing differentiated counts is not recommended unless otherwise prescribed.

EXPRESSION OF THE RESULTS

The results of CFC culture are usually expressed as the arithmetic mean of the number of colonies counted in at least 3 plates in the test. The mean number of colonies is then related to 10^4 or 10^5 viable nucleated cells placed in culture.

N3. Nucleated Cell Count and Viability

(Ph. Eur. method 2.7.29)

The determination of the quality of cell suspensions requires accurate measurements of both cell concentration and percentage of viable cells. These data are essential to the decision-making process for preparing cellular products and for maintaining optimum culture conditions. The cell count may be expressed as the number of cells per volume of cell suspension and the cell viability as the number of viable cells per volume of cell suspension. The cell-count procedure may be performed manually (haemocytometer) or with an automated apparatus (for example, particle counter, flow cytometer). Other methods than that described below may be used.

CELL NUMBER

MANUAL COUNTING

Description of the apparatus and test principle The following materials are required:

- a haemocytometer: a specialised microscope counting chamber available in different designs. It consists of a thick slide and a coverslip mounted to delimit a chamber with a specific volume for each design. The thick slide of the various haemocytometers consists of counting chambers separated by deep grooves to avoid cross-filling. The counting chamber is etched in the glass and contains a grid which is specific for each model;
- a light microscope - low power 10× to 40× magnification;
- pipettes of a suitable volume range.

The haemocytometer is used to quantify the number of cells in a given solution by calculation of the cell concentration per millilitre (C) using the following expression:

$$a \times 10^n \times d$$

a = number of cells counted;
d = dilution factor (where applicable);
n = factor varying with the volume of the haemocytometer chamber.

It is possible to distinguish between mixed cell populations provided they differ in size or pigmentation (for example, leukocytes and erythrocytes).

Preparation of the counting chamber and analysis Mount the coverslip (slightly moistened on the edges) on the slide. Move the coverslip back and forth over the slide, pressing slightly on the sides. Prepare a suitable dilution of the cell suspension in isotonic buffer or in haemolysis buffer.

Add an appropriate volume of the dilution to the counting chamber. The liquid is added to the border of the coverslip and is drained inside the chamber by capillarity. Carefully place the haemocytometer under the microscope and focus. Count the cells in a zone of the grid. Calculate the cell concentration in the diluted and original samples.

To increase the accuracy of the measurement, it is important to respect the following basic precautions:

- use only suitably thickened coverslips;
- wherever possible, count more than 100 cells (if necessary, count more areas);
- where cell clustering is detected (i.e. the cell suspension is not monocellular), resuspend the cells before sampling and count again;
- avoid underfilling or overflowing the chamber, otherwise the volume will no longer be accurate.

AUTOMATED COUNTING METHODS

Particle counters based on conductivity variation Electronic particle counting devices measure the size and number of particles in a solution.

Particle counters are calibrated before use with a solution of particles of known concentration and size. To allow the counting of larger particles, tubes fitted with differently calibrated orifices are available. These apparatuses do not allow the discrimination between dead and live cells. As cell debris may also generate pulses that may cause errors, counters are also fitted with a threshold control allowing only larger particles to be counted.

The apparatus must be qualified for the counting of cellular products (in terms of linearity, accuracy, etc.).

Particle counters based on flow cytometry (*2.7.24*). The flow cytometer is calibrated with reference particles of known concentration and size to give an absolute cell number per volume. However, a calibrating solution is no longer necessary in instruments using 2 electrodes inserted in the sampling chamber where the fixed size of the sampling chamber and distance between the 2 electrodes allow the measurement of the content of a fixed volume. This type of instrument rarely needs to be calibrated after the initial setting.

VIABILITY

This section applies to cell staining by viability dyes and manual or automated analysis, under a light microscope or by flow cytometry, of a cell suspension in order to determine the percentage of viable cells.

Depending on the type of cells and the method used, the results may differ.

MANUAL DYE-EXCLUSION METHOD

Test principle This test is based on the exclusion of the dye from viable cells whereas dead or damaged cells absorb the dye and are coloured. It provides information on the

cytoplasmic membrane integrity but its results do not necessarily reflect cell functionality. Recently trypsinised or thawed viable cells may have leaky membranes, causing them to absorb the dye.

Dye Trypan blue is the stain most commonly used to distinguish between viable and non-viable cells, but other suitable dyes such as erythrosin B or nigrosin may also be used. It is an acid dye (M_r 961), an anion with 4 sulfonate groups that can easily bind to proteins; therefore the protein concentration of the preparation to be tested must be as low as possible.

Test conditions Dye fixation is strongly influenced by pH, within a range of 6.6 to 7.6. Fixation is optimal at pH 7.5. The other conditions, such as the dye concentration and the staining time are validated.

Storage conditions of the dye Generally a 0.4 or 0.5 per cent trypan blue solution in sterile phosphate-buffered saline is used. Store protected from light and air.

Test preparation and analysis Stain the cell suspension at the required dilution (usually in phosphate-buffered saline) with, for example, a trypan blue solution having a final concentration of 0.1 to 0.2 per cent. Mix gently. Incubate for not more than 2-4 min at room temperature. Mix gently and place a suitable volume in a counting chamber. Count without delay.

Determine the percentage of viable cells from the ratio of the number of unstained cells to the total number of cells under a light microscope, considering all stained cells as dead cells. Viability (V) is calculated as a percentage using the following expression:

$$\frac{n}{N} \times 100$$

n = number of unstained (viable) cells;
N = total number of cells (stained and unstained).

It is essential that the incubation time be not more than 4 min as the number of stained cells may increase significantly afterwards. For a new determination, it may therefore be necessary to prepare a new test.

AUTOMATED METHODS

Flow cytometry

Test principle The test is based on the ability of certain dyes to cross damaged membranes and bind to DNA by intercalating between bases so that dead cells may fluoresce and be detected by flow cytometry (*2.7.24*). Non-viable cells are evaluated and discriminated by focusing on positive staining whereas viable cells remain unstained. This analysis is generally performed with 7-aminoactinomycin D (7-AAD) or propidium iodide (PI) but other suitable dyes may also be used.

Dye 7-AAD and PI are given as examples of membrane-impermeants that may be used as viability dyes.

7-AAD is an analogue of actinomycin D that contains a substituted amino group at position 7 of the chromophore. It intercalates between cytosine and guanine DNA bases. The spectral properties of 7-AAD make this molecule particularly suitable for flow-cytometry analysis.
The maximum absorption of the 7-AAD/DNA complex is situated in the green spectral region and is thus suitable for an argon laser-equipped cytometer (excitation wavelength of 488 nm). The deep red fluorescence emission of the 7-AAD viability dye (635 nm to 675 nm) eases the use of the probe in combination with fluorescein isothiocyanate (FITC)-and phycoerythrin (PE)-conjugated antibodies, because in contrast to PI, the 7-AAD/DNA complex shows minimal overlap with FITC and PE.

PI binds to double-stranded DNA by intercalating between bases with little or no sequence preference and with a stoichiometry of 1 dye molecule per 4-5 DNA base pairs. Once the dye is bound to nucleic acids, its fluorescence is enhanced 20- to 30-fold, the fluorescence excitation maximum is shifted around 30-40 nm towards the red and the fluorescence emission maximum (615 nm) is shifted around 15 nm towards the blue. Although its absorptivity is quite low, PI exhibits a sufficiently large Stokes shift to allow simultaneous detection of nucleic acids and fluorescein-labelled antibodies, provided that the suitable optical filters are used.

Storage conditions of nucleic acid dye solution 5 ± 3 °C.

Test preparation and analysis In the case of haematopoietic cells, the dye may be added after CD45 labelling to obtain a better separation of cells from debris and platelets with a side scatter (SS)/CD45+ gating region. The incubation conditions of the cell suspension with the dye are validated previously.

Incubation is performed at room temperature protected from light. Where necessary, lysis of red blood cells is performed using, for example, ammonium chloride. If not, add buffer alone.

Percentages of viable cells are directly given by the flow cytometer and deduced from the analysis of positive cells (dead cells) in the SS/7-AAD or SS/PI cytogram (dot plots).

Positive controls may consist of stabilised cells (dead cells) mixed with fresh viable cells at a target value.

Digital imaging of stained cells

Digital imaging allows the automation of dye-exclusion methods. The cell suspension and viability-dye solution are directly mixed by a machine. The system, which allows sample aspiration, reagent handling, and subsequent instrument cleaning is fully automated. Once the cellular suspension has been aspirated and mixed with the dye solution, it is pumped to the flow cell for imaging.
The stained cell suspension is aspirated through a chamber where stroboscopic light allows a camera to photograph the flowing cells. The images are digitalised and the number of dead or live cells counted by the software.

O. Host-cell Protein Assays

(Ph. Eur. method 2.6.34)

This general chapter provides guidance for the development and validation of host-cell protein (HCP) assays used to test products obtained by recombinant DNA technology. It does not exclude the use of alternative approaches that are acceptable to the competent authority.

INTRODUCTION

Host-cell proteins (HCPs) are process-related impurities derived from the host organism used for the production of a medicinal product by recombinant DNA technology.
In order to mitigate their potential adverse effects (e.g. immunogenicity), HCP content is expected to be reduced to the lowest possible level.

HCP clearance during the purification process must be assessed and the HCP content determined using an HCP

assay that has been evaluated and validated for a given product.

The HCP acceptance limit, typically expressed in nanograms of HCP per milligram of active substance (ppm), must be justified with regard to the HCP clearance capacity of the purification process and with regard to the potential impact of residual HCP on patients, taking into account the worst-case quantity of HCP that could be administered with the product.

HCPs are generally measured using an immuno-based assay containing, as reagents, the HCP antigen preparation (hereinafter 'the HCP antigens') or HCP reference standard and the corresponding polyclonal antibodies (antisera). Antisera must cover a broad spectrum of HCPs representative of the product concerned.

Sandwich-type enzyme-linked immunosorbent assays (ELISA) are the most commonly employed assays to assess quantitatively the level of HCPs. It should be noted that HCP content measured by ELISA does not represent absolute HCP mass content. The sensitivity is the result of the observed cumulative responses of many individual HCPs in comparison to the response of an HCP reference standard. The use of orthogonal analytical methods (e.g. electrophoresis, HPLC, Western blot, mass spectrometry) to characterise the various HCPs in the product is recommended to support the development and selection of the assay.

ASSAY SELECTION

Several types of assay are available, with selection taking into account several factors, including the stage of development of the product, the nature of the host cell and the protein immunogenicity, the expression mode, the manufacturing process, and prior knowledge. When selecting and developing the assay, its life cycle (e.g. reagent supply, consistency, assay validation, process change) must also be considered.

TYPES OF ASSAY

Process-specific assays

Process-specific HCP assays (also called product-specific HCP assays) are developed and validated taking into account the specificity of the production process, and using the same host organism expressing the recombinant product.

The HCP antigens are derived from a mock run of the active substance manufacturing process (or a process representative of it) up to a step capable of generating a broad spectrum of HCPs in sufficient quantities.

The antisera raised must cover a broad range of HCPs, in order to detect as many different HCPs as possible and also to accommodate process variations.

Platform assays

Platform assays are developed by individual manufacturers and customised for the processes and host organism used by the manufacturer for production. The same sets of reference standards and reagents may be used to monitor HCPs in several products manufactured in the same host organism, provided that upstream processes (and downstream, if relevant) are sufficiently similar for these products. The suitability of the antiserum should be evaluated as described above for process-specific assays.

Generic assays

Commercially available HCP test kits are commonly referred to as generic HCP assays. They are intended to work broadly across similar expression hosts. Detailed information on the preparation of the reagents may not be disclosed by the vendor. For instance, the HCP antigens may be derived from a combination of strains of an expression host species, and the process(es) used may not mimic the process applied for the product of interest. The suitability of the antiserum should be evaluated as described above for process-specific assays.

CRITERIA FOR ASSAY SELECTION

In view of the potential safety issues associated with residual HCPs in the active substance, a risk assessment is performed to support the choice between a generic, a platform or a process-specific strategy, taking into account the stage of development of the product.

For early development a generic assay or a platform assay may be used. For later development phases, process-specific assays must be considered, as they are generally regarded as superior, especially when compared to generic assays. This is because process-specific assays are more likely to show immunoreactivity against representative HCPs.

Platform or generic assays may be used, provided that the assay is appropriately characterised and validated against process-specific HCPs.

PRODUCTION AND TESTING OF THE HCP ANTIGEN PREPARATION

The HCP reagents (HCP antigens and anti-HCP antibodies) are produced in such a way as to facilitate replication of the production when a replenishment for the HCP assay is needed.

The HCP antigens are used to generate the polyclonal antibody reagent for the HCP immunoassay by immunising one or more suitable animal species. In addition, they serve as the HCP reference standard in the HCP immunoassay.

As far as possible, the HCP antigens must cover the relevant HCP population expected to be derived from the manufacturing process of the protein of interest.

The HCP population must also be broad enough to cover worst-case purification scenarios and to provide robustness against potential manufacturing process changes during the life cycle of the product.

PROCESS-SPECIFIC ASSAYS

Null cell line

Development of a process-specific assay involves the selection of a null cell line that does not contain the expression gene for the product of interest and is derived from the same cell line that has been used to establish the production cell line. This null cell line may be non-transfected or mock-transfected. A mock-transfected cell line is created by transfecting the parental cell line with a blank plasmid, i.e. the plasmid used to create the production cell line, but missing the gene coding for the protein of interest.

Mock production process

Upstream

The antigens produced for process-specific assays are obtained by a mock production process that mimics the intended manufacturing process, using the null cell line and, as far as possible, the same operating conditions.

As for any mock production, the process used represents an approximation of the intended manufacturing process and leads to differences (e.g. different scale, operating parameters, product interaction). However, the impact of those differences needs to be considered carefully because they may affect the composition of the HCP population.

For example, a mock fermentation of an inclusion body manufacturing process may not deliver the desired inclusion bodies if the product is not present. Therefore, depending on

the null cell line used (e.g. mock-transfected or not), the antigens may need to be isolated differently compared to the intended manufacturing process.

In some situations, operating parameters for the mock production may be adjusted to cover worst-case scenarios (e.g. to deliver antigens covering a broad spectrum of different HCP species). For example, the antigen-containing cell culture supernatant may be harvested beyond the minimum level of cell viability in order to include more cytosolic proteins, which are released by additional cell lysis.

Downstream

The HCP antigens derived from the upstream process are usually only minimally processed (filtration, concentration), in order to obtain a representative spectrum of HCPs. Further purification is generally not recommended as there will be a risk of losing HCP species.

However, in cases where the antigens are not representative (e.g. resulting in low coverage), mixing of mock materials from different processing steps can be considered. Enrichment may also be achieved by pooling materials from mock fermentation or purification runs using different operating conditions, or from selective purification steps (e.g. to reduce large amounts of the few immunodominant HCPs).

Cross-contamination with the protein of interest

The HCP antigens must be produced in a manner that avoids contamination with even minute traces of the product in order to avoid cross-reactivity with the polyclonal antibodies.

To achieve this goal, dedicated or single-use equipment is used as much as possible. Where multi-purpose equipment is used, it must be cleaned appropriately. In addition, the risk of contamination when filling or handling the antigens in the laboratory environment must also be considered.

Characterisation and testing

Before using the HCP antigens for immunisation, the protein content is assessed (total protein assay) and the absence of the protein of interest verified.

Comparison of the HCP population with the mock and the intended production process is performed, typically by SDS-PAGE and/or two-dimensional (2D) electrophoresis with a high sensitivity stain. The aim of this comparison is to show that the HCP antigens resulting from the mock production process contain most of the representative HCP species of the intended manufacturing process. Where necessary, complementary information may be gathered by orthogonal methods, e.g. mass spectrometry.

PLATFORM ASSAYS

Null cell line

Development of a platform assay involves a null cell line that does not contain the expression gene for the product of interest, and uses the same host species. This null cell line may be non-transfected or mock-transfected, and may be used for the production of HCP antigens for products from a given company's manufacturing platform.

Mock production process

Upstream

The HCP antigens produced for platform assays are obtained by a mock production process that mimics the platform upstream process that is used for several products, and typically uses the same media components. As for any mock production, the process used represents an approximation of the intended manufacturing process, which may impact the composition of the HCP population (see process-specific assays).

Downstream

As for other assays, the HCP antigens derived from the upstream process are, in general, only minimally processed (e.g. no or limited number of purification steps) to obtain a broad spectrum of HCPs, although mixing and pooling strategies may also be used to widen the spectrum of HCP species.

Characterisation and testing

As for process-specific assays, both the protein content and the absence of the protein of interest are tested. Comparison of the HCP population with the mock and the intended production process is performed.

GENERIC ASSAYS

Generic assays are commercially available and are developed by the vendor.

Detailed information on the preparation of the reagents may not be disclosed by the vendor. For instance, the null cell line may be derived from a combination of strains of an expression host species, and the process(es) used may not mimic the process applied for the product of interest.

Nevertheless, the generic assay must be selected with consideration given to the intended manufacturing process (e.g. appropriate host cell line), and be appropriately validated for the product of interest and phase of development. As a consequence, if generic assays are used in later stages of development or during commercial manufacturing, it is recommended to validate the assay and control lot-to-lot reagent consistency using either appropriate upstream fractions from the production process or a mock preparation generated using a null cell line.

PRODUCTION AND CHARACTERISATION OF THE ANTI-HCP ANTIBODY REAGENT

PROCESS-SPECIFIC AND PLATFORM ASSAYS

Immunisation

One of the challenges of the immunisation step is to generate polyclonal antibodies that are highly specific and sensitive for each of the antigenic proteins in the complex mixture of HCPs used as an immunogen. An animal's immune response must be stimulated against both the stronger and the weaker antigens.

An animal host that yields a sufficient quantity and diversity of HCP-specific immunoglobulin G (IgG) is selected.

Where both the polyclonal capture and the polyclonal detection antibodies are from the same source, it can be assumed that they recognise different epitopes on the same HCP in the assay. Alternatively, polyclonal anti-HCP antibodies from different animal species may be used. Using several animals for a given species may reduce the impact of individual variations in immune competence and provide additional response diversity, resulting in maximised antibody coverage against the HCP antigens.

An immune response to a limited number of HCP antigens may be obtained rapidly, particularly when adjuvants are used to boost the immune response. However, in complex mixtures, differential enhancement of the immune response towards weaker antigens or those at lower concentrations may be necessary.

It usually takes several immunisations to reach a maximum immunological response and, depending on the frequency of immunisation, the process can take 3-6 months to complete.

The immune response against the HCPs for a given immunisation scheme has to be monitored by determining the antibody titre using, for example, an ELISA, and by

comparing the results of 1D or 2D electrophoresis after protein staining and a Western blot, where the polyclonal anti-HCP antibodies are used as primary antibody.
In practice, some minor proteins that elicit a strong immune response may not be visible in the protein-stained gel, and some poorly antigenic proteins that are detectable by protein staining may not elicit a detectable immune response.
To achieve sample-dilution linearity in complex multi-analyte immunoassays, it is essential that the immune reagent simultaneously and specifically recognises as many individual analytes as possible in an assay sample and that it is present in stoichiometric excess. For this purpose, a series of sample dilutions from different process steps may be tested by ELISA using purified anti-HCP antibodies from bleedings that have shown suitable coverage by Western blot.

Finally, based on the results of the tests described above, antisera from different animals are pooled, retested and purified.

Purification and preparation
The HCP antibodies must be purified before an assay can be developed.

Typically, this is achieved by protein A- or protein G-chromatography and/or HCP antigen affinity chromatography. In the case of HCP antigen affinity chromatography, the antigens used for immunisation are immobilised on column chromatography media and the specific antibodies are captured by applying the antisera onto the column.

Additional purification to remove potential aggregates might be required by gel permeation chromatography.

For the ELISA, a part of the purified anti-HCP antibodies is conjugated to a detection label (e.g. biotin or horseradish peroxidase).

The purified anti-HCP antibodies and the sera must be stored at a temperature that ensures their stability.

Characterisation and testing
The suitability of the derived HCP assay reagent is assessed by demonstrating the coverage of the HCPs representative of the manufacturing process by the anti-HCP antibodies.

For this purpose, 2D electrophoresis of the HCP antigens is performed. The protein pattern of the immunostain is compared with the protein pattern of the total stain.
The anti-HCP antibodies must recognise a broad range of HCPs over the full range of charge and molecular size. Other methods using native conditions may be considered.

GENERIC ASSAYS
Immunisation, purification and preparation of the anti-HCP antibody reagent are carried out by the vendor and details may not be available.

Characterisation and testing of anti-HCP antibodies are performed as for other assay types. Typically, there is limited control over lot-to-lot reagent consistency. Appropriate comparative lot testing is therefore required.

VALIDATION OF THE HCP ASSAY
The HCP ELISA is developed to detect and quantify a heterogeneous mixture of antigens at varying concentrations, and with a reagent containing antibodies that are not represented at a one-to-one ratio. The section below is intended to target the specifics in development and validation of this type of ELISA.

HCP assays such as ELISA are validated with regard to accuracy, specificity, precision, quantitation and detection limits, linearity, range and robustness.

During the life cycle of the product, a full or partial revalidation of the assay may be required, for example when implementing a manufacturing process change that may impact the suitability of the HCP reagent.

Accuracy
Accuracy is demonstrated by spike/recovery analysis of the HCP reference standard in a relevant background matrix (e.g. the active substance or a sample from a relevant purification step).

Specificity
Specificity is demonstrated by the absence of interference from the matrix background (including the active substance). For instance, data from the accuracy study can be used to assess specificity.

Precision
As for any other quantitative assay, repeatability, intermediate precision and reproducibility are appropriately demonstrated.

Quantitation and detection limits
Sensitivity is usually in the ppm range and is normally described through the quantitation limit (QL). QL is typically determined by HCP spike recovery studies in the active substance or an appropriate sample matrix, and is calculated from the minimal spike providing a response with predefined accuracy and precision from replicate analyses.

Detection limit (DL) is often not determined (optional validation parameter).

Linearity
The linearity of the HCP assay is demonstrated using dilution series of the HCP standard and spike/recovery experiments (accuracy study).

Additionally, due to the nature of HCP assays, the multiple HCP analytes and polyclonal anti-HCP antibodies, sample-dilution non-linearity may be observed, i.e. back-calculated results increase with increasing dilutions of samples, which in most cases is related to the excess of one or more individual HCPs in the sample when compared to the available antibodies in the HCP immunoassay. As a consequence, dilution linearity must be properly assessed for the relevant process steps by comparison of target versus measured HCP concentrations at varying sample dilutions. Dilution linearity is demonstrated if the acceptance criteria for assay variation are met for different sample dilutions. Studies demonstrating dilution linearity can be carried out either during method development or at the latest during method validation.

If a sample shows dilution non-linearity, multiple sample dilutions are prepared beyond the range where non-linear behaviour is observed.

The final HCP value is typically reported as the average HCP concentration obtained for a minimum of 2 dilutions within the linear dilution range. If justified, 1 dilution may be sufficient.

Range
The range of the assay is typically defined by the HCP concentrations for which a suitable level of precision, accuracy and linearity has been demonstrated.

Robustness
The evaluation of robustness is considered during the development phase.

CHANGE OF HCP ASSAY AND/OR REAGENT
The quantities of antigens and antibodies must be large enough to supply the HCP assay for several years. Therefore, the supply, quality and consistency of reagents must be appropriately managed throughout the life cycle of the assay.

Table 2.6.34.-1

	Depleted reagents		Process change
	HCP reference standard	Anti-HCP antibody	
Reagent characterisation	The protein concentration of the new reference standard is determined preferably using the same method as for the current reference standard to ensure that the protein concentrations are comparable. Using suitable methods (e.g. 1D-/2D-PAGE, 2D-DIGE), the similarity in protein composition between the new and current HCP reference standards is assessed.	Total protein concentration of the new antibody is determined. The final assay concentration must be titrated for the new lot in order to achieve a similar standard curve as for the current lot. For detection antibodies, the detection label: protein stoichiometry is controlled and ensured to be similar to the current antibody lot. Immunoreactivity of the new antibody is compared qualitatively (by visual comparison) or semi-quantitatively (coverage determination) against the current lot by suitable methods (e.g. 1D or 2D Western blot). Due to the variability of the method, it is particularly advisable to perform this characterisation side-by-side with the current antibody lot.	The effects of process changes that have the potential to impact the HCP composition are analysed by suitable methods (e.g. 1D-/2D-PAGE, Western blot, HCP assay). If the process change does not lead to a relevant change in HCP composition, the current HCP reagents are also suitable for the new process. If the process change does lead to a relevant change in HCP composition, but the suitability of the current HCP reagents was demonstrated, the current HCP reagents are also suitable for the new process. If the process change does lead to a relevant change in HCP composition, but the current HCP reagents were shown to be unsuitable for the new process, a new assay must to be developed including a mock fermentation according to the new process and a new immunisation.
Testing of reagents in HCP assays	The new HCP reference standard is quantitatively tested against the current reference standard for spike recovery at different concentrations covering the validated assay range. Standard curves obtained with the new versus the current reference standard are assessed for similarity.	Standard curves obtained when using new versus current antibody lots are compared. A bridging study is performed with testing of relevant process samples (e.g. purification steps from harvest to the final active substance). In a side-by-side experiment, new antibodies must detect HCP levels at different process steps equally or with an improved quantitation limit.	A mock run harvest from the new process is tested for spike recovery using the current HCP assay. Relevant process samples (e.g. purification steps from harvest to the final active substance) from the new and the previous process are tested side-by-side.
Assessment of validation status	If reagent characterisation and ELISA testing demonstrate suitability of the new HCP reference standard, the current reference standard can be replaced. No revalidation of the test method is required. If the new HCP reference standard differs significantly in protein composition and/or assay performance from the current reference standard, revalidation is required.	If reagent characterisation and ELISA testing demonstrate that the new antibody is suitable, the current antibody can be replaced. No revalidation of the test method is required. If the new antibody differs significantly in Western blot immunoreactivity or immunoassay sensitivity and/or assay performance compared to the current antibody, revalidation is required.	If, for the new process, the antibody shows similar or higher immunoreactivity compared to the previous process, and the HCP assay shows adequate recovery from the mock harvest of the new process and also similar or higher sensitivity for samples from the relevant process steps, then the current assay and reagents are considered suitable for the new process. No revalidation of the test method is required. If reagents appear suitable to detect HCP from the new process, but the ELISA indicates significant differences in spike recovery of the mock sample or of HCP levels at relevant process steps, then revalidation is required. The process change might also impact dilution linearity of test samples from certain process steps; if these steps are essential for the HCP control strategy, revalidation or even generation of new antibody reagents might be required. In case of a major change in HCP composition with the new process that leads to either a mismatch in protein composition compared to the current assay standard, reduced immunoreactivity of the antibody, or significantly decreased immunoassay sensitivity, then new assay reagents are prepared and the HCP assay is validated with the new reagents.

PAGE: polyacrylamide gel electrophoresis
DIGE: differential gel electrophoresis

For generic HCP assays, in order to ensure the consistency and quality of the reagents, recharacterisation or revalidation of the assay may be required for each new batch of reagent, as their quality may change from one batch to another.

For process-specific and for platform assays, there are generally 2 situations where new HCP assay reagents may be required:

— the HCP reference standard and/or antibody are depleted; antibody may then be purified from a frozen serum stock or a new immunisation is required;

— a manufacturing process change can impact the HCP composition for the purification intermediates or the final product; the assay reagent may not be properly suited to detect and quantify the modified HCPs; a manufacturing process change can therefore render the reagents unsuitable for assay use.

Newly prepared reagents must be thoroughly characterised (e.g. by 2D-SDS-PAGE/Western blot for coverage, 2D-SDS-PAGE/differential gel electrophoresis (DIGE)/identification by MS). Afterwards, the validation status of the assay using the new reagents must be assessed. It is recommended to perform these experiments side-by-side with the currently used reagents.

Table 2.6.34.-1 outlines a recommendation for reagent characterisation and assessment of immunoassay validation, as a consequence of a depletion of assay reagents or a process change.

O2. Quantification and Characterisation of Residual Host-cell DNA

(Ph. Eur. method 2.6.35)

This general chapter describes analytical methods that may be used to measure the content and to characterise the size of residual host-cell DNA in biological products produced in cell substrates. It does not exclude the use of alternative approaches that are acceptable to the competent authority.

INTRODUCTION

Several sensitive analytical methods exist for the quantification of residual host-cell DNA, including real-time quantitative PCR (qPCR) (Method A) and an immunoenzymatic method (Method B).

qPCR may also be used to assess the residual host-cell DNA size distribution, as a characterisation test depending on the nature of the cell substrate (e.g. continuous cell lines) and on the amount of residual host-cell DNA.

A suitable method is selected depending on the nature of the biological product to be tested and taking into account the characteristics and limitations of each method as summarised in Table 2.6.35.-1.

Table 2.6.35.-1 - *Comparison of the characteristics of qPCR and immunoenzymatic methods*

Characteristics	qPCR (Method A)	Immunoenzymatic method (Method B)
Can be used to assess DNA size distribution	Yes	No
Limit of quantification *(may vary depending on the matrix, method and interfering substances)*	0.01-10 pg/mL	2-10 pg/mL
Specificity (total DNA versus specific DNA)	Specific DNA	Total DNA
Interfering substances	Proteins	Detergents/proteins/solvents/RNA
Limitations	Fragments smaller than the PCR product can not be detected and quantified.	Not applicable for DNA-based products. The DNA content is underestimated for fragments below 1000 base pairs (bp). Detectability depends on DNA size. Undetectable short DNA fragments (below 80 nucleotides) can interfere with the assay through reagent consumption. Products must be free of bacterial DNA. Narrow quantification range: 5-150 pg/well.

SAMPLE PREPARATION

The concentration of residual host-cell DNA may vary depending on the type of biological product and the DNA clearance capacity of the manufacturing process.

Depending on the sample matrix, pretreatment of the sample may be necessary to ensure appropriate recovery of the residual host-cell DNA.

When analysing highly purified protein samples such as recombinant proteins or monoclonal antibodies, simple digestion with proteinase or affinity chromatography may be sufficient to recover the residual host-cell DNA. For more complex matrices such as viral vaccines and viral vectors, an additional virus lysis step may be required to release the residual host-cell DNA from the viral particles.

The immunoenzymatic method is especially sensitive to interference from proteins. This can be avoided by performing an initial pretreatment step, which may include digestion with proteinase K and sodium dodecyl sulfate (SDS). This step may suffice to recover the residual host-cell DNA. However, in other cases, the residual host-cell DNA may be bound to the sample components, and/or soluble interfering substances may be present, in which case it may be necessary to extract the residual host-cell DNA from the sample.

The DNA can be extracted using protocols which have demonstrated a satisfactory recovery rate during spiking experiments. Several suitable methods exist, including DNA precipitation or DNA-specific binding to a matrix (e.g. magnetic beads or silica columns). Commercial kits can be used to extract residual host-cell DNA samples. Some of these kits use a chaotrope (sodium iodide) and a detergent (sodium lauroylsarcosinate) to disrupt the association between the residual host-cell DNA and the sample components. The residual host-cell DNA in the sample is then recovered by co-precipitation with a carrier molecule such as glycogen in the presence of ethanol or 2-propanol. Several independent extraction procedures may be required, depending on the reproducibility of the spike recovery. Negative controls must be included in each extraction procedure. In some cases, dilution of the samples may be recommended to reduce the matrix effect. A correction factor may also be applied to take into account the recovery rate of the spike.

METHOD A – REAL-TIME QUANTITATIVE PCR (QPCR)

This method can be used to quantify a cellular DNA target sequence from a variety of samples. For the quantification of residual host-cell DNA, qPCR targeting either a stable sequence within a highly conserved host-cell region or targeting repetitive elements to enhance the sensitivity of the test can be used. When repetitive elements are targeted, it may be difficult to eliminate potential background noise due to environmental DNA (e.g. when using Alu human sequences). The specificity of the qPCR method must be established during the validation studies by demonstrating the absence of cross-reactivity with unrelated sequences.

Alternatively, digital PCR methods may be used.

qPCR amplification

The detection and quantification of residual host-cell DNA by qPCR may involve the use of either a non-specific fluorescent dye that intercalates with any double-stranded DNA, or sequence-specific DNA probes. The qPCR principle described in general chapter *2.6.21. Nucleic acid amplification techniques* applies.

The number of cycles required for the fluorescent measurement to exceed a threshold value (Ct or Cp) correlates to the starting amount of residual host-cell DNA in the sample.

If several extractions are performed, the resulting samples must be analysed at an appropriate dilution.

PCR negative controls are used.

A standard curve is plotted using serial dilutions of host-cell genomic DNA in order to allow residual host-cell DNA levels in biological products to be determined based on their Ct or Cp values. The use of carefully characterised representative genomic DNA extracted from the cells used for the production of the biological product is recommended for the preparation of the standard.

The same methodology is applied for residual host-cell DNA size evaluation. At least 2 sets of primers can be designed to amplify overlapping fragments of different sizes in the target sequence.

Suitability criteria

Control samples In order to control the risk of contamination and to ensure adequate sensitivity, each PCR assay includes the following controls:

- *a negative control for qPCR* and *a negative control for extraction*, composed of a sample of a suitable matrix already proven to be free of the target sequence(s);
- *a positive control for qPCR*, which contains a defined number of target sequence copies or a defined DNA concentration which is determined individually for each assay system;
- *a control for extraction*, typically an internal control added to the test material as a defined concentration or number of target-sequence copies. In this case, the amplicons must be clearly discernible and may be detected in a separate qPCR. Alternatively, an external control consisting of test sample spiked with a well-characterised level of genomic DNA may be used.

Extraction recovery must fall within predetermined values based on the performance of the assay as demonstrated during assay validation.

Genomic DNA standard curve The standard curve is linear over the chosen range.

The coefficient of determination R^2 associated with the standard curve must be greater or equal to 0.98. The PCR efficiency falls within pre-established limits.

The coefficient of variation for the different extracts or replicates is not higher than a predefined criterion.

Calculation

If several extractions are performed, each extracted sample is analysed individually. The residual host-cell DNA content is calculated from the genomic standard curve by averaging the values obtained for the different extractions or replicates.

A correction factor may also be applied to take into account the recovery rate for total DNA quantification in the samples.

For the characterisation of residual host-cell DNA size, the distribution of the overlapping fragments of different sizes is calculated as the ratio of the number of copies for each amplicon size to the number of copies of the smallest amplicon size.

METHOD B – IMMUNOENZYMATIC METHOD

The immunoenzymatic method is a non-specific technique for the quantification of residual host-cell DNA (regardless of its origin). It is therefore also a total DNA assay, and consequently, it is not only critical to avoid contamination through environmental DNA, but all materials and reagents used must be DNA-free. The samples to be tested must be free of microbial contamination and all samples, controls and standards must be processed under controlled conditions until the denaturation step.

By design, the method detects single-stranded DNA.

Principle

This total DNA assay consists of 4 steps:

- *denaturation and formation of complexes*, where the DNA is denatured into single-stranded DNA by heating the sample. The denatured DNA is mixed with a single reagent that contains a single-stranded DNA-binding protein conjugated to streptavidin and a monoclonal anti-DNA antibody conjugated to urease. The DNA-binding protein and the monoclonal antibody are specific for single-stranded DNA but are not sequence-specific. The liquid phase, in the presence of streptavidin, facilitates the formation of a complex with the single-stranded DNA from the sample.
- *filtration*, where the complex is filtered through a biotinylated nitrocellulose membrane. The biotin in the membrane captures the complexes by binding to streptavidin. The membrane is washed to remove any unbound reagents. Non-specific binding is avoided by the use of an albumin-coated nitrocellulose membrane.
- *detection*, where the membrane is placed in a reader, which contains a urea solution that reacts with the urease in the DNA complex and produces ammonia. The associated change in pH is measured by a potentiometric sensor in µV/s and is directly proportional to the amount of DNA in the sample.
- *analysis*, where the raw data from the sample and from the standard curve are analysed using appropriate software to determine the residual host-cell DNA content in the sample.

All samples and negative controls are tested spiked and unspiked. The spike solution (1000 pg/mL) is prepared by dilution of a concentrated standard (calf thymus DNA) at 5000 pg/mL.

Suitability criteria

Control samples

- the amount of DNA in the positive control falls within the range indicated on the batch certificate provided by the supplier;

— spike recovery in the negative control is between 80 per cent and 120 per cent.

Samples

— when several replicates are analysed, the coefficient of variation for the different replicates is not higher than a predefined criterion;
— spike recovery is between 80 per cent and 120 per cent.

Calculation

The content of residual host-cell DNA is calculated in picograms per millilitre using the following expression:

$$\frac{ID \times (C - A)}{V}$$

ID = ratio for dilution and sampling;
C = raw mean value (in picograms per tube) for the test tubes containing the diluted sample;
A = raw mean value (in picograms per tube) for the test tubes containing the negative control;
V = volume in the test tube, in millilitres (usually 0.5 mL per tube).

Where necessary, this result may be corrected by the extraction recovery (e.g. mean recovery for a given product).

P. Determination of Bactericidal, Fungicidal or Yeasticidal Activity of Antiseptic Medicinal Products

(Ph. Eur. method 5.1.11)

This general chapter describes a test that can be used for the determination of antimicrobial activity in antiseptic medicinal products that are miscible with water and intended for administration by direct contact with the skin or mucous membranes. The extent of testing is dependent on the declared antimicrobial activity of the product.

The test determines whether a product exhibits bactericidal, fungicidal or yeasticidal activity and complies with an established specification for such activity.

This test cannot replace or confirm the assessment of the clinical efficacy of such preparations.

1 PRINCIPLE

Antimicrobial activity is determined by adding test suspensions of micro-organisms (bacteria, fungi or yeasts, separately) to the sample antiseptic product. The mixture is maintained at 33 ± 1 °C for contact times of 5 min for bactericidal activity and 15 min for fungicidal or yeasticidal activity. Additional contact times may be chosen, according to the stated use of the antiseptic medicinal product. At the end of the contact time, an aliquot is taken and the antimicrobial activity in this aliquot is immediately stopped by a validated method. 2 methods are available: dilution-neutralisation and membrane filtration.

The procedure is validated to verify its ability to demonstrate the required reduction in the count of viable micro-organisms by the use of appropriate controls.

2 TEST MICRO-ORGANISMS AND GROWTH CONDITIONS

Prepare standardised stable suspensions of test strains as stated in section 2-1. Seed-lot culture maintenance techniques (seed-lot systems) are used so that the viable micro-organisms used for inoculation are not more than 5 passages removed from the original master seed-lot. Grow each of the microbial test strains separately as described in Table 5.1.11.-1.

Table 5.1.11.-1. – *Test micro-organisms and growth conditions*

Strains for bactericidal activity testing	
Staphylococcus aureus	such as ATCC 6538, NCIMB 9518, CIP 4.83, NBRC 13276
Enterococcus hirae	such as ATCC 10541, NCIMB 8192, CIP 58.55, DSM 3320
Escherichia coli	such as NCIMB 10083, CIP 54.117, NCTC 10538, DSM 11250
Pseudomonas aeruginosa	such as ATCC 15442, NCIMB 8626, CIP 103467, NBRC 13275
Bacterial growth conditions	
Casein soya bean digest agar or casein soya bean digest broth - for preparation of test strains: 30-35 °C, 18-24 h and subculture at least twice - for testing of the product and validation of the test: 30-35 °C, ≤ 3 days	number of CFU in test suspension: 1-5 × 10^8 CFU/mL
Strain for yeasticidal activity testing	
Candida albicans	such as ATCC 10231, NCPF 3179, CIP 48.72, NBRC 1594
Yeast growth conditions	
Sabouraud-dextrose agar or Sabouraud-dextrose broth - for preparation of test strains: 20-25 °C, 2-3 days and subculture at least twice - for testing of the product and validation of the test: 20-25 °C, ≤ 5 days	number of CFU in test suspension: 1-5 × 10^7 CFU/mL
Strains for fungicidal activity testing	
Candida albicans	such as ATCC 10231, NCPF 3179, CIP 48.72, NBRC 1594
Aspergillus brasiliensis	such as ATCC 16404, IMI 149007, CIP 1431.83, NBRC 9455
Fungal growth conditions	
Sabouraud-dextrose agar or Sabouraud-dextrose broth - for preparation of test suspension of *C. albicans*: 20-25 °C, 2-3 days - for preparation of test suspension of *A. brasiliensis* spores: 20-25 °C, at least 5 days until good sporulation - for testing of the product and validation of the test with *C. albicans* and *A. brasiliensis*: 20-25 °C, ≤ 5 days	number of CFU in test suspension: 1-5 × 10^7 CFU/mL

The recommended solutions and media are described in general chapter *2.6.13*. Purified water is used. All reagents are sterile prior to use.

The test for bactericidal, fungicidal or yeasticidal activity is performed with the designated strains as described in Table 5.1.11.-1. In addition to these micro-organisms, it may be necessary to add other bacterial or fungal strains that represent the indications of the antiseptic medicinal product tested.

Single-strain challenges are used. The counts are performed in duplicate and the arithmetic mean of the results is calculated and expressed in CFU/mL.

2-1 PREPARATION OF TEST SUSPENSION

For harvesting the micro-organisms use a sufficient volume of a 9 g/L solution of *sodium chloride R* (for bacteria and *C. albicans*) or a solution containing 9 g/L of *sodium chloride R* and 0.5 g/L of *polysorbate 80 R* (for *A. brasiliensis*), to obtain a test suspension with the number of CFU described in Table 5.1.11.-1. Use the suspension within 2 h or within 24 h if stored at 2-8 °C.

2-2 PREPARATION OF ANTISEPTIC PRODUCT TEST SOLUTION

The concentration of the antiseptic product test solution shall be, if possible, 1.25 times the in-use test concentration because it is diluted to 80 per cent during the test and the method validation.

2-3 NEUTRALISING AGENTS

Neutralising agents are used to neutralise the antimicrobial activity of the antiseptic product. The common neutralising agents are listed in Table 2.6.12.-2 of general chapter *2.6.12. Microbiological examination of non-sterile products: microbial enumeration tests*. The neutralisation time is not less than 10 s and not more than 60 s.

3 METHODS

Prior to testing, equilibrate the temperature of all reagents to 33 ± 1 °C.

3-1 DILUTION-NEUTRALISATION METHOD

Transfer 1.0 mL of a 3 g/L solution of *bovine albumin R* into a tube, add 1.0 mL of the test suspension and maintain at 33 ± 1 °C for 2 min. Add 8.0 mL of the antiseptic product test solution and maintain at 33 ± 1 °C for the chosen contact time. Then, take a 1.0 mL sample of the test mixture and transfer into a tube containing 1.0 mL of *water R* and 8.0 mL of the neutralising agent and maintain at 33 ± 1 °C for the appropriate neutralisation time. Take 1.0 mL of the neutralised test mixture, in duplicate, and inoculate using the pour-plate or surface-spread method. For incubation conditions, see Table 5.1.11.-1. After incubation, perform the count.

3-1-1 Suitability of the test/controls

For all methods, prepare a validation suspension containing 100-1000 CFU of the test micro-organisms per millilitre.

3-1-1-1 Experimental conditions control

Transfer 1.0 mL of a 3 g/L solution of *bovine albumin R* into a tube, add 1.0 mL of the validation suspension and maintain at 33 ± 1 °C for 2 min. Add 8.0 mL of *water R* and maintain at 33 ± 1 °C for the chosen contact time. Take 1.0 mL of this mixture, in duplicate, and inoculate using the pour-plate or surface-spread method. For incubation conditions, see Table 5.1.11.-1. After incubation, perform the count. The number of CFU recovered following incubation is not less than 0.5 × (number of CFU in the validation suspension)/10.

3-1-1-2 Neutralising agent control

Transfer 1.0 mL of a 3 g/L solution of *bovine albumin R* into a tube, add 1.0 mL of the validation suspension and 8.0 mL of the neutralising agent used in the test and maintain at 33 ± 1 °C for the appropriate neutralisation time. Take 1.0 mL of this mixture, in duplicate, and inoculate using the pour-plate or surface-spread method. For incubation conditions, see Table 5.1.11.-1. After incubation, perform the count. The number of CFU recovered following incubation is not less than 0.5 × (number of CFU in the validation suspension)/10.

3-1-1-3 Dilution-neutralisation method control

Transfer 1.0 mL of a 3 g/L solution of *bovine albumin R* into a tube, add 1.0 mL of a 9 g/L solution of *sodium chloride R* and 8.0 mL of the product test solution and maintain at 33 ± 1 °C for the chosen contact time. Transfer 1.0 mL of this mixture into a tube containing 8.0 mL of the neutralising agent and maintain at 33 ± 1 °C for the appropriate neutralisation time. Then add 1.0 mL of the validation suspension and mix. After 30 min, take a sample of 1.0 mL of the mixture, in duplicate, and inoculate using the pour-plate or surface-spread method. For incubation conditions, see Table 5.1.11.-1. After incubation, perform the count. The number of CFU recovered following incubation is not less than 0.5 × (number of CFU in the validation suspension)/10.

3-2 MEMBRANE FILTRATION METHOD

Proceed as described in section 3-1, carrying out immediately the filtration step in place of the neutralisation step.

Use membrane filters having a nominal pore size not greater than 0.45 µm. The type of filter material is chosen such that the microbe-retaining efficiency is not affected by the components of the sample to be investigated. For each of the micro-organisms listed, a single membrane filter is used. Appropriately dilute 0.1 mL of the test solution and immediately filter the total volume, then rinse the membrane filter with an appropriate volume of the diluent. Perform the test in duplicate. For incubation conditions, see Table 5.1.11.-1. After incubation, perform the count.

3-2-1 Verification of the selected experimental conditions and of the membrane filtration method

3-2-1-1 Experimental conditions control

Proceed as described in section 3-1-1-1, except at the end of the contact time, take the sample in duplicate, and transfer into a separate membrane filtration apparatus. Filter immediately and then transfer each of the membrane filters to the surface of separate plates. For incubation conditions, see Table 5.1.11.-1. After incubation, perform the count. The number of CFU recovered following incubation is not less than 0.5 × (number of CFU in the validation suspension)/10.

3-2-1-2 Membrane filtration method control

Proceed as described in section 3-1-1-3, except at the end of the chosen contact time, take that sample in duplicate, and transfer into a separate membrane filtration apparatus. Filter and rinse as described in section 3-2, then cover the membranes with rinsing liquid and add a sample of the validation suspension. Filter again and transfer each of the membrane filters to the surface of separate plates. For incubation conditions, see Table 5.1.11.-1. After incubation, perform the count. The number of CFU recovered following incubation is not less than 0.5 × (number of CFU in the validation suspension)/10.

4 ACCEPTANCE CRITERIA

Unless otherwise justified and authorised, the preparation has a:

- bactericidal activity if the defined number of CFU is reduced by at least 5 $\log_{10}$;
- fungicidal activity if the defined number of CFU is reduced by at least 4 $\log_{10}$;
- yeasticidal activity if the defined number of CFU is reduced by at least 4 $\log_{10}$.

Appendix XV

Production and Testing of Vaccines

A. Terminology used in Monographs on Biological Products

(Ph. Eur. general texts 5.2.1)

Terminology: Vaccines

For some items, alternative terms commonly used in connection with veterinary vaccines are shown in parenthesis.

Seed-lot system
A seed-lot system is a system according to which successive batches of a product are derived from the same master seed lot. For routine production, a working seed lot may be prepared from the master seed lot. The origin and the passage history of the master seed lot and the working seed lot are recorded.

Master seed lot
A culture of a micro-organism distributed from a single bulk into containers and processed together in a single operation in such a manner as to ensure uniformity and stability and to prevent contamination. A master seed lot in liquid form is usually stored at or below −70 °C. A freeze-dried master seed lot is stored at a temperature known to ensure stability.

Working seed lot
A culture of a micro-organism derived from the master seed lot and intended for use in production. Working seed lots are distributed into containers and stored as described above for master seed lots.

Cell-bank system (Cell-seed system)
A system whereby successive final lots (batches) of a product are manufactured by culture in cells derived from the same master cell bank (master cell seed). A number of containers from the master cell bank (master cell seed) are used to prepare a working cell bank (working cell seed). The cell-bank system (cell-seed system) is validated for the highest passage level achieved during routine production.

Master cell bank (Master cell seed)
A culture of cells distributed into containers in a single operation, processed together and stored in such a manner as to ensure uniformity and stability and to prevent contamination. A master cell bank (master cell seed) is usually stored at −70 °C or lower.

Working cell bank (Working cell seed)
A culture of cells derived from the master cell bank (master cell seed) and intended for use in the preparation of production cell cultures. The working cell bank (working cell seed) is distributed into containers, processed and stored as described for the master cell bank (master cell seed).

Primary cell cultures
Cultures of cells obtained by trypsination of a suitable tissue or organ. The cells are essentially identical to those of the tissue of origin and are no more than 5 *in vitro* passages from the initial preparation from the animal tissue.

Cell lines
Cultures of cells that have a high capacity for multiplication *in vitro*. In diploid cell lines, the cells have essentially the same characteristics as those of the tissue of origin.
In continuous cell lines, the cells are able to multiply indefinitely in culture and may be obtained from healthy or tumoral tissue. Some continuous cell lines have oncogenic potential under certain conditions.

Production cell culture
A culture of cells intended for use in production; it may be derived from one or more containers of the working cell bank (working cell seed) or it may be a primary cell culture.

Control cells
A quantity of cells set aside, at the time of virus inoculation, as uninfected cell cultures. The uninfected cells are incubated under similar conditions to those used for the production cell cultures.

Single harvest
Material derived on one or more occasions from a single production cell culture inoculated with the same working seed lot or a suspension derived from the working seed lot, incubated, and harvested in a single production run.

Monovalent pooled harvest
Pooled material containing a single strain or type of micro-organism or antigen and derived from a number of eggs, cell culture containers etc. that are processed at the same time.

Final bulk vaccine
Material that has undergone all the steps of production except for the final filling. It consists of one or more monovalent pooled harvests, from cultures of one or more species or types of micro-organism, after clarification, dilution or addition of any adjuvant or other auxiliary substance. It is treated to ensure its homogeneity and is used for filling the containers of one or more final lots (batches).

Final lot (Batch)
A collection of closed, final containers or other final dosage units that are expected to be homogeneous and equivalent with respect to risk of contamination during filling or preparation of the final product. The dosage units are filled, or otherwise prepared, from the same final bulk vaccine, freeze-dried together (if applicable) and closed in one continuous working session. They bear a distinctive number or code identifying the final lot (batch). Where a final bulk vaccine is filled and/or freeze-dried on several separate sessions, there results a related set of final lots (batches) that are usually identified by the use of a common part in the distinctive number or code; these related final lots (batches) are sometimes referred to as sub-batches, sub-lots or filling lots.

Combined vaccine
A multicomponent preparation formulated so that different antigens are administered simultaneously. The different antigenic components are intended to protect against different strains or types of the same organism and/or different organisms. A combined vaccine may be supplied by the manufacturer either as a single liquid or freeze-dried preparation or as several constituents with directions for admixture before use.

B. Aluminium in Adsorbed Vaccines

(Ph. Eur. method 2.5.13)

Homogenise the preparation to be examined and transfer a suitable quantity, presumed to contain 5 mg to 6 mg of aluminium, to a 50 mL combustion flask. Add 1 mL of *sulfuric acid R*, 0.1 mL of *nitric acid R* and some glass beads. Heat the solution until thick, white fumes are evolved.

If there is charring at this stage add a few more drops of *nitric acid R* and continue boiling until the colour disappears. Allow to cool for a few minutes, carefully add 10 mL of *water R* and boil until a clear solution is obtained. Allow to cool, add 0.05 mL of *methyl orange solution R* and neutralise with *strong sodium hydroxide solution R* (6.5 mL to 7 mL). If a precipitate forms dissolve it by adding, dropwise, sufficient *dilute sulfuric acid R*. Transfer the solution to a 250 mL conical flask, rinsing the combustion flask with 25 mL of *water R*. Add 25.0 mL of *0.02 M sodium edetate*, 10 mL of *acetate buffer solution pH 4.4 R* and a few glass beads and boil gently for 3 min. Add 0.1 mL of *pyridylazonaphthol solution R* and titrate the hot solution with *0.02 M copper sulfate* until the colour changes to purplish-brown. Carry out a blank titration omitting the vaccine.

1 mL of *0.02 M sodium edetate* is equivalent to 0.5396 mg of Al.

C. Calcium in Adsorbed Vaccines

(Ph. Eur. method 2.5.14)

All solutions used for this test must be prepared using water R.

Determine the calcium by atomic emission spectrometry (*2.2.22, Method I*). Homogenise the preparation to be examined. To 1.0 mL add 0.2 mL of *dilute hydrochloric acid R* and dilute to 3.0 mL with *water R*. Measure the absorbance at 620 nm.

D. Free Formaldehyde

(Ph. Eur. method 2.4.18)

Use method A, unless otherwise prescribed. Method B is suitable for vaccines where sodium metabisulfite has been used to neutralise excess formaldehyde.

METHOD A

For vaccines for human use, prepare a 1 in 10 dilution of the vaccine to be examined. For bacterial toxoids for veterinary use, prepare a 1 in 25 dilution of the vaccine to be examined.

To 1 mL of the dilution, add 4 mL of *water R* and 5 mL of *acetylacetone reagent R1*. Place the tube in a water-bath at 40 °C for 40 min. Examine the tubes down their vertical axes. The solution is not more intensely coloured than a standard, prepared at the same time and in the same manner, using 1 mL of a dilution of *formaldehyde solution R* containing 20 µg of formaldehyde (CH_2O) per millilitre, instead of the dilution of the vaccine to be examined.

METHOD B

Test solution Prepare a 1 in 200 dilution of the vaccine to be examined with *water R*. If the vaccine is an emulsion, prepare an equivalent dilution using the aqueous phase separated by a suitable procedure (see below). If one of the methods described below is used for separation of the aqueous phase, a 1 in 20 dilution of the latter is used.

Reference solutions Prepare solutions containing 0.25 g/L, 0.50 g/L, 1.00 g/L and 2.00 g/L of CH_2O by dilution of *formaldehyde solution R* with *water R*. Prepare a 1 in 200 dilution of each solution with *water R*.

To 0.5 mL of the test solution and of each of the reference solutions in test-tubes, add 5.0 mL of a freshly prepared 0.5 g/L solution of *methylbenzothiazolone hydrazone hydrochloride R*. Close the tubes, shake and allow to stand for 60 min. Add 1 mL of ferric chloride-sulfamic acid reagent R and allow to stand for 15 min. Measure the absorbance (*2.2.25*) of the solutions at 628 nm. Calculate the content of formaldehyde in the vaccine to be examined from the calibration curve established using the reference solutions. The test is invalid if the correlation coefficient (r) of the calibration curve is less than 0.97.

Emulsions If the vaccine to be examined is an emulsion, the aqueous phase is separated using a suitable procedure and used for preparation of the test solution. The following procedures have been found suitable.

(a) Add 1.0 mL of the vaccine to be examined to 1.0 mL of *isopropyl myristate R* and mix. Add 1.3 mL of *1 M hydrochloric acid*, 2.0 mL of *chloroform R* and 2.7 mL of a 9 g/L solution of *sodium chloride R*. Mix thoroughly. Centrifuge at 15 000 *g* for 60 min. Transfer the aqueous phase to a 10 mL volumetric flask and dilute to volume with *water R*. If this procedure fails to separate the aqueous phase, add 100 g/L of *polysorbate 20 R* to the sodium chloride solution and repeat the procedure but centrifuge at 22 500 *g*.

(b) Add 1.0 mL of the vaccine to be examined to 1.0 mL of a 100 g/L solution of *sodium chloride R* and mix. Centrifuge at 1000 *g* for 15 min. Transfer the aqueous phase to a 10 mL volumetric flask and dilute to volume with *water R*.

(c) Add 1.0 mL of the vaccine to be examined to 2.0 mL of a 100 g/L solution of *sodium chloride R* and 3.0 mL of *chloroform R* and mix. Centrifuge at 1000 *g* for 5 min. Transfer the aqueous phase to a 10 mL volumetric flask and dilute to volume with *water R*.

E. Phenol in Immunosera (Antisera) and Vaccines

(Ph. Eur. method 2.5.15)

Homogenise the preparation to be examined. Dilute an appropriate volume with *water R* so as to obtain a solution presumed to contain 15 µg of phenol per millilitre. Prepare a series of reference solutions with *phenol R* containing 5 µg, 10 µg, 15 µg, 20 µg and 30 µg of phenol per millilitre respectively. To 5 mL of the solution to be examined and to 5 mL of each of the reference solutions respectively, add 5 mL of *buffer solution pH 9.0 R*, 5 mL of *aminopyrazolone solution R* and 5 mL of *potassium ferricyanide solution R*. Allow to stand for 10 min and measure the intensity of colour at 546 nm.

Plot the calibration curve and calculate the phenol content of the preparation to be examined.

F. Neurovirulence

Test for Neurovirulence of Live Virus Vaccines

(Ph. Eur. method 2.6.18)

For each test, use not fewer than ten monkeys that are seronegative for the virus to be tested. For each monkey, inject not more than 0.5 mL of the material to be examined into the thalamic region of each hemisphere, unless otherwise prescribed. The total amount of virus inoculated in each monkey must be not less than the amount contained in the recommended single human dose of the vaccine. As a check against the introduction of wild neurovirulent virus, keep a

group of not fewer than four control monkeys as cage-mates or in the immediate vicinity of the inoculated monkeys. Observe the inoculated monkeys for 17 to 21 days for symptoms of paralysis and other evidence of neurological involvement; observe the control monkeys for the same period plus 10 days. Animals that die within 48 h of injection are considered to have died from non-specific causes and may be replaced. The test is not valid if: more than 20 per cent of the inoculated monkeys die from nonspecific causes; serum samples taken from the control monkeys at the time of inoculation of the test animals and 10 days after the latter are euthanised show evidence of infection by wild virus of the type to be tested or by measles virus. At the end of the observation period, carry out autopsy and histopathological examinations of appropriate areas of the brain for evidence of central nervous system involvement. The material complies with the test if there is no unexpected clinical or histopathological evidence of involvement of the central nervous system attributable to the inoculated virus.

G. Composition of Polysaccharide Vaccines

Protein

(Ph. Eur. method 2.5.16)

Test solution Use a volumetric flask with a suitable volume for preparation of a solution containing about 5 mg per millilitre of dry polysaccharide. Transfer the contents of a container quantitatively to the flask and dilute to volume with *water R*. Place 1 mL of the solution in a glass tube and add 0.15 mL of a 400 g/L solution of *trichloroacetic acid R*. Shake, allow to stand for 15 min, centrifuge for 10 min at 5000 r/min and discard the supernatant. Add 0.4 mL of *0.1 M sodium hydroxide* to the centrifugation residue.

Reference solutions Dissolve 0.100 g of *bovine albumin R* in 100 mL of *0.1 M sodium hydroxide* (stock solution containing 1 g of protein per litre). Dilute 1 mL of the stock solution to 20 mL with *0.1 M sodium hydroxide* (working dilution 1: 50 mg of protein per litre). Dilute 1 mL of the stock solution to 4 mL with *0.1 M sodium hydroxide* (working dilution 2: 250 mg of protein per litre). Place in 6 glass tubes 0.10 mL, 0.20 mL and 0.40 mL of working dilution 1 and 0.15 mL, 0.20 mL and 0.25 mL of working dilution 2. Make up the volume in each tube to 0.40 mL using *0.1 M sodium hydroxide*.

Prepare a blank using 0.40 mL of *0.1 M sodium hydroxide*.

Add 2 mL of *cupri-tartaric solution R3* to each tube, shake and allow to stand for 10 min. Add to each tube 0.2 mL of a mixture of equal volumes of *phosphomolybdotungstic reagent R* and *water R*, prepared immediately before use. Stopper the tubes, mix by inverting and allow to stand in the dark for 30 min. The blue colour is stable for 60 min. If necessary, centrifuge to obtain clear solutions.

Measure the absorbance (*2.2.25*) of each solution at 760 nm using the blank as the compensation liquid. Draw a calibration curve from the absorbances of the 6 reference solutions and the corresponding protein contents and read from the curve the content of protein in the test solution.

Nucleic Acids

(Ph. Eur. method 2.5.17)

Test solution Use a volumetric flask with a suitable volume for preparation of a solution containing about 5 mg per millilitre of dry polysaccharide. Transfer the contents of a container quantitatively to the flask and dilute to volume with *water R*.

Dilute the test solution if necessary to obtain an absorbance value suitable for the instrument used. Measure the absorbance (*2.2.25*) at 260 nm using *water R* as the compensation liquid.

The absorbance of a 1 g/L solution of nucleic acid at 260 nm is 20.

Phosphorus

(Ph. Eur. method 2.5.18)

Test solution Use a volumetric flask with a suitable volume for preparation of a solution containing about 5 mg per millilitre of dry polysaccharide. Transfer the contents of a container quantitatively to the flask and dilute to volume with *water R*. Dilute the solution so that the volume used in the test (1 mL) contains about 6 µg of phosphorus. Transfer 1.0 mL of the solution to a 10 mL ignition tube.

Reference solutions Dissolve 0.2194 g of *potassium dihydrogen phosphate R* in 500 mL of *water R* to give a solution containing the equivalent of 0.1 mg of phosphorus per millilitre. Dilute 5.0 mL of the solution to 100.0 mL with *water R*. Introduce 0.5 mL, 1.0 mL and 2.0 mL of the dilute solution into 3 ignition tubes.

Prepare a blank solution using 2.0 mL of *water R* in an ignition tube.

To all the tubes add 0.2 mL of *sulfuric acid R* and heat in an oil bath at 120 °C for 1 h and then at 160 °C until white fumes appear (about 1 h). Add 0.1 mL of *perchloric acid R* and heat at 160 °C until the solution is decolorised (about 90 min). Cool and add to each tube 4 mL of *water R* and 4 mL of *ammonium molybdate reagent R*. Heat in a water-bath at 37 °C for 90 min and cool. Adjust the volume to 10.0 mL with *water R*. The blue colour is stable for several hours.

Measure the absorbance (*2.2.25*) of each solution at 820 nm using the blank solution as the compensation liquid. Draw a calibration curve with the absorbances of the 3 reference solutions as a function of the quantity of phosphorus in the solutions and read from the curve the quantity of phosphorus in the test solution.

O-Acetyl Groups

(Ph. Eur. method 2.5.19)

Test solution Use a volumetric flask with a suitable volume for preparation of a solution containing about 5 mg per millilitre of dry polysaccharide. Transfer the contents of a container quantitatively to the flask and dilute to volume with *water R*. Dilute the solution so that the volumes used in the test contain 30 µg to 600 µg of acetylcholine chloride (*O*-acetyl). Introduce 0.3 mL, 0.5 mL and 1.0 mL in duplicate into 6 tubes (3 reaction solutions and 3 correction solutions).

Reference solutions Dissolve 0.150 g of *acetylcholine chloride R* in 10 mL of *water R* (stock solution containing 15 g of acetylcholine chloride per litre). Immediately before use, dilute 1 mL of the stock solution to 50 mL with *water R* (working dilution 1: 300 µg of acetylcholine chloride per millilitre). Immediately before use, dilute 1 mL of the stock solution to 25 mL with *water R* (working dilution 2: 600 µg of acetylcholine chloride per millilitre). Introduce 0.1 mL and 0.4 mL of working dilution 1 in duplicate (reaction and correction solutions) into 4 tubes and 0.6 mL and 1.0 mL of working dilution 2 in duplicate (reaction and correction solutions) into another 4 tubes.

Prepare a blank using 1 mL of *water R*.

Make up the volume in each tube to 1 mL with *water R*. Add 1.0 mL of a 4 M solution of hydrochloric acid prepared from *hydrochloric acid R* to each of the correction tubes and to the blank. Add 2.0 mL of *alkaline hydroxylamine solution R* to each tube. Allow the reaction to proceed for exactly 2 min and add 1.0 mL of the 4 M solution of hydrochloric acid to each of the reaction tubes. To each tube, add 1.0 mL of a 100 g/L solution of *ferric chloride R* in a 0.1 M solution of hydrochloric acid prepared from *hydrochloric acid R*, stopper the tubes and shake vigorously to remove bubbles.

Measure the absorbance (*2.2.25*) of each solution at 540 nm using the blank as the compensation liquid. For each reaction solution, subtract the absorbance of the corresponding correction solution. Draw a calibration curve from the corrected absorbances for the 4 reference solutions and the corresponding content of acetylcholine chloride and read from the curve the content of acetylcholine chloride in the test solution for each volume tested. Calculate the mean of the 3 values.

1 mole of acetylcholine chloride (181.7 g) is equivalent to 1 mole of *O*-acetyl (43.05 g).

Hexosamines

(Ph. Eur. method 2.5.20)

Test solution Use a volumetric flask with a suitable volume for preparation of a solution containing about 5 mg per millilitre of dry polysaccharide. Transfer the contents of a container quantitatively to the flask and dilute to volume with *water R*. Dilute the solution so that the volumes used in the test contain 125 µg to 500 µg of glucosamine (hexosamine). Introduce 1.0 mL of the diluted solution into a graduated tube.

Reference solutions Dissolve 60 mg of *glucosamine hydrochloride R* in 100 mL of *water R* (stock solution containing 0.500 g of glucosamine per litre). Introduce 0.25 mL, 0.50 mL, 0.75 mL, and 1.0 mL of the working dilution into 4 graduated tubes.

Prepare a blank using 1 mL of *water R*.

Make up the volume in each tube to 1 mL with *water R*. Add 1 mL of a solution of *hydrochloric acid R* (292 g/L) to each tube. Stopper the tubes and place in a water-bath for 1 h. Cool to room temperature. Add to each tube 0.05 mL of a 5 g/L solution of *thymolphthalein R* in *alcohol R*; add a solution of *sodium hydroxide R* (200 g/L) until a blue colour is obtained and then *1 M hydrochloric acid* until the solution is colourless. Dilute the volume in each tube to 10 mL with *water R* (neutralised hydrolysates).

In a second series of 10 mL graduated tubes, place 1 mL of each neutralised hydrolysate. Add 1 mL of acetylacetone reagent (a mixture, prepared immediately before use, of 1 volume of *acetylacetone R* and 50 volumes of a 53 g/L solution of *anhydrous sodium carbonate R*) to each tube. Stopper the tubes and place in a water-bath at 90 °C for 45 min. Cool to room temperature. Add to each tube 2.5 mL of *alcohol R* and 1.0 mL of dimethylaminobenzaldehyde solution (immediately before use dissolve 0.8 g of *dimethylaminobenzaldehyde R* in 15 mL of *alcohol R* and add 15 mL of *hydrochloric acid R*) and dilute the volume in each tube to 10 mL with *alcohol R*. Stopper the tubes, mix by inverting and allow to stand in the dark for 90 min. Measure the absorbance (*2.2.25*) of each solution at 530 nm using the blank as the compensation liquid.

Draw a calibration curve from the absorbances for the 4 reference solutions and the corresponding content of hexosamine and read from the curve the quantity of hexosamine in the test solution.

Methylpentoses

(Ph. Eur. method 2.5.21)

Test solution Use a volumetric flask with a suitable volume for preparation of a solution containing about 5 mg per millilitre of dry polysaccharide. Transfer the contents of a container quantitatively to the flask and dilute to volume with *water R*. Dilute the solution so that the volumes used in the test contain 2 µg to 20 µg of rhamnose (methylpentoses). Introduce 0.25 mL, 0.50 mL and 1.0 mL of the diluted solution into 3 tubes.

Reference solutions Dissolve 0.100 g of *rhamnose R* in 100 mL of *water R* (stock solution containing 1 g of methylpentose per litre). Immediately before use, dilute 1 mL of the stock solution to 50 mL with *water R* (working dilution: 20 mg of methylpentose per litre). Introduce 0.10 mL, 0.25 mL, 0.50 mL, 0.75 mL and 1.0 mL of the working dilution into 5 tubes.

Prepare a blank using 1 mL of *water R*.

Make up the volume in each tube to 1 mL with *water R*. Place the tubes in iced water and add dropwise and with continuous stirring to each tube 4.5 mL of a cooled mixture of 1 volume of *water R* and 6 volumes of *sulfuric acid R*. Warm the tubes to room temperature and place in a water-bath for a few minutes. Cool to room temperature. Add to each tube 0.10 mL of a 30 g/L solution of *cysteine hydrochloride R*, prepared immediately before use. Shake and allow to stand for 2 h.

Measure the absorbance (*2.2.25*) of each solution at 396 nm and at 430 nm using the blank as compensation liquid. For each solution, calculate the difference between the absorbance measured at 396 nm and that measured at 430 nm. Draw a calibration curve from the absorbance differences for the 5 reference solutions and the corresponding content of methylpentose and read from the curve the quantity of methylpentose in the test solution for each volume tested. Calculate the mean of the 3 values.

Uronic Acids

(Ph. Eur. method 2.5.22)

Test solution Use a volumetric flask with a suitable volume for preparation of a solution containing about 5 mg per millilitre of dry polysaccharide. Transfer the contents of a container quantitatively to the flask and dilute to volume with *water R*. Dilute the solution so that the volumes used in the test contain 4 µg to 40 µg of glucuronic acid (uronic acids). Introduce 0.25 mL, 0.50 mL and 1.0 mL of the diluted solution into 3 tubes.

Reference solutions Dissolve 50 mg of *sodium glucuronate R* in 100 mL of *water R* (stock solution containing 0.4 g of glucuronic acid per litre). Immediately before use, dilute 5 mL of the stock solution to 50 mL with *water R* (working dilution: 40 mg of glucuronic acid per litre). Introduce 0.10 mL, 0.25 mL, 0.50 mL, 0.75 mL, and 1.0 mL of the working dilution into 5 tubes.

Prepare a blank using 1 mL of *water R*.

Make up the volume in each tube to 1 mL with *water R*. Place the tubes in iced water and add dropwise and with continuous stirring to each tube 5.0 mL of *borate solution R*. Stopper the tubes and place in a water-bath for 15 min. Cool to room temperature. Add 0.20 mL of a 1.25 g/L solution of *carbazole R* in *ethanol R* to each tube. Stopper the tubes and place in a water-bath for 15 min. Cool to room temperature.

Measure the absorbance (*2.2.25*) of each solution at 530 nm using the blank as the compensation liquid.

Draw a calibration curve from the absorbances for the 5 reference solutions and the corresponding content of glucuronic acid and read from the curve the quantity of glucuronic acid in the test solution for each volume tested. Calculate the mean of the 3 values.

Sialic Acid

(Ph. Eur. method 2.5.23)

Test solution Transfer quantitatively the contents of one or several containers to a volumetric flask of a suitable volume that will give a solution with a known concentration of about 250 µg per millilitre of polysaccharide and dilute to volume with *water R*. Using a syringe, transfer 4.0 mL of this solution to a 10 mL ultrafiltration cell suitable for the passage of molecules of relative molecular mass less than 50 000. Rinse the syringe twice with *water R* and transfer the rinsings to the ultrafiltration cell. Carry out the ultrafiltration, with constant stirring, under *nitrogen R* at a pressure of about 150 kPa. Refill the cell with *water R* each time the volume of liquid in it has decreased to 1 mL and continue until 200 mL has been filtered and the remaining volume in the cell is about 2 mL. Using a syringe, transfer this residual liquid to a 10 mL volumetric flask. Wash the cell with 3 quantities, each of 2 mL, of *water R*, transfer the washings to the flask and dilute to 10.0 mL with *water R* (test solution). In each of 2 test-tubes place 2.0 mL of the test solution.

Reference solutions Use the reference solutions prescribed in the monograph.

Prepare 2 series of 3 test-tubes, place in the tubes of each series 0.5 mL, 1.0 mL and 1.5 mL respectively, of the reference solution corresponding to the type of vaccine to be examined and adjust the volume in each tube to 2.0 mL with *water R*.

Prepare blank solutions using 2.0 mL of *water R* in each of 2 test-tubes.

To all the tubes add 5.0 mL of *resorcinol reagent R*. Heat at 105 °C for 15 min, cool in cold water and transfer the tubes to a bath of iced water. To each tube add 5 mL of *isoamyl alcohol R* and mix thoroughly. Place in the bath of iced water for 15 min. Centrifuge the tubes and keep them in the bath of iced water until the examination by absorption spectrophotometry. Measure the absorbance (*2.2.25*) of each supernatant solution at 580 nm and 450 nm using *isoamyl alcohol R* as the compensation liquid. For each wavelength, calculate the absorbance as the mean of the values obtained with 2 identical solutions. Subtract the mean value for the blank solution from the mean values obtained for the other solutions.

Draw a graph showing the difference between the absorbances at 580 nm and 450 nm of the reference solutions as a function of the content of *N*-acetylneuraminic acid and read from the graph the quantity of *N*-acetylneuraminic acid (sialic acid) in the test solution.

Ribose

(Ph. Eur. method 2.5.31)

Test solution Use a volumetric flask with a suitable volume for preparation of a solution containing about 5 mg per millilitre of dry polysaccharide. Transfer the contents of a container quantitatively to the flask and dilute to volume with *water R*. Dilute the solution so that the volumes used in the test contain 2.5 µg to 25 µg of ribose. Introduce 0.20 mL and 0.40 mL of the diluted solution into tubes in triplicate.

Reference solutions Dissolve 25 mg of *ribose R* in *water R* and dilute to 100.0 mL with the same solvent (stock solution containing 0.25 g/L of ribose). Immediately before use, dilute 1 mL of the stock solution to 10.0 mL with *water R* (working dilution: 25 mg/L of ribose). Introduce 0.10 mL, 0.20 mL, 0.40 mL, 0.60 mL, 0.80 mL and 1.0 mL of the working dilution into 6 tubes.

Prepare a blank using 2 mL of *water R*.

Make up the volume in each tube to 2 mL with *water R*. Shake. Add 2 mL of a 0.5 g/L solution of *ferric chloride R* in *hydrochloric acid R* to each tube. Shake. Add 0.2 mL of a 100 g/L solution of *orcinol R* in *ethanol R*. Place the tubes in a water-bath for 20 min. Cool in iced water. Measure the absorbance (*2.2.25*) of each solution at 670 nm using the blank as the compensation liquid. Draw a calibration curve from the absorbance readings for the 6 reference solutions and the corresponding content of ribose and read from the curve the quantity of ribose in the test solution for each volume tested. Calculate the mean of the 3 values.

H. Chicken Flocks Free from Specified Pathogens for the Production and Quality Control of Vaccines

(Ph. Eur. method 5.2.2)

Where specified, chickens, embryos or cell cultures used for the production or quality control of vaccines are derived from eggs produced by chicken flocks free from specified pathogens (SPF). The SPF status of a flock is ensured by means of the system described below. The list of micro-organisms given is based on current knowledge and will be updated as necessary.

A flock is defined as a group of birds sharing a common environment and having their own caretakers who have no contact with non-SPF flocks. Once a flock is defined, no non-SPF birds are added to it.

Each flock is housed so as to minimise the risk of contamination. The facility in which the flock is housed must not be sited near to any non-SPF flocks of birds with the exception of flocks that are in the process of being established as SPF flocks and that are housed in facilities and conditions appropriate to SPF flocks. The SPF flock is housed within an isolator or in a building with filtered air under positive pressure. Appropriate measures are taken to prevent entry of rodents, wild birds, insects and unauthorised personnel.

Personnel authorised to enter the facility must have no contact with other birds or with agents potentially capable of infecting the flock. It is advisable for personnel to shower and change clothing or to wear protective clothing before entering the controlled facility.

Wherever possible, items taken into the facility are sterilised. In particular it is recommended that the feed is suitably treated to avoid introduction of undesirable micro-organisms and that water is at least of potable quality, for example from a chlorinated supply. No medication is administered to birds within the flock that might interfere with detection of any disease.

A permanent record is kept of the general health of the flock and any abnormality is investigated. Factors to be monitored include morbidity, mortality, general physical condition, feed consumption, daily egg production and egg quality, fertility

and hatchability. Records are maintained for a period of at least 5 years. Details of any deviation from normal in these performance parameters or detection of any infection are notified to the users of the eggs as soon as practicable.

The tests or combination of tests described below must have suitable specificity and sensitivity with respect to relevant serotypes of the viruses. Samples for testing are taken at random.

A positive result for chicken anaemia virus (CAV) does not necessarily exclude use of material derived from the flock, but live vaccines for use in birds less than 7 days old shall be produced using material from CAV-negative flocks. Inactivated vaccines for use in birds less than 7 days old may be produced using material from flocks that have not been shown to be free from CAV, provided it has been demonstrated that the inactivation process inactivates CAV.

ESTABLISHMENT OF AN SPF FLOCK

A designated SPF flock is derived from chickens shown to be free from vertically-transmissible agents listed in Table 5.2.2-1. This is achieved by testing of 2 generations prior to the designated SPF flock. A general scheme for the procedure to be followed in establishing and maintaining an SPF flock is shown diagrammatically in Table 5.2.2.-2. In order to establish a new SPF flock, a series of tests must be conducted on 3 generations of birds. All birds in the 1st generation must be tested at least once before the age of 20 weeks for freedom from avian leucosis group-antigen and tested by an enzyme immunoassay (EIA) or by virus neutralisation (VN) for freedom of antibodies to avian leucosis virus subtypes A, B and J. All birds must also be tested for freedom from antibodies to the vertically-transmissible agents listed in Table 5.2.2-1. From the age of 8 weeks the flock is tested for freedom from *Salmonella*. Clinical examination is carried out on the flock from 8 weeks of age and the birds must not exhibit any signs of infectious disease. The test methods to be used for these tests are given in the table and further guidance is also given in the section below on routine testing of designated SPF flocks. From 20 weeks of age, the flock is tested as described under Routine testing of designated SPF flocks. All stages of this testing regime are also applied to the subsequent 2 generations, except the testing of every bird before lay for vertically-transmissible agents. All test results must indicate freedom from pathogens in all 3 generations for the flock consisting of the 3rd generation to be designated as SPF.

SPF embryos derived from another designated SPF flock contained within a separate facility on the same site may be introduced. From 8 weeks of age, these replacement birds are regarded as a flock and are tested in accordance with test procedures described above.

INITIAL TESTING REQUIREMENTS FOR SUBSEQUENT GENERATIONS DERIVED FROM A DESIGNATED SPF FLOCK

Where a replacement flock is derived exclusively from a fully established SPF flock the new generation is tested prior to being designated as SPF. In addition to the tests for *Salmonella* and monitoring of the general health and performance of the flock, further specific testing from the age of 8 weeks is required. Tests are performed on two 5 per cent samples of the flock (minimum 10, maximum 200 birds) taken with an interval of at least 4 weeks between the ages of 12-16 weeks and 16-20 weeks.

All samples are collected and tested individually. Blood samples for antibody tests and suitable samples for testing for leucosis antigen are collected. The test methods to be used are as described under Routine testing of designated SPF flocks. Only when all tests have confirmed the absence of infection may the new generation be designated as SPF.

ROUTINE TESTING OF DESIGNATED SPF FLOCKS

General examination and necropsy Clinical examination is carried out at least once per week throughout the life of the flock in order to verify that the birds are free from fowl-pox virus and signs of any other infection. In the event of mortality exceeding 0.2 per cent per week, necropsy is performed on all available carcasses to verify that there is no sign of infection. Where appropriate, histopathological and/or microbiological/virological studies are performed to confirm diagnosis. Specific examination for tuberculosis lesions is carried out and histological samples from any suspected lesions are specifically stained to verify freedom from *Mycobacterium avium*. Caecal contents of all available carcasses are examined microbiologically for the presence of *Salmonella* spp. using the techniques described below. Where appropriate, caecal samples from up to 5 birds may be pooled.

Cultural testing for Salmonella spp Cultural testing for *Salmonella* spp. is performed either by testing samples of droppings or cloacal swabs or by testing of drag swabs. Where droppings or cloacal swabs are tested, a total of 60 samples within each 4-week period is tested throughout the entire life of the flock. Tests may be performed on pools of up to 10 samples. Where drag swabs are tested, a minimum of 2 drag swabs are tested during each 4-week period throughout the entire life of the flock. Detection of *Salmonella* spp. in these samples is performed by pre-enrichment of the samples followed by culture using *Salmonella*-selective media.

Tests for avian leucosis antigen Prior to the commencement of laying, cloacal swabs or blood samples (using buffy coat cultivation) are tested for the presence of group-specific leucosis antigen. A total of 5 per cent (minimum 10, maximum 200) of the flock is sampled during each 4-week period. During lay, albumen samples from 5 per cent (minimum 10, maximum 200) of the eggs are tested in each 4-week period. Tests are performed by EIA for group-specific antigen using methods that are capable of detecting antigen from subgroups A, B and J.

Test for antibodies to other agents Tests for antibodies to all agents listed in Table 5.2.2.-1 are performed throughout the laying period of the flock. In each 4-week period, samples are taken from 5 per cent (minimum 10, maximum 200) of the flock. It is recommended that 1.25 per cent of the flock is sampled each week since some test methods for some agents must be conducted on a weekly basis. Table 5.2.2.-1 classifies the agents into those that spread rapidly through the flock and those that spread slowly or may not infect the entire flock. For those agents listed as slowly spreading, each sample is tested individually. For those agents listed as rapidly spreading, at least 20 per cent of the samples collected in each 4-week period are tested individually or, where serum neutralisation or ELISA tests are employed, all of the samples may be tested individually or by preparing pools of 5 samples, collected at the same time.

Suitable methods to be used for detection of the agents are shown in Table 5.2.2.-1. Subject to agreement by the competent authority, other test methods may be used provided they are shown to be at least as sensitive as those indicated and of appropriate specificity.

Table 5.2.2.-1

Agent	Test to be used**	Vertical transmission	Rapid/slow spread
Avian adenoviruses, group I	AGP, EIA	yes	slow
Avian encephalomyelitis virus	AGP, EIA	yes	rapid
Avian infectious bronchitis virus	HI, EIA	no	rapid
Avian infectious laryngotracheitis virus	VN, EIA	no	slow
Avian leucosis viruses	EIA for virus, VN, EIA for antibody	yes	slow
Avian nephritis virus	IS	no	slow
Avian orthoreoviruses	IS, EIA	yes	slow
Avian reticuloendotheliosis virus	AGP, IS, EIA	yes	slow
Chicken anaemia virus	IS, EIA, VN	yes	slow
Egg drop syndrome virus	HI, EIA	yes	slow
Infectious bursal disease virus	Serotype 1: AGP, EIA, VN Serotype 2: VN	no	rapid
Influenza A virus	AGP, EIA, HI	no	rapid
Marek's disease virus	AGP	no	rapid
Newcastle disease virus	HI, EIA	no	rapid
Turkey rhinotracheitis virus	EIA	no	slow
Mycoplasma gallisepticum	Agg and HI to confirm a positive test, EIA, HI	yes	slow
Mycoplasma synoviae	Agg and HI to confirm a positive test, EIA, HI	yes	rapid
Salmonella pullorum	Agg	yes	slow

Agg: agglutination
AGP: agar gel precipitation; the technique is suitable where testing is carried out weekly
EIA: enzyme immunoassay
HI: haemagglutination inhibition
IS: immunostaining
VN: virus neutralisation

**Subject to agreement by the competent authority, other types of test may be used provided they are at least as sensitive as those indicated and of appropriate specificity.

Table 5.2.2-2. – *Schematic description of the establishment and maintenance of SPF flocks*

NEW STOCK	Establish freedom from vertically-transmissible agents
	Test all birds for avian leucosis antigen and antibodies prior to 20 weeks of age
	Test for *Salmonella* spp. and perform general clinical observation from 8 weeks of age
	Carry out routine testing for specified agents from 20 weeks of age
2nd GENERATION	Test all birds for avian leucosis antigen and antibodies prior to 20 weeks of age
	Test for *Salmonella* spp. and perform general clinical observation from 8 weeks of age
	Carry out routine testing for specified agents from 20 weeks of age
3rd GENERATION	Test all birds for avian leucosis antigen and antibodies prior to 20 weeks of age
	Test for *Salmonella* spp. and perform general clinical observation from 8 weeks of age
	DESIGNATE FLOCK AS SPF IF ALL TESTS ARE SATISFACTORY
3rd GENERATION	Carry out routine testing for specified agents from 20 weeks of age
	Carry out post-lay testing for vertically-transmissible agents
SUBSEQUENT GENERATIONS	Test two 5 per cent samples for avian leucosis antigen and for antibodies against specified agents between 12 and 20 weeks of age
	Test for *Salmonella* spp. and perform general clinical observation from 8 weeks of age
	Carry out routine testing for specified agents from 20 weeks of age
	Carry out post-lay testing for vertically-transmissible agents

TESTS TO BE CONDUCTED AT THE END OF THE LAYING PERIOD

Following the last egg collection, final testing to confirm the absence of vertically-transmissible agents indicated in Table 5.2.2.-1 is performed. After the last egg collection, a minimum of 5 per cent of the flock (minimum 10, maximum 200) is retained for at least 4 weeks. Blood samples are collected from every bird in the group during the 4-week period with at least 1.25 per cent of the birds (25 per cent of the sample) being bled not earlier than 4 weeks after the final egg collection. Serum samples are tested for vertically-transmissible agents (as defined by Table 5.2.2.-1) using the

methods indicated. Where sampling is performed on a weekly basis, at least 1.25 per cent of the birds (25 per cent of the sample) are tested each week during this period. Alternatively, within 4 weeks of the final egg collection blood and/or other suitable sample materials are collected from at least 5 per cent of the flock and tested for the presence of vertically-transmissible agents using validated nucleic acid amplification techniques (*2.6.21*).

ACTION TO BE TAKEN IN THE EVENT OF DETECTION OF A SPECIFIED AGENT

If evidence is found of contamination of the flock by an agent listed as slowly spreading in Table 5.2.2.-1, all materials derived from the flock during the 4-week period immediately preceding the date on which the positive sample was collected are considered unsatisfactory. Similarly, if evidence is found of contamination of the flock by an agent listed as rapidly spreading in Table 5.2.2.-1, all materials derived from the flock during the 2-week period immediately preceding the date on which the positive sample was collected are considered unsatisfactory. Any product manufactured with such materials, and for which the use of SPF materials is required, is considered unsatisfactory and must be discarded; any quality control tests conducted using the materials are invalid.

Producers must notify users of all eggs of the evidence of contamination as soon as possible following the outbreak.

Any flock in which an outbreak of any specified agent is confirmed may not be redesignated as an SPF flock. Any progeny derived from that flock during or after the 4-week period prior to the last negative sample being collected may not be designated as SPF.

J. Cell Substrates for the Production of Vaccines for Human Use

(Ph. Eur. general texts 5.2.3)

This general chapter deals with diploid cell lines and continuous cell lines used as cell substrates for the production of vaccines for human use; additional issues specifically related to vaccines prepared by recombinant DNA technology are covered by the monograph *Products of recombinant DNA technology (0784)*. The testing to be carried out at the various stages (cell seed, master cell bank (MCB), working cell bank (WCB), end of productions cells (EOPC) or extended cell bank (ECB) corresponding to cells at or beyond the maximum population doubling level used for production) is indicated in Table 5.2.3.-1. General provisions for the use of cell lines and test methods are given below. Where primary cells or cells that have undergone a few passages without constitution of a cell bank are used for vaccine production, requirements are given in the individual monograph for the vaccine concerned.

Diploid cell lines

A diploid cell line has a high but finite capacity for multiplication *in vitro*.

Continuous cell lines

A continuous cell line has the capacity to multiply indefinitely *in vitro*; the cells often have differences in karyotype compared to the original cells; they may be obtained from healthy or tumour tissue either from mammals or from insects.

There are perceived theoretical risks associated with the use of continuous cell lines, especially if their tumorigenic potential has been demonstrated experimentally. These risks are linked to the potential biological activity of the residual host-cell DNA present in the vaccine. The residual host-cell DNA may be associated with an infectivity risk if the genome of a DNA virus or a provirus is present in the cellular DNA (either integrated or extra chromosomal). In addition, there is a potential risk of oncogenicity if the cell substrate is tumorigenic.

For vaccines produced in continuous cell lines, whether tumorigenic or not, risk assessment and risk mitigation must be performed to evaluate the suitability of the cell substrate, to define the acceptable criteria for residual host-cell DNA in the final product and to evaluate the consistency of host-cell proteins.

Cell-bank system

Production of vaccines in diploid or continuous cell lines is based on a cell-bank system. The *in vitro* age of the cells is counted from the MCB. Each WCB is prepared from one or more containers of the MCB. The use, identity and inventory control of the containers is carefully documented.

Media and substances of human or animal origin

The composition of media used for isolation and all subsequent culture is recorded in detail, and if substances of human or animal origin are used they must be free from extraneous agents (*2.6.16*) and must comply with general chapter *5.1.7. Viral safety.*

If human albumin is used, it complies with the monograph *Human albumin solution (0255)*.

If bovine serum is used, it complies with the monograph *Bovine serum (2262)*.

Unless of recombinant origin, trypsin used for the preparation of cell cultures is tested by suitable methods and shown to be sterile and free from mycoplasmas and viruses.

Cell seed

The data used to assess the suitability of the cell seed comprises information, where available, on source, history and characterisation.

Source of the cell seed For human cell lines, the following information concerning the donor is recorded: ethnic and geographical origin; age; sex; general physiological condition; tissue or organ used; results of any tests for pathogens.

For animal cell lines, the following information concerning the source of the cells is recorded: species; strain; breeding conditions; geographical origin; age; sex; general physiological condition; tissue or organ used; results of any tests for pathogens.

Cells of neural origin, such as neuroblastoma and P12 cell lines are not used for vaccine production since they may contain substances that concentrate agents of spongiform encephalopathies.

History of the cell seed The following information is recorded: the method used to isolate the cell seed; culture methods; any other procedures used to establish the MCB, notably any that might expose the cells to extraneous agents.

Full information may not be available on the media ingredients used in the past for cultivation of cells, for example on the source of substances of animal origin; where justified and authorised, cell banks already established using such media may be used for vaccine production.

Characterisation of the cell seed The following properties are investigated:

(1) the identity of the cells, using methods such as isoenzyme analysis, *in vitro* immunochemical assays, nucleic acid fingerprinting and nucleic acid amplification techniques (NAT);

(2) the growth characteristics of the cells and their morphological properties (optical and electron microscopy);

(3) for diploid cell lines, karyotype;

(4) for diploid cell lines, the *in vivo* life span in terms of population doubling level.

Cell substrate stability

Suitable viability of the cell line in the intended storage conditions must be demonstrated. For a given product to be prepared in the cell line, it is necessary to demonstrate that consistent production can be obtained with cells at passage and/or population doubling levels at the beginning and end of the intended period of use.

Infectious extraneous agents

For cell lines for vaccine production, the testing for infectious extraneous agents must be carried out based on a risk assessment. The origin of the cell substrate as well as the potential extraneous agents that may be inadvertently introduced during production processes or through the use of animal or plant derived raw materials must be taken into account in the choice of suitable permissive cells. One such strategy is given in Table 5.2.3.-1, but alternative strategies could focus on more extensive testing at the MCB or WCB level. In any case, any strategy must be justified and lead to the same level of safety as outlined in Table 5.2.3-1. New, sensitive molecular techniques with broad detection capabilities are available, including massive parallel sequencing (MPS) methods, degenerate polymerase chain reaction (PCR) for whole virus families or random-priming methods (associated or not with sequencing), hybridisation to oligonucleotide arrays and mass spectrometry. These methods may be used either as an alternative to *in vivo* or specific NAT tests or as a supplement/alternative to *in vitro* culture tests, in agreement with the competent authority. The capacity of the process to remove/inactivate specific viruses must take into account the origin and culture history of the cell line and adventitious viruses that are known to persistently infect the species of origin, for example, simian virus 40 in rhesus monkeys, Flock house virus in insect cells or viruses that may inadvertently be introduced during production processes or through the use of raw materials of animal or plant origin. For cell lines of insect origin, tests for specific viruses relevant to the species of origin of the insect cells and for arboviruses (arthropod-borne viruses) are carried out. The panel of viruses tested is chosen according to the current state of scientific knowledge. For cell lines shown to express endogenous retroviral particles (e.g. rodent cells), the test for reverse transcriptase is not needed because it is expected to be positive, and thus infectivity tests must be performed to determine whether these endogenous retroviral particles are infectious or not.

Cell lines that show the presence of infectious retroviruses are not acceptable for production of vaccines, unless otherwise justified and authorised.

Tumorigenicity

Tumorigenicity is defined as the potential of a given cell line to induce a tumour after injection of intact live cells into immunodeficient/immunosuppressed animals (usually rodents). The tumorigenicity test is carried out using cells at or beyond the maximum population doubling level that will be used for vaccine production.

The MRC-5, WI-38 and FRhL-2 cell lines are recognised as being non-tumorigenic and further testing is not necessary. Known tumorigenic cell lines (e.g. CHO) do not need to be documented further.

When a previously uncharacterised cell line is tumorigenic, an oncogenicity study must be performed using purified DNA from the cell line and/or cell line lysate to demonstrate the absence of oncogenic components. The results are used as part of the risk analysis performed to support the use of the cell line for vaccine production. The determination of the TPD_{50} (tumour-producing dose in 50 per cent of animals) and the capacity to form metastases are characteristic properties that must be determined as part of the risk analysis.

Despite the difficulty in demonstrating a perfect and conclusive correlation with a tumorigenic phenotype, additional *in vitro* characterisation tests may be performed to document other cell substrate properties, such as the ability to grow in soft agar gels, the ability to induce invasive cell growth in muscle and/or the ability of the cell substrate to induce transformation of 3T3 cells.

Residual host-cell DNA

For each particular vaccine produced on continuous cell lines, residual host-cell DNA content must be tested and an acceptable upper limit, based on a risk assessment, must be established in the final product taking into consideration:

(1) the nature of the cell substrate (non-tumorigenic, level of tumorigenicity) and its origin (human/non-human);

(2) the presence in the production process of any steps to inactivate the potential biological activity (oncogenicity, infectivity) of the residual host-cell DNA (e.g. chemical agents such as betapropiolactone and/or DNase treatment);

(3) the capacity of the process to reduce the amount and size of the contaminating residual host-cell DNA;

(4) the intended use of the vaccine (e.g. route of administration);

(5) the method used to measure the residual host-cell DNA.

In general, a purification process for parenteral vaccines is able to reduce residual host-cell DNA in final products to less than 10 ng per dose, but the acceptance limits must be approved by the competent authority.

Once validation studies (e.g. spiking studies using an adequate size distribution of DNA) have been performed and the reproducibility of the production process in reducing residual host-cell DNA to the level expected has been demonstrated, residual host-cell DNA testing may be omitted after agreement from the competent authority.

Chromosomal characterisation

Diploid cell lines shall be shown to be diploid. More extensive characterisation of a diploid cell line by karyotype analysis is required if the removal of intact cells during post-harvest processing has not been validated. Samples from 4 passage levels evenly spaced over the life span of the cell line are examined. A minimum of 200 cells in metaphase are examined for exact chromosome count and for the frequency of hyperploidy, hypoploidy, polyploidy, breaks and structural abnormalities.

The MRC-5, WI-38 and FRhL-2 cell lines are recognised as being diploid and well characterised; where they are not genetically modified, further characterisation is not necessary.

Table 5.2.3.-1. – *Testing of cell lines*

Test	Cell seed	Master cell bank (MCB)	Working cell bank (WCB)	EOPC/ECB (Cells at or beyond the maximum population doubling level used for production)
	1. IDENTITY AND PURITY			
Morphology	+	+	+	+
Identification	+	+	+	+
Karyotype (diploid cell lines)	+	+	+(1)	+(1)
Life span (diploid cell lines)	–	+	+	–
	2. EXTRANEOUS AGENTS			
Bacterial and fungal contamination	–	+	+	–
Mycobacteria	–	+(2)	+(2)	–
Mycoplasmas	–	+	+	–
Spiroplasmas(3)	–	+	+	–
Electron microscopy	–	+(4)	–	+(4)
Tests for extraneous agents in cell cultures (with viable cells or equivalent cell lysate)	–	+	+	+
Tests in suckling mice and eggs	–	–	+(5)	+(5)
Test for specific viruses by NAT	–	+(6)	+(6)	+(6)
Test for viruses using broad molecular methods	+(7)	+(7)	+(7)	+(7)
Retroviruses	–	+(4)	–	+(4)
	3. TUMORIGENICITY			
Tumorigenicity	+(8, 9)	–	–	+(8)

(1) The diploid character is established for each WCB but using cells at or beyond the maximum population doubling level used for production.

(2) If the cells are susceptible to infection with *Mycobacterium tuberculosis* or other species.

(3) If insect cells or raw materials of plant origin are used.

(4) Testing is carried out for the MCB, but using cells at or beyond the maximum population doubling level used for production.

(5) Testing is carried out for each WCB, but using cells at or beyond the maximum population doubling level used for production.

(6) Specific tests for possible contaminants (e.g. viruses) defined according to a risk assessment based on the origin of the cells and on the potential extraneous agents inadvertently introduced during production processes or through the use of animal or plant derived raw materials. The appropriate testing stages should be selected based on the risk assessment.

(7) These methods may be used either as alternative to *in vivo* tests and specific NAT or as supplement or alternative to *in vitro* culture tests based on the risk assessment and in agreement with the competent authority. The appropriate testing stages should be selected based on the risk assessment.

(8) The MRC-5, WI-38 and FRhL-2 cell lines are recognised as being non-tumorigenic and they do not need to be tested. Tests are not carried out on cell lines that are known or assumed to be tumorigenic, for example CHO and BHK-21.

(9) Testing is carried out on the cell seed, but using cells at or beyond the maximum population doubling level used for production.

TEST METHODS FOR CELL CULTURES

Morphology

The morphology of the cells is adequately described and documented.

Identification

Nucleic acid fingerprint analysis and a relevant selection of the following are used to establish the identity of the cells:

(1) biochemical characteristics (isoenzyme analysis);

(2) immunological characteristics (histocompatibility antigens, *in vitro* immunochemical assays);

(3) cytogenetic markers;

(4) NAT.

Contaminating cells

The nucleic acid fingerprint analysis carried out for identification also serves to demonstrate freedom from contaminating cells.

Bacterial and fungal contamination

The MCB and each WCB comply with the test for sterility (*2.6.1*), carried out using, for each medium, 10 mL of supernatant from cell cultures. Carry out the test on 1 per cent of the containers, with a minimum of 2 containers.

Mycobacteria

If the cells are susceptible to infection with *Mycobacterium tuberculosis* or other species, the MCB and each WCB comply with the test for mycobacteria (*2.6.2*). NAT (*2.6.21*) may be used as an alternative to this culture method provided such an assay is validated and shown to be comparable to the culture method.

Mycoplasmas (*2.6.7*)

The MCB and each WCB comply with the test for mycoplasmas. Use one or more containers for the test.

Spiroplasmas

Spiroplasmas may be introduced into cell substrates through contamination of raw materials of plant origin or when insect cell lines are used. When appropriate, the MCB and each WCB are demonstrated to be free of spiroplasmas using a validated method approved by the competent authority. NAT methods for detection of mycoplasmas (*2.6.7*) may be used to detect spiroplasmas after validation and agreement from the competent authority. Use one or more containers for the test.

Electron microscopy

The MCB is examined by electron microscopy for the presence of extraneous agents. Cell lines are maintained at the temperature routinely used for production and taken at or beyond the maximum population doubling level used for production. In addition, insect cell lines are maintained at temperatures above and below those routinely used for production and may also be subjected to other treatments such as exposure to chemical stressors. For insect cell lines the maintenance temperatures and treatments used are agreed with the competent authority, along with the number of sectioned cells to be examined.

Test for extraneous agents in cell cultures

For mammalian cells, viable cells (at least 10^7 cells) or the equivalent cell lysate, in their culture supernatant, are either co-cultivated (for viable cells) or inoculated (for cell lysate) onto monolayer cultures of:

(1) human diploid cells;

(2) continuous simian kidney cells; and

(3) for cell substrates other than human or simian, cells of that species, from a separate batch.

For insect cell lines, cell lysates are inoculated onto monolayer cultures of other cell systems, including human, simian and, in addition, at least 1 cell line that is different from that used in production, is permissible to insect viruses and allows detection of human arboviruses (e.g. BHK-21).

The resulting co-cultivated cell culture (for viable cells) or inoculated cell cultures (for cell lysate) are observed for evidence of viruses by cytopathic effect for at least 2 weeks. If the cell line is known to be capable of supporting the growth of human or simian cytomegalovirus, the human diploid cultures are observed for at least 4 weeks.

The extended 4-week cell culture of human diploid cells, for the purpose of detecting human or simian cytomegalovirus, can be replaced by the use of NAT (*2.6.21*). In cases where it is difficult to keep the cell cultures healthy for the additional 2 weeks, it may be necessary to introduce fresh medium or to subculture after 2 weeks onto fresh cultures in order to be able to detect viral agents. At the end of the observation period, carry out tests on the cell culture supernatants for haemagglutinating viruses, or on the viable cells for haemadsorbing viruses using guinea-pig red blood cells. If the guinea-pig red blood cells have been stored, they shall have been stored at 5 ± 3 °C for not more than 7 days. Analyse half of the cultures after incubation at 5 ± 3 °C for 30 min and the other half after incubation at 20-25 °C for 30 min. The test for haemagglutinating viruses is not valid for arboviruses.

The test is not valid unless at least 80 per cent of the cell cultures remain viable. The cell substrate complies with the test if no evidence of any extraneous agent is found.

Retroviruses

If the cell line is not known to produce retroviral particles, examine for the presence of retroviruses using:

(1) product-enhanced reverse transcriptase (PERT) assay (*2.6.21*) carried out for cell bank supernatants using cells at or beyond the maximum population doubling level that will be used for production;

(2) transmission electron microscopy.

If tests (1) and/or (2) give a positive result, infectivity assays are carried out on permissible human cells with a PERT assay end-point on the supernatant.

If the cell line is shown to produce retroviral particles (e.g. rodent cell lines), examine for the presence of retroviruses using:

- transmission electron microscopy;
- infectivity assays carried out on permissible human cells and on relevant additional cells (e.g. *Mus dunni* cells or SC-1 cells for CHO cell substrate) with a PERT assay end-point on the supernatant, except when the amplification cells are positive for reverse transcriptase, in which case the readout is performed using plaque assay or a fluorescent focus assay.

Since the sensitivity of PERT assays is very high, interpretation of a positive signal may be equivocal and a decision on the acceptability of a cell substrate is based on all available data.

Tests in suckling mice

The test is carried out if a risk assessment indicates that it provides a risk mitigation taking into account the overall testing package applied to a given cell substrate.

Inject 10^7 viable cells or the equivalent cell lysate, in their culture supernatant into 2 litters of suckling mice less than 24 h old, comprising not fewer than 10 animals;

Inject at least 0.1 mL intraperitoneally and 0.01 mL intracerebrally.

Observe the suckling mice for at least 4 weeks. Investigate suckling mice that become sick or show any abnormality to establish the cause of illness. The cell substrate complies with the test if no evidence of any extraneous agent is found. The test is invalid if fewer than 80 per cent of the suckling mice in each group remain healthy and survive to the end of the observation period.

Tests in eggs (only required for avian cell substrates)

The test is carried out if a risk assessment indicates that it provides a risk mitigation taking into account the overall testing package applied to a given cell substrate. Inject an inoculum of 10^6 viable cells or the equivalent cell lysate, in their culture supernatant, into the allantoic cavity of ten 9- to 11-day-old SPF embryonated hens' eggs (*5.2.2*) and into the yolk sac of ten 5- to 7-day-old SPF embryonated hens' eggs. Incubate for not less than 5 days. Test the allantoic fluids for the presence of haemagglutinins using mammalian and avian red blood cells; carry out the test at 5 ± 3 °C and 20-25 °C and read the results after 30-60 min. The cell substrate complies with the test if no evidence of any extraneous agent is found. The test is invalid if fewer than 80 per cent of the embryos remain healthy and survive to the end of the observation period.

Tests for specific viruses

The list of specific viruses to be tested is defined based on a viral contamination risk assessment in accordance with the principles detailed in general chapter *5.1.7. Viral Safety*, and takes into account (but is not limited to) the origin of the cells and the potential sources of viral contamination (e.g. raw material of animal or plant origin). NAT tests (*2.6.21*) are carried out with or without prior amplification in cells. For cell lines of rodent origin, NAT (*2.6.21*) or antibody production tests in mice, rats or hamsters are used to detect species-specific viruses.

Tests for viruses using broad molecular methods

In agreement with the competent authority, broad molecular methods (e.g. High Throughput Sequencing) may be used either as an alternative to *in vivo* tests and specific NAT or as a supplement or alternative to *in vitro* culture tests based on the risk assessment.

For both NAT (*2.6.21*) and broad molecular methods, the stage at which testing is to be conducted (e.g. MCB, WCB, EOPC/ECB) is also based on the risk assessment and depends on the steps where viral contaminants may be introduced. In case of positive results with either broad molecular methods or NAT tests, a follow-up investigation must be conducted to determine whether detected nucleic acids are due to the presence of infectious extraneous agents and/or are known to constitute a risk to human health.

Tests for tumorigenicity *in vivo*

The test establishes a comparison between the continuous cell line and a suitable positive control cell line as reference (for example, HeLa or Hep2 cells).

Animal systems that have been shown to be suitable for this test include:

(1) athymic mice (Nu/Nu genotype);

(2) newborn mice, rats or hamsters that have been treated with antithymocyte serum or globulin;

(3) thymectomised and irradiated mice that have been reconstituted (T^-, B^+) with bone marrow from healthy mice.

Whichever animal system is selected, the cell line and the positive control cells are injected into separate groups of 10 animals each. In both cases, the inoculum for each animal is 10^7 cells suspended in a volume of 0.2 mL, and the injection may be given by the intramuscular or the subcutaneous route. Newborn animals are treated with 0.1 mL of antithymocyte serum or globulin on days 0, 2, 7 and 14 after birth. A potent serum or globulin is one that suppresses the immune mechanisms of growing animals to the extent that the subsequent inoculum of 10^7 positive control cells regularly produces tumours and metastases. Severely affected animals showing evident, progressively growing tumours are euthanised before the end of the test to avoid unnecessary suffering.

At the end of the observation period all animals, including the positive control group, are euthanised and examined for gross and microscopic evidence of the proliferation of inoculated cells at the site of injection and in other organs (for example, lymph nodes, lungs, kidneys and liver).

In all test systems, the animals are observed and palpated at regular intervals for the formation of nodules at the sites of injection. Any nodules formed are measured in 2 perpendicular directions, the measurements being recorded regularly to determine whether there is progressive growth of the nodule. Animals showing nodules that begin to regress during the period of observation are euthanised before the nodules are no longer palpable, and processed for histological examination. Animals with progressively growing nodules are observed for 1-2 weeks. Among those without nodule formation, half are observed for 3 weeks and half for 12 weeks before they are euthanised and processed for histological examination. A necropsy is performed on each animal and includes examination for gross evidence of tumour formation at the site of injection and in other organs such as lymph nodes, lungs, brain, spleen, kidneys and liver. All tumour-like lesions and the site of injection are examined histologically. In addition, since some cell lines may give rise to metastases without evidence of local tumour growth, any detectable regional lymph nodes and the lungs of all animals are examined histologically.

The test is invalid if fewer than 9 of the 10 animals injected with the reference positive-control cells show progressively growing tumours.

For a new tumorigenic cell line, in order to document the level of tumorigenicity, a dose range of cell substrate (e.g. dose of cells in the range of 10^5, 10^6 and 10^7) is injected in different groups of 10 animals. The number of animals showing progressively growing nodules within the animal groups is monitored to calculate the TPD_{50}.

K. Carrier Proteins for the Production of Conjugated Polysaccharide Vaccines for Human Use

(Ph. Eur. general text 5.2.11)

The use of alternative carrier proteins, production methods and tests are acceptable provided they have been authorised by the competent authority.

Bacterial polysaccharides are not able to induce a T-cell-dependent B-cell immune response, which is needed to obtain an immunological memory response, and are generally poorly immunogenic in children under 2 years of age. The limitations are overcome by conjugating polysaccharides to carrier proteins. Carrier proteins are highly immunogenic and, when conjugated to bacterial polysaccharides, increase the capacity of polysaccharides to induce a protective response in infants.

Carrier proteins currently used in polysaccharide vaccines for human use are toxoids, non-toxic mutated toxins, surface or outer membrane proteins extracted from micro-organisms. Micro-organisms used for the production of the protein may be of genetically modified origin.

The production method used for a carrier protein shall have been shown to yield consistently batches suitable for conjugation of the carrier protein to a polysaccharide antigen.

Appropriate acceptance criteria for low bioburden before conjugation with the polysaccharide may be established. It is a prerequisite that the carrier protein is filtered through a bacteria-retentive filter prior to storage and that adequate measures are in place to avoid contamination and growth of micro-organisms during storage.

The production of carrier proteins is based on a seed-lot system. The seed lots are shown to be free from contamination using suitable methods of appropriate sensitivity. The culture may be inactivated and the carrier protein is purified by a suitable method.

The protein is characterised by one or more suitable method(s) (such as SDS-PAGE, isoelectric focusing, HPLC, size-exclusion chromatography with multiple-angle laser light scattering detection (MALLS), amino-acid analysis, amino-acid sequencing, circular dichroism, fluorescence spectroscopy, peptide mapping and mass spectrometry) and its purity is verified by a suitable method. Suitable tests are carried out, for validation or routinely, to demonstrate that where applicable, the product is free from specific toxins. If purification steps are present, the reduction of selected process-related impurities and residuals is monitored to establish consistency of the purification process. In the case of recombinant carrier proteins, tests for at least the following impurities are also carried out:

— residual host-cell proteins, including proteins derived from the expression vector;
— residual cellular DNA.

Only a carrier protein that complies with the following tests may be used in the preparation of the conjugate.

Identification

The carrier protein is identified using a suitable method.

pH *(2.2.3)*

Where applicable, the pH of the carrier protein prior to conjugation is monitored and is within the limits approved for the particular product.

Protein content (*2.5.16*)
The content of the carrier protein is determined using a suitable method and is within the limits approved by the competent authority.

Bacterial endotoxins (*2.6.14*)
The content is within the limits approved for the particular product.

In addition, the following requirements apply for the carrier proteins listed below.

Diphtheria toxoid
It is produced as described in the monograph *Diphtheria vaccine (adsorbed) (0443)* and complies with the requirements prescribed therein for bulk purified toxoid, except that the test for sterility (*2.6.1*) is not required.

Tetanus toxoid
It is produced as described in the monograph *Tetanus vaccine (adsorbed) (0452)* and complies with the requirements prescribed therein for bulk purified toxoid, except that the antigenic purity is not less than 1500 Lf per milligram of protein nitrogen and that the test for sterility (*2.6.1*) is not required.

CRM 197 diphtheria protein
It may be prepared from the growth of genetically modified (C7/β197) or non-genetically modified (mCRM) *Corynebacterium diphtheriae* or prepared by recombinant DNA technology in organisms such as *Escherichia coli*. The culture supernatant may be concentrated by ultrafiltration and purified by successive precipitation, filtration, and chromatography steps. When it is produced on the same premises as diphtheria toxin, CRM 197 diphtheria protein should be distinguished from the active toxin by a suitable method. The purity is not less than 90 per cent of diphtheria protein.

OMP (*Neisseria meningitidis* group B outer membrane protein complex)
Neisseria meningitidis group B outer membrane protein complex is extracted from bacterial cell cultures with a buffer containing a detergent. Cell debris is first removed.
The membrane protein complex may be concentrated and purified by successive filtration and additional suitable purification steps. The lipopolysaccharide content does not exceed 8 per cent. The relative quantity of the major OMP is set and approved by the competent authority. The OMP complex complies with the test for pyrogens (*2.6.8*): inject into each rabbit 0.25 µg of OMP per kilogram of body mass.

Recombinant protein D
Protein D is a surface protein of nontypable *Haemophilus influenzae*. It is produced using a specific strain of *E. coli* carrying a plasmid containing the coding sequence for protein D. In order to express protein D, the modified strain is grown in a suitable liquid medium. At the end of cultivation, a purification step is carried out. The purity of the product is not less than 95 per cent of protein D.

L. Immunonephelometry for Vaccine Component Assay

(Ph. Eur. method 2.7.35)

GENERAL PRINCIPLE

The general principle of nephelometry is described in general chapter *2.2.1. Clarity and degree of opalescence of liquids.* Immunonephelometry measures turbidity that is mainly due to immune complexes formed by the antigen-antibody reaction. Although immunonephelometry is applicable for the quantification of both antibodies and antigens, this general chapter describes the quantification of antigens as vaccine components.

APPARATUS

The nephelometer used is described in general chapter *2.2.1. Clarity and degree of opalescence of liquids.* However the measurement may be made at a non-zero angle different from 90°.

METHODS

In general, the progress of the reaction measured by light scattering can be described in 3 steps.

Initially, a baseline level of light scattering due to the reaction medium is detected. After the 1st reagent (antigen) is added, an increase in the signal is observed followed by a plateau. When the 2nd reagent (antibody) is added, a 2nd increase of the signal is observed with a 2nd plateau followed by a final increase in the intensity of the signal, which continues until a 3rd plateau is reached. The measurement zone starts from the addition of the 2nd reagent until the higher intensity of light scattering (the 3rd plateau) is reached depending on the concentrations of the component to be assayed.

The following methods can be used to quantify the immune complexes formed during this type of reaction.

END-POINT NEPHELOMETRY
The light scattering is measured after the immune complex has formed, i.e. after about 60 min. The value of the end-point is in this case directly proportional to the content of the component to be assayed when an excess of antibodies is used. A blank is necessary in order to subtract the value of nonspecific scattering due to the reaction mixture and to the reaction/measurement cell.

RATE NEPHELOMETRY
Rate nephelometry is based on the rate of immune complex formation (rate of increase in light scattering), which is proportional to the concentration of the component to be assayed.

There are 2 types of rate nephelometry.

Fixed-time rate nephelometry
A 1st measurement of light scattering is carried out a few seconds after the last reagent has been added. A 2nd measurement is carried out after a fixed time interval established during development of the method.
The difference between the 2 values is proportional to the quantity of the component to be assayed.

Rate nephelometry using the peak of the first derivative
The formation of the immune complex is described by the curve of the first derivative of the variation in light scattering with time ($d\Theta_D/dt$). Two successive peaks are observed due to the addition of the 1st and then the 2nd reagent.

The height of the 3rd peak of the first derivative is proportional to the quantity of component to be assayed in the reaction medium. Successive measurements are carried out a few seconds after the 2nd reagent has been added.

In this case, the light scattered by the reaction medium, the component and the reagent does not affect the initial value of the derived signal.

OPERATING CONDITIONS

REAGENTS AND REFERENCE STANDARDS
The reagents, diluents and samples to be examined must be clear and free from any suspended particles. To this end, a

preliminary filtration or centrifugation step may be considered.

The antisera or antibodies are selected based on their specificity, their precipitating capacity and their avidity for the antigen. Indeed, high avidity results in clearly detectable signals being generated rapidly, which thus improves the quality of the results.

In addition, it is important to define the range of antigen concentrations to be assayed with an excess of antibodies and to verify the absence of a zone effect in this range.

It is recommended to ensure that the profiles of response will be similar to that of an appropriate reference standard.

PRE-TREATMENT OF THE SAMPLES TO BE EXAMINED

An appropriate pre-treatment could be envisaged to eliminate an adjuvant or another interfering substance.

ASSAY

Ensure that all the reagents are at a suitable temperature to optimise the reaction.

To the samples diluted with a suitable medium in the reaction measurement cell, add a fixed quantity of antibodies. Carry out several determinations of the dilution being examined.

In some cases, the progress of the reaction may be improved by introducing additional substances into the reaction medium (e.g. polyethylene glycol).

The reference standards and samples must be treated in the same manner.

PERFORMANCE OF A BLANK TEST

To ensure that the assays are specific, only the scattering due to immune complexes must be measured. Other particles, the matrix of the sample, the reagents or the reaction measurement cell may interfere. In this case and if the end-point method is used, it is necessary to carry out a blank test to avoid taking non-specific scattering into account.

CALIBRATION

The calibration curve is obtained by preparing a set of dilutions of a reference standard. The concentrations are chosen in order to encompass the working range.

METHODS OF CALCULATION

The parallel line method (see general chapter *5.3*) or a calibration curve is used for the calculations.

VALIDITY OF THE TESTS

VALIDITY OF THE CALIBRATION CURVES

Only calibration curves that comply with the defined validity criteria may be used.

The following parameters are examples to be used to define validity criteria:

- minimum of light scattering units (LSU) obtained for the lowest concentration point of the calibration curve;
- coefficient of variation for the LSU measured for each point on the curve;
- coefficients of regression or of correlation of the calibration curve;
- slope and intercept of the calibration curve.

VALIDITY OF THE RESULTS

The following criteria are examples of validity criteria of the samples to be assayed:

- coefficient of variation or the range of values for the determinations carried out on the sample;
- assay of an in-house reference standard with trend monitoring or a control chart.

M. Substitution of in Vivo Method(s) by in Vitro Method(s) for the Quality Control of Vaccines

(Ph. Eur. general texts 5.2.14)

PURPOSE

The purpose of this general chapter is to provide guidance to facilitate the implementation of *in vitro* methods as substitutes for existing *in vivo* methods, in cases where a typical one-to-one assay comparison is not appropriate for reasons unrelated to the suitability of one or more *in vitro* methods. This general chapter will not discuss the details of assay validation as such, since those principles are described elsewhere.

The general chapter applies primarily to vaccines for human or veterinary use, however the principles described may also apply to other biologicals such as sera.

CONTEXT

The test methods used for routine quality control of vaccines are intended to monitor production consistency and to ensure comparability of the quality attributes between commercial batches and those batches originally found to be safe and efficacious in clinical studies or, for veterinary vaccines, in the target species.

While the *in vivo* potency and safety assays described within Ph. Eur. vaccine monographs have historically played a central role in safeguarding the quality of vaccines, the inherent variability of *in vivo* assays can make them less suitable than appropriately designed *in vitro* assays for monitoring consistency of production and for assessing the potential impact of manufacturing changes. As a result, it is essential continually to challenge the scientific value and relevance of these *in vivo* test methods. When *in vivo* tests are found to be of limited or no value, it is imperative to eliminate them, given the ethical considerations and the obligations under the relevant conventions. In addition, there is a substantial effort to develop *in vitro* methods (including immunological, molecular and physico-chemical tests) to replace the animal tests. In several cases this has led to the successful introduction of new *in vitro* methods in vaccine monographs. The use of appropriate *in vitro* methods not only reduces the use of animals while maintaining or improving the scientific relevance of the assays involved, but also substantially reduces assay variability and the time and resources required, and enhances the predictability of the release of safe and effective vaccine lots for use.

In addition to the benefits resulting from the substitution of appropriate *in vitro* methods for existing *in vivo* methods, under the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes, the Ph. Eur. Commission has committed to the reduction of animal usage wherever possible in pharmacopoeial testing. Under the convention, those associated with the work of the European Pharmacopoeia are encouraged to develop and/or implement *in vitro* procedures, and the General Notices support the introduction of alternatives to *in vivo* methods described in Ph. Eur. monographs.

GENERAL CONSIDERATIONS

One of the consequences associated with the inherent variability of *in vivo* assays is the problem this poses with their replacement by the more-consistent *in vitro* methods, which typically requires one-to-one assay comparison. This

can be a challenge in some cases as repeated efforts through multicentre international collaborative studies can fail due to the variability inherent in the *in vivo* methods. Another consideration is that many of the legacy *in vivo* safety and potency assays for vaccines were generally shown to be fit for purpose and have historically proven their value in ensuring the efficacy and safety of vaccines. However, this was in an era when validation requirements, such as ICH Q2 (R1) or VICH GL2 guideline, were not in place, making a formal one-to-one comparison challenging or even impossible in some cases. Since precision, reproducibility, limits of detection and quantification were not established for the *in vivo* method, the comparability of one method to another becomes difficult to evaluate. It should also be noted that, because Ph. Eur. methods are considered validated under the General Notices, it is not only impractical and excessively costly now to undertake a retrospective ICH/VICH validation of these methods, but it would also be unethical given the above-mentioned convention on animal use in pharmacopoeial testing.

When considering the transition from an *in vivo*-based to an *in vitro*-based quality control assay system, it is important to understand what *in vivo* assays can and cannot offer. Although properly established *in vivo* potency assays in laboratory animals have the potential to measure complex functional responses for demonstrating proof of concept, these do not necessarily predict the actual responses in the target population. In addition, *in vitro* bioassays have the potential to mimic specific elements of complex *in vivo* responses with generally lower variability and higher sensitivity.

Another key consideration is that when an *in vivo* test for a given product is to be replaced with an *in vitro* test, the quality attribute(s) of the product will likely be assessed differently. Examples include: the determination of antigen content or a functional response (e.g. virus or toxin neutralisation) in an *in vitro* bioassay instead of *in vivo* potency; molecular consistency instead of *in vivo* neurovirulence or attenuated phenotype; absence of the extraneous agent genomes using molecular methods instead of absence of micro-organisms through *in vivo* testing; and demonstration of toxin binding and enzyme activity instead of *in vivo* specific toxicity. As a consequence, a demonstration of agreement between the 2 methods is generally not scientifically justified and should not always be expected. Even where pass/fail results from the 2 test procedures are in agreement, the correlation between 2 quantitative methods across the assay range may still be low. Regardless, the *in vitro* method(s) or testing strategy must provide at least the same confidence that the key quality attributes, which are necessary to ensure the consistency of a product's safety and effectiveness, are adequately controlled.

While the focus of this general chapter is on the replacement of existing methods for approved products, it is important to consider the use of *in vitro* methods for quality control during product development and to understand that the use of *in vivo* assays is not mandatory.

ALTERNATIVE APPROACHES FOR THE SUBSTITUTION OF *IN VIVO* METHODS

The primary focus for the implementation of any proposed *in vitro* methods within a quality control system should be the scientific relevance of *in vitro* assays for control of the relevant quality attributes. Additionally, any *in vitro* methods will have to meet the current validation requirements.

In the Ph. Eur., *in vivo* assays for vaccines are typically replaced by *in vitro* assays following multicentre collaborative studies, but this should not be a prerequisite for *in vivo* assay replacement initiatives for individual products. Additionally, while it may be desirable to have assays that are widely applicable to a class of products, this should not be a requirement.

As explained in the guidance below, in some cases an existing method may need to be substituted by more than 1 *in vitro* test, in order to characterise the key qualitative and quantitative attributes measured by the existing test.

POTENCY TESTS

When it is not possible to show agreement between the *in vitro* and *in vivo* methods due to low discriminating power and/or high variability of the *in vivo* assay, the following approach can be used. It is assumed that the product under consideration has a well-established safety and efficacy profile, with consistent manufacturing.

The *in vitro* test(s) should be able to detect differences that are relevant to the control of the production process as justified scientifically. This should be supported by data demonstrating the capability of the proposed assay(s) to control key quality attributes of the vaccine and maintain the link between the quality of the batches to be released and those batches found to be safe and efficacious through clinical studies or routine use. With the setting of appropriate specifications, the consistency of manufacturing with the *in vitro* method(s) will be maintained.

The design of an assay/assay system for vaccine quality control needs to reflect both antigen content and functionality. If a single method is used, it should preferably measure the content and integrity of the antigen by targeting epitope(s) relevant to the protection offered by the vaccine. An example of this would be a monoclonal antibody or monoclonal antibodies against an epitope or epitopes as the main target for generating neutralising antibodies.
The epitope or epitopes should preferably be conformational in order to have a stability-indicating assay (as is the case for rabies vaccine). In some cases, a single *in vitro* method may not adequately reflect the content and functionality. This can be remedied through the use of multiple assays, as is the case with conjugate polysaccharide vaccines, where molecular size, conjugate integrity, and total and free polysaccharides are examples of relevant measures.

To establish quantitative measurements with an *in vitro* method, samples that differ in the magnitude of the response will be needed. In most cases, samples that are below the minimum approved potency specification with the *in vivo* method will not be available because production consistency is generally well maintained, and potency between batches does not differ significantly and/or the precision of the *in vivo* assay is such that it cannot discriminate between batches unless the difference is very large. Therefore, initial assay evaluation should be performed with samples at different concentrations, which could be followed by testing of samples subjected to different types of stress conditions to assess further the stability-indicating potential of the new method. The inability to demonstrate agreement between an *in vitro* and an *in vivo* method does not necessarily mean that the *in vitro* method is not suitable/relevant. In many cases, an *in vitro* method will detect changes in the product profile that would not be detected by the *in vivo* method. In such cases, the *in vitro* method may be considered superior for monitoring the consistency of production and may be more relevant to assess the impact of manufacturing changes.

SAFETY TESTS

Specific toxicity

An *in vitro* method for detection of residual toxic components should be specific and at least as sensitive as the existing *in vivo* method. Where possible, a fully functional *in vitro* system should be used (e.g. toxin-sensitive cell line). Where no functional *in vitro* system is available, an *in vitro* testing strategy could be based on the detection/measurement of more than 1 parameter, sequentially where relevant, that together reflect the mode of action for the toxic components in question. Examples include the use of assays with immunological and biochemical steps to detect receptor binding and enzyme activity. In most cases, where an *in vivo* assay is to be replaced there will be data available on the sensitivity of that model for detection of the toxin in question. Therefore, new *in vitro* methods can be characterised to demonstrate that they are sufficiently sensitive using spiking experiments and referring to historic data for the *in vivo* assay. Such assays, in conjunction with the appropriate time and temperature conditions, could also be used to demonstrate the absence of reversion of a specific toxoid.

Molecular consistency by deep sequencing versus the neurovirulence test

An *in vitro* genotypic method to assess the molecular consistency of a viral vaccine has the potential to replace an existing *in vivo* neurovirulence test. A prerequisite for any *in vitro* genotypic method is an in-depth knowledge of the molecular markers responsible for the attenuation of the live viral vaccine (as is the case for oral poliovirus vaccine, for example). In such a case, monitoring the consistency of the vaccine lots would be achieved by confirming the presence of the required molecular attenuation markers and percentage of mutants with methods such as deep sequencing.

Detection of viral extraneous agents by novel molecular methods

Detection of viral extraneous agents in cell banks, seed lots and cell culture harvests is currently conducted using a panel of *in vivo* and *in vitro* methods at different stages of the manufacturing process. Novel, sensitive molecular techniques with broad detection capabilities are available, including deep sequencing or high-throughput sequencing methods, degenerate polymerase chain reaction (PCR) for whole virus families or random-priming methods (associated or not with sequencing), hybridisation to oligonucleotide arrays and mass spectrometry. The use of these new molecular methods has highlighted gaps in the existing testing strategy by identifying previously undetected viral contaminants in final product, the cell banks from which it was produced and intermediate manufacturing stages. These new molecular methods (e.g. deep sequencing or high-throughput sequencing) detect genomes while the existing *in vivo* methods are based on observations of the effects viruses have on experimental animals. The implementation of such new molecular methods as substitutes for *in vivo* methods requires a comparison of the specificity (breadth of detection) and the sensitivity of the new and existing methods. For this purpose, an appropriate panel of representative, well-characterised model viruses should be used to assess the ability of the new method to detect viruses that are (or are not) detected by the *in vivo* methods, and to determine if the sensitivity is at least equivalent to the sensitivity of the *in vivo* methods. This last element is particularly complex since these new molecular methods do not detect the same characteristic of the viral contaminant (genome for molecular methods versus infectious virus for *in vivo* methods) and also since no or limited validation data exist for the *in vivo* methods.
It should also be emphasised that the outcome of the new molecular methods is not the final result since the detection of a genome or fragments of a genome does not necessarily indicate the presence of an infectious virus.

Appendix XVI

A. Test for Sterility

Sterility[1]

(Ph. Eur. method 2.6.1)

The test is applied to substances, preparations or articles which, according to the Pharmacopoeia, are required to be sterile. However, a satisfactory result only indicates that no contaminating micro-organism has been found in the sample examined in the conditions of the test.

PRECAUTIONS AGAINST MICROBIAL CONTAMINATION

The test for sterility is carried out under aseptic conditions. In order to achieve such conditions, the test environment has to be adapted to the way in which the sterility test is performed. The precautions taken to avoid contamination are such that they do not affect any micro-organisms which are to be revealed in the test. The working conditions in which the tests are performed are monitored regularly by appropriate sampling of the working area and by carrying out appropriate controls.

CULTURE MEDIA AND INCUBATION TEMPERATURES

Media for the test may be prepared as described below, or equivalent commercial media may be used provided that they comply with the growth promotion test.

The following culture media have been found to be suitable for the test for sterility. Fluid thioglycollate medium is primarily intended for the culture of anaerobic bacteria; however, it will also detect aerobic bacteria. Soya-bean casein digest medium is suitable for the culture of both fungi and aerobic bacteria.

Fluid thioglycollate medium

L-Cystine	0.5 g
Agar	0.75 g
Sodium chloride	2.5 g
Glucose monohydrate/Glucose	5.5 g/5.0 g
Yeast extract (water-soluble)	5.0 g
Pancreatic digest of casein	15.0 g
Sodium thioglycollate or	0.5 g
Thioglycollic acid	0.3 mL
Resazurin sodium solution (1 g/L of resazurin sodium), freshly prepared	1.0 mL
Water R	1000 mL
pH after sterilisation 7.1 ± 0.2	

Mix the L-cystine, agar, sodium chloride, glucose, water-soluble yeast extract and pancreatic digest of casein with the *water R* and heat until solution is effected. Dissolve the sodium thioglycollate or thioglycollic acid in the solution and, if necessary, add *1 M sodium hydroxide* so that, after sterilisation, the solution will have a pH of 7.1 ± 0.2. If filtration is necessary, heat the solution again without boiling and filter while hot through moistened filter paper. Add the resazurin sodium solution, mix and place the medium in suitable vessels which provide a ratio of surface to depth of medium such that not more than the upper half of the medium has undergone a colour change indicative of oxygen uptake at the end of the incubation period. Sterilise using a validated process. If the medium is stored, store at a temperature between 2 °C and 25 °C in a sterile, airtight container. If more than the upper one-third of the medium has acquired a pink colour, the medium may be restored once by heating the containers in a water-bath or in free-flowing steam until the pink colour disappears and cooling quickly, taking care to prevent the introduction of non-sterile air into the container. Do not use the medium for a longer storage period than has been validated.

Fluid thioglycollate medium is to be incubated at 30-35 °C.

For products containing a mercurial preservative that cannot be tested by the membrane-filtration method, fluid thioglycollate medium incubated at 20-25 °C may be used instead of soya-bean casein digest medium provided that it has been validated as described in growth promotion test.

Where prescribed or justified and authorised, the following alternative thioglycollate medium may be used. Prepare a mixture having the same composition as that of the fluid thioglycollate medium, but omitting the agar and the resazurin sodium solution, sterilise as directed above. The pH after sterilisation is 7.1 ± 0.2. Heat in a water-bath prior to use and incubate at 30-35 °C under anaerobic conditions.

Soya-bean casein digest medium

Pancreatic digest of casein	17.0 g
Papaic digest of soya-bean meal	3.0 g
Sodium chloride	5.0 g
Dipotassium hydrogen phosphate	2.5 g
Glucose monohydrate/Glucose	2.5 g/2.3 g
Water R	1000 mL
pH after sterilisation 7.3 ± 0.2	

Dissolve the solids in *water R*, warming slightly to effect solution. Cool the solution to room temperature. Add *1 M sodium hydroxide*, if necessary, so that after sterilisation the solution will have a pH of 7.3 ± 0.2. Filter, if necessary, to clarify, distribute into suitable vessels and sterilise using a validated process. Store at a temperature between 2 °C and 25 °C in a sterile well-closed container, unless it is intended for immediate use. Do not use the medium for a longer storage period than has been validated.

Soya-bean casein digest medium is to be incubated at 20-25 °C.

The media used comply with the following tests, carried out before or in parallel with the test on the product to be examined.

Sterility

Incubate portions of the media for 14 days. No growth of micro-organisms occurs.

Growth promotion test of aerobes, anaerobes and fungi

Test each batch of ready-prepared medium and each batch of medium prepared either from dehydrated medium or from ingredients. Suitable strains of micro-organisms are indicated in Table 2.6.1.-1.

Inoculate portions of fluid thioglycollate medium with a small number (not more than 100 CFU) of the following micro-organisms, using a separate portion of medium for each of the following species of micro-organism: *Clostridium sporogenes, Pseudomonas aeruginosa, Staphylococcus aureus.* Inoculate portions of soya-bean casein digest medium with a small number (not more than 100 CFU) of the following micro-organisms, using a separate portion of medium for each of the following species of micro-organism: *Aspergillus brasiliensis, Bacillus subtilis, Candida albicans.* Incubate for not more than 3 days in the case of bacteria and not more than 5 days in the case of fungi.

[1] *This chapter has undergone pharmacopoeial harmonisation. See chapter 5.8 Pharmacopoeial harmonisation.*

Table 2.6.1.-1. – *Strains of the test micro-organisms suitable for use in the growth promotion test and the method suitability test*

Aerobic bacteria	
Staphylococcus aureus	ATCC 6538, CIP 4.83, NCTC 10788, NCIMB 9518, NBRC 13276
Bacillus subtilis	ATCC 6633, CIP 52.62, NCIMB 8054, NBRC 3134
Pseudomonas aeruginosa	ATCC 9027, NCIMB 8626, CIP 82.118, NBRC 13275
Anaerobic bacterium	
Clostridium sporogenes	ATCC 19404, CIP 79.3, NCTC 532, ATCC 11437, NBRC 14293
Fungi	
Candida albicans	ATCC 10231, IP 48.72, NCPF 3179, NBRC 1594
Aspergillus brasiliensis	ATCC 16404, IP 1431.83, IMI 149007, NBRC 9455

Seed lot culture maintenance techniques (seed-lot systems) are used so that the viable micro-organisms used for inoculation are not more than 5 passages removed from the original master seed-lot.

The media are suitable if a clearly visible growth of the micro-organisms occurs.

METHOD SUITABILITY TEST

Carry out a test as described below under Test for sterility of the product to be examined using exactly the same methods except for the following modifications.

Membrane filtration

After transferring the contents of the container or containers to be tested to the membrane add an inoculum of a small number of viable micro-organisms (not more than 100 CFU) to the final portion of sterile diluent used to rinse the filter.

Direct inoculation

After transferring the content of the container or containers to be tested (for catgut and other surgical sutures for veterinary use: strands) to the culture medium add an inoculum of a small number of viable micro-organisms (not more than 100 CFU) to the medium.

In both cases use the same micro-organisms as those described above under Growth promotion test of aerobes, anaerobes and fungi. Perform a growth promotion test as a positive control. Incubate all the containers containing medium for not more than 5 days.

If clearly visible growth of micro-organisms is obtained after the incubation, visually comparable to that in the control vessel without product, either the product possesses no antimicrobial activity under the conditions of the test or such activity has been satisfactorily eliminated. The test for sterility may then be carried out without further modification.

If clearly visible growth is not obtained in the presence of the product to be tested, visually comparable to that in the control vessels without product, the product possesses antimicrobial activity that has not been satisfactorily eliminated under the conditions of the test. Modify the conditions in order to eliminate the antimicrobial activity and repeat the method suitability test.

This method suitability test is performed:

a) when the test for sterility has to be carried out on a new product;

b) whenever there is a change in the experimental conditions of the test.

The method suitability test may be performed simultaneously with the test for sterility of the product to be examined.

TEST FOR STERILITY OF THE PRODUCT TO BE EXAMINED

The test may be carried out using the technique of membrane filtration or by direct inoculation of the culture media with the product to be examined. Appropriate negative controls are included. The technique of membrane filtration is used whenever the nature of the product permits, that is, for filterable aqueous preparations, for alcoholic or oily preparations and for preparations miscible with or soluble in aqueous or oily solvents provided these solvents do not have an antimicrobial effect in the conditions of the test.

Membrane filtration

Use membrane filters having a nominal pore size not greater than 0.45 µm whose effectiveness to retain micro-organisms has been established. Cellulose nitrate filters, for example, are used for aqueous, oily and weakly alcoholic solutions and cellulose acetate filters, for example, for strongly alcoholic solutions. Specially adapted filters may be needed for certain products, e.g. for antibiotics.

The technique described below assumes that membranes about 50 mm in diameter will be used. If filters of a different diameter are used the volumes of the dilutions and the washings should be adjusted accordingly. The filtration apparatus and membrane are sterilised by appropriate means. The apparatus is designed so that the solution to be examined can be introduced and filtered under aseptic conditions; it permits the aseptic removal of the membrane for transfer to the medium or it is suitable for carrying out the incubation after adding the medium to the apparatus itself.

Aqueous solutions If appropriate, transfer a small quantity of a suitable, sterile diluent such as a 1 g/L neutral solution of meat or casein peptone pH 7.1 ± 0.2 onto the membrane in the apparatus and filter. The diluent may contain suitable neutralising substances and/or appropriate inactivating substances for example in the case of antibiotics.

Transfer the contents of the container or containers to be tested to the membrane or membranes, if necessary after diluting to the volume used in the method suitability test with the chosen sterile diluent but in any case using not less than the quantities of the product to be examined prescribed in Table 2.6.1.-2. Filter immediately. If the product has antimicrobial properties, wash the membrane not less than 3 times by filtering through it each time the volume of the chosen sterile diluent used in the method suitability test. Do not exceed a washing cycle of 5 times 100 mL per filter, even if during the method suitability test it has been demonstrated that such a cycle does not fully eliminate the antimicrobial activity. Transfer the whole membrane to the culture medium or cut it aseptically into 2 equal parts and

Table 2.6.1.-2. – *Minimum quantity to be used for each medium*

Quantity per container	Minimum quantity to be used for each medium unless otherwise justified and authorised
Liquids	
— less than 1 mL	The whole contents of each container
— 1-40 mL	Half the contents of each container but not less than 1 mL
— greater than 40 mL and not greater than 100 mL	20 mL
— greater than 100 mL	10 per cent of the contents of the container but not less than 20 mL
Antibiotic liquids	1 mL
Insoluble preparations, creams and ointments to be suspended or emulsified	Use the contents of each container to provide not less than 200 mg
Solids	
— less than 50 mg	The whole contents of each container
— 50 mg or more but less than 300 mg	Half the contents of each container but not less than 50 mg
— 300 mg to 5 g	150 mg
— greater than 5 g	500 mg
Catgut and other surgical sutures for veterinary use	3 sections of a strand (each 30 cm long)

transfer one half to each of 2 suitable media. Use the same volume of each medium as in the method suitability test. Alternatively, transfer the medium onto the membrane in the apparatus. Incubate the media for not less than 14 days.

Soluble solids Use for each medium not less than the quantity prescribed in Table 2.6.1.-2 of the product dissolved in a suitable solvent such as the solvent provided with the preparation, water for injections, saline or a 1 g/L neutral solution of meat or casein peptone and proceed with the test as described above for aqueous solutions using a membrane appropriate to the chosen solvent.

Oils and oily solutions Use for each medium not less than the quantity of the product prescribed in Table 2.6.1.-2. Oils and oily solutions of sufficiently low viscosity may be filtered without dilution through a dry membrane. Viscous oils may be diluted as necessary with a suitable sterile diluent such as isopropyl myristate shown not to have antimicrobial activity in the conditions of the test. Allow the oil to penetrate the membrane by its own weight then filter, applying the pressure or suction gradually. Wash the membrane at least 3 times by filtering through it each time about 100 mL of a suitable sterile solution such as 1 g/L neutral meat or casein peptone containing a suitable emulsifying agent at a concentration shown to be appropriate in the method suitability test, for example polysorbate 80 at a concentration of 10 g/L. Transfer the membrane or membranes to the culture medium or media or vice versa as described above for aqueous solutions, and incubate at the same temperatures and for the same times.

Ointments and creams Use for each medium not less than the quantities of the product prescribed in Table 2.6.1.-2. Ointments in a fatty base and emulsions of the water-in-oil type may be diluted to 1 per cent in isopropyl myristate as described above, by heating, if necessary, to not more than 40 °C. In exceptional cases it may be necessary to heat to not more than 44 °C. Filter as rapidly as possible and proceed as described above for oils and oily solutions.

Direct inoculation of the culture medium

Transfer the quantity of the preparation to be examined prescribed in Table 2.6.1.-2 directly into the culture medium so that the volume of the product is not more than 10 per cent of the volume of the medium, unless otherwise prescribed.

If the product to be examined has antimicrobial activity, carry out the test after neutralising this with a suitable neutralising substance or by dilution in a sufficient quantity of culture medium. When it is necessary to use a large volume of the product it may be preferable to use a concentrated culture medium prepared in such a way that it takes account of the subsequent dilution. Where appropriate, the concentrated medium may be added directly to the product in its container.

Oily liquids Use media to which have been added a suitable emulsifying agent at a concentration shown to be appropriate in the method suitability test, for example polysorbate 80 at a concentration of 10 g/L.

Ointments and creams Prepare by diluting to about 1 in 10 by emulsifying with the chosen emulsifying agent in a suitable sterile diluent such as a 1 g/L neutral solution of meat or casein peptone. Transfer the diluted product to a medium not containing an emulsifying agent.

Incubate the inoculated media for not less than 14 days. Observe the cultures several times during the incubation period. Shake cultures containing oily products gently each day. However when fluid thioglycollate medium is used for the detection of anaerobic micro-organisms keep shaking or mixing to a minimum in order to maintain anaerobic conditions.

Catgut and other surgical sutures for veterinary use

Use for each medium not less than the quantities of the product prescribed in Table 2.6.1.-2. Open the sealed package using aseptic precautions and remove 3 sections of the strand for each culture medium. Carry out the test on 3 sections, each 30 cm long, cut off from the beginning, the centre and the end of the strand. Use whole strands from freshly opened cassette packs. Transfer each section of the strand to the selected medium. Use sufficient medium to cover adequately the material to be tested (20 mL to 150 mL).

Table 2.6.1.-3. —*Minimum number of items to be tested*

Number of items in the batch*	Minimum number of items to be tested for each medium, unless otherwise justified and authorised**
Parenteral preparations	
— Not more than 100 containers	10 per cent or 4 containers, whichever is the greater
— More than 100 but not more than 500 containers	10 containers
— More than 500 containers	2 per cent or 20 containers (10 containers for large-volume parenterals) whichever is less
Ophthalmic and other non-injectable preparations	
— Not more than 200 containers	5 per cent or 2 containers, whichever is the greater
— More than 200 containers	10 containers
— If the product is presented in the form of single-dose containers, apply the scheme shown above for preparations for parenteral administration	
Catgut and other surgical sutures for veterinary use	2 per cent or 5 packages whichever is the greater, up to a maximum total of 20 packages
Bulk solid products	
— Up to 4 containers	Each container
— More than 4 containers but not more than 50 containers	20 per cent or 4 containers, whichever is the greater
— More than 50 containers	2 per cent or 10 containers, whichever is the greater

* If the batch size is not known, use the maximum number of items prescribed.
**If the contents of one container are enough to inoculate the 2 media, this column gives the number of containers needed for both the media together.

OBSERVATION AND INTERPRETATION OF RESULTS

At intervals during the incubation period and at its conclusion, examine the media for macroscopic evidence of microbial growth. If the material being tested renders the medium turbid so that the presence or absence of microbial growth cannot be readily determined by visual examination, 14 days after the beginning of incubation transfer portions (each not less than 1 mL) of the medium to fresh vessels of the same medium and then incubate the original and transfer vessels for not less than 4 days.

If no evidence of microbial growth is found, the product to be examined complies with the test for sterility. If evidence of microbial growth is found the product to be examined does not comply with the test for sterility, unless it can be clearly demonstrated that the test was invalid for causes unrelated to the product to be examined. The test may be considered invalid only if one or more of the following conditions are fulfilled:

a) the data of the microbiological monitoring of the sterility testing facility show a fault;

b) a review of the testing procedure used during the test in question reveals a fault;

c) microbial growth is found in the negative controls;

d) after determination of the identity of the micro-organisms isolated from the test, the growth of this species or these species may be ascribed unequivocally to faults with respect to the material and/or the technique used in conducting the sterility test procedure.

If the test is declared to be invalid it is repeated with the same number of units as in the original test.

If no evidence of microbial growth is found in the repeat test the product examined complies with the test for sterility.
If microbial growth is found in the repeat test the product examined does not comply with the test for sterility.

APPLICATION OF THE TEST TO PARENTERAL PREPARATIONS, OPHTHALMIC AND OTHER NON-INJECTABLE PREPARATIONS REQUIRED TO COMPLY WITH THE TEST FOR STERILITY

When using the technique of membrane filtration, use, whenever possible, the whole contents of the container, but not less than the quantities indicated in Table 2.6.1.-2, diluting where necessary to about 100 mL with a suitable sterile solution, such as 1 g/L neutral meat or casein peptone.

When using the technique of direct inoculation of media, use the quantities shown in Table 2.6.1.-2, unless otherwise justified and authorised. The tests for bacterial and fungal sterility are carried out on the same sample of the product to be examined. When the volume or the quantity in a single container is insufficient to carry out the tests, the contents of 2 or more containers are used to inoculate the different media.

MINIMUM NUMBER OF ITEMS TO BE TESTED

The minimum number of items to be tested in relation to the size of the batch is given in Table 2.6.1.-3.

Guidelines on the test for sterility are given in general chapter 5.1.9.

B. Microbiological Examination of Non-sterile Products

1. Test for Specified Micro-organisms[1]

(Ph. Eur. method 2.6.13)

1 INTRODUCTION

The tests described hereafter will allow determination of the absence or limited occurrence of specified micro-organisms that may be detected under the conditions described.

The tests are designed primarily to determine whether a substance or preparation complies with an established specification for microbiological quality. When used for such purposes, follow the instructions given below, including the number of samples to be taken, and interpret the results as stated below.

Alternative microbiological procedures, including automated methods, may be used, provided that their equivalence to the Pharmacopoeia method has been demonstrated.

2 GENERAL PROCEDURES

The preparation of samples is carried out as described in general chapter *2.6.12.*

If the product to be examined has antimicrobial activity, this is insofar as possible removed or neutralised as described in general chapter *2.6.12.*

If surface-active substances are used for sample preparation, their absence of toxicity for micro-organisms and their compatibility with inactivators used must be demonstrated as described in general chapter *2.6.12.*

3 GROWTH-PROMOTING AND INHIBITORY PROPERTIES OF THE MEDIA, SUITABILITY OF THE TEST AND NEGATIVE CONTROLS

The ability of the test to detect micro-organisms in the presence of the product to be tested must be established. Suitability must be confirmed if a change in testing performance, or the product, which may affect the outcome of the test is introduced.

3-1 PREPARATION OF TEST STRAINS

Use standardised stable suspensions of test strains or prepare them as stated below. Seed lot culture maintenance techniques (seed-lot systems) are used so that the viable micro-organisms used for inoculation are not more than 5 passages removed from the original master seed-lot.

3-1-1 Aerobic micro-organisms

Grow each of the bacterial test strains separately in casein soya bean digest broth or on casein soya bean digest agar at 30-35 °C for 18-24 h. Grow the test strain for *Candida albicans* separately on Sabouraud-dextrose agar or in Sabouraud-dextrose broth at 20-25 °C for 2-3 days.

- *Staphylococcus aureus* such as ATCC 6538, NCIMB 9518, CIP 4.83 or NBRC 13276;
- *Pseudomonas aeruginosa* such as ATCC 9027, NCIMB 8626, CIP 82.118 or NBRC 13275;
- *Escherichia coli* such as ATCC 8739, NCIMB 8545, CIP 53.126 or NBRC 3972;
- *Salmonella enterica* subsp. *enterica* serovar Typhimurium, such as ATCC 14028 or, as an alternative, *Salmonella enterica* subsp. *enterica* serovar Abony such as NBRC 100797, NCTC 6017 or CIP 80.39;
- *Candida albicans* such as ATCC 10231, NCPF 3179, IP 48.72 or NBRC 1594.

Use buffered sodium chloride-peptone solution pH 7.0 or phosphate buffer solution pH 7.2 to make test suspensions. Use the suspensions within 2 h or within 24 h if stored at 2-8 °C.

3-1-2 Clostridia

Use *Clostridium sporogenes* such as ATCC 11437 (NBRC 14293, NCIMB 12343, CIP 100651) or ATCC 19404 (NCTC 532 or CIP 79.03). Grow the clostridial test strain under anaerobic conditions in reinforced medium for clostridia at 30-35 °C for 24-48 h. As an alternative to preparing and then diluting down a fresh suspension of vegetative cells of *C. sporogenes*, a stable spore suspension is used for test inoculation. The stable spore suspension may be maintained at 2-8 °C for a validated period.

3-2 NEGATIVE CONTROL

To verify testing conditions, a negative control is performed using the chosen diluent in place of the test preparation. There must be no growth of micro-organisms. A negative control is also performed when testing the products as described in section 4. A failed negative control requires an investigation.

3-3 GROWTH PROMOTION AND INHIBITORY PROPERTIES OF THE MEDIA

Test each batch of ready-prepared medium and each batch of medium prepared either from dehydrated medium or from ingredients.

Verify suitable properties of relevant media as described in Table 2.6.13.-1.

Test for growth promoting properties, liquid media

Inoculate a portion of the appropriate medium with a small number (not more than 100 CFU) of the appropriate micro-organism. Incubate at the specified temperature for not more than the shortest period of time specified in the test. Clearly visible growth of the micro-organism comparable to that previously obtained with a previously tested and approved batch of medium occurs.

Test for growth promoting properties, solid media

Perform the surface-spread method, inoculating each plate with a small number (not more than 100 CFU) of the appropriate micro-organism. Incubate at the specified temperature for not more than the shortest period of time specified in the test. Growth of the micro-organism comparable to that previously obtained with a previously tested and approved batch of medium occurs.

Test for inhibitory properties, liquid or solid media

Inoculate the appropriate medium with at least 100 CFU of the appropriate micro-organism. Incubate at the specified temperature for not less than the longest period of time specified in the test. No growth of the test micro-organism occurs.

Test for indicative properties Perform the surface-spread method, inoculating each plate with a small number (not more than 100 CFU) of the appropriate micro-organism. Incubate at the specified temperature for a period of time within the range specified in the test. Colonies are comparable in appearance and indication reactions to those previously obtained with a previously tested and approved batch of medium.

3-4 SUITABILITY OF THE TEST METHOD

For each product to be tested, perform the sample preparation as described in the relevant paragraph in section 4. Add each test strain at the time of mixing, in the prescribed growth medium. Inoculate the test strains

[1] *This chapter has undergone pharmacopoeial harmonisation. See chapter 5.8 Pharmacopoeial harmonisation.*

Table 2.6.13.-1 – *Growth promoting, inhibitory and indicative properties of media*

	Medium	Property	Test strains
Test for bile-tolerant gram-negative bacteria	Enterobacteria enrichment broth-Mossel	Growth promoting	*E. coli* *P. aeruginosa*
		Inhibitory	*S. aureus*
	Violet red bile glucose agar	Growth promoting + indicative	*E. coli* *P. aeruginosa*
Test for *Escherichia coli*	MacConkey broth	Growth promoting	*E. coli*
		Inhibitory	*S. aureus*
	MacConkey agar	Growth promoting + indicative	*E. coli*
Test for *Salmonella*	Rappaport Vassiliadis *Salmonella* enrichment broth	Growth promoting	*Salmonella enterica* subsp. *enterica* serovar Typhimurium or *Salmonella enterica* subsp. *enterica* serovar Abony
		Inhibitory	*S. aureus*
	Xylose, lysine, deoxycholate agar	Growth promoting + indicative	*Salmonella enterica* subsp. *enterica* serovar Typhimurium or *Salmonella enterica* subsp. *enterica* serovar Abony
Test for *Pseudomonas aeruginosa*	Cetrimide agar	Growth promoting	*P. aeruginosa*
		Inhibitory	*E. coli*
Test for *Staphylococcus aureus*	Mannitol salt agar	Growth promoting + indicative	*S. aureus*
		Inhibitory	*E. coli*
Test for clostridia	Reinforced medium for clostridia	Growth promoting	*C. sporogenes*
	Columbia agar	Growth promoting	*C. sporogenes*
Test for *Candida albicans*	Sabouraud dextrose broth	Growth promoting	*C. albicans*
	Sabouraud dextrose agar	Growth promoting + indicative	*C. albicans*

individually. Use a number of micro-organisms equivalent to not more than 100 CFU in the inoculated test preparation.

Perform the test as described in the relevant paragraph in section 4 using the shortest incubation period prescribed.

The specified micro-organisms must be detected with the indication reactions as described in section 4.

Any antimicrobial activity of the product necessitates a modification of the test procedure (see 4-5-3 of general chapter *2.6.12*).

If for a given product the antimicrobial activity with respect to a micro-organism for which testing is prescribed cannot be neutralised, then it is to be assumed that the inhibited micro-organism will not be present in the product.

4 TESTING OF PRODUCTS

4-1 BILE-TOLERANT GRAM-NEGATIVE BACTERIA

4-1-1 Sample preparation and pre-incubation

Prepare a sample using a 1 in 10 dilution of not less than 1 g of the product to be examined as described in general chapter *2.6.12*, but using casein soya bean digest broth as the chosen diluent, mix and incubate at 20-25 °C for a time sufficient to resuscitate the bacteria but not sufficient to encourage multiplication of the organisms (usually 2 h but not more than 5 h).

4-1-2 Test for absence

Unless otherwise prescribed, use the volume corresponding to 1 g of the product, as prepared in 4-1-1, to inoculate enterobacteria enrichment broth-Mossel. Incubate at 30-35 °C for 24-48 h. Subculture on plates of violet red bile glucose agar. Incubate at 30-35 °C for 18-24 h.

The product complies with the test if there is no growth of colonies.

4-1-3 Quantitative test

4-1-3-1 Selection and subculture. Inoculate suitable quantities of enterobacteria enrichment broth-Mossel with the preparation as described under 4-1-1 and/or dilutions of it containing respectively 0.1 g, 0.01 g and 0.001 g (or 0.1 mL, 0.01 mL and 0.001 mL) of the product to be examined. Incubate at 30-35 °C for 24-48 h. Subculture each of the cultures on a plate of violet red bile glucose agar. Incubate at 30-35 °C for 18-24 h.

4-1-3-2 Interpretation. Growth of colonies constitutes a positive result. Note the smallest quantity of the product that gives a positive result and the largest quantity that gives a negative result. Determine from Table 2.6.13.-2 the probable number of bacteria.

Table 2.6.13.-2 – *Interpretation of results*

Results for each quantity of product			Probable number of bacteria per gram or millilitre of product
0.1 g or 0.1 mL	0.01 g or 0.01 mL	0.001 g or 0.001 mL	
+	+	+	$> 10^3$
+	+	–	$< 10^3$ and $> 10^2$
+	–	–	$< 10^2$ and > 10
–	–	–	< 10

4-2 ESCHERICHIA COLI

4-2-1 Sample preparation and pre-incubation

Prepare a sample using a 1 in 10 dilution of not less than 1 g of the product to be examined as described in general chapter *2.6.12*, and use 10 mL or the quantity corresponding to 1 g or 1 mL to inoculate a suitable amount (determined as described under 3-4) of casein soya bean digest broth, mix and incubate at 30-35 °C for 18-24 h.

◊When testing orodispersible films, filter the volume of sample corresponding to 1 film of the preparation described under 4-5-1 in general chapter *2.6.12* through a sterile filter membrane and place in 100 mL of casein soya bean digest broth. Incubate at 30-35 °C for 18-24 h.◊

4-2-2 Selection and subculture

Shake the container, transfer 1 mL of casein soya bean digest broth to 100 mL of MacConkey broth and incubate at 42-44 °C for 24-48 h. Subculture on a plate of MacConkey agar at 30-35 °C for 18-72 h.

4-2-3 Interpretation

Growth of colonies indicates the possible presence of *E. coli*. This is confirmed by identification tests.

The product complies with the test if no colonies are present or if the identification tests are negative.

4-3 SALMONELLA

4-3-1 Sample preparation and pre-incubation

Prepare the product to be examined as described in general chapter *2.6.12*, and use the quantity corresponding to not less than 10 g or 10 mL to inoculate a suitable amount (determined as described under 3-4) of casein soya bean digest broth, mix and incubate at 30-35 °C for 18-24 h.

4-3-2 Selection and subculture

Transfer 0.1 mL of casein soya bean digest broth to 10 mL of Rappaport Vassiliadis *Salmonella* enrichment broth and incubate at 30-35 °C for 18-24 h. Subculture on plates of xylose, lysine, deoxycholate agar. Incubate at 30-35 °C for 18-48 h.

4-3-3 Interpretation

The possible presence of *Salmonella* is indicated by the growth of well-developed, red colonies, with or without black centres. This is confirmed by identification tests.

The product complies with the test if colonies of the types described are not present or if the confirmatory identification tests are negative.

4-4 PSEUDOMONAS AERUGINOSA

4-4-1 Sample preparation and pre-incubation

Prepare a sample using a 1 in 10 dilution of not less than 1 g of the product to be examined as described in general chapter *2.6.12*, and use 10 mL or the quantity corresponding to 1 g or 1 mL to inoculate a suitable amount (determined as described under 3-4) of casein soya bean digest broth and mix. When testing transdermal patches ◊or orodispersible films◊, filter the volume of sample corresponding to 1 patch ◊or 1 film◊ of the preparation described under 4-5-1 in general chapter *2.6.12* through a sterile filter membrane and place in 100 mL of casein soya bean digest broth. Incubate at 30-35 °C for 18-24 h.

4-4-2 Selection and subculture

Subculture on a plate of cetrimide agar and incubate at 30-35 °C for 18-72 h.

4-4-3 Interpretation

Growth of colonies indicates the possible presence of *P. aeruginosa*. This is confirmed by identification tests.

The product complies with the test if colonies are not present or if the confirmatory identification tests are negative.

4-5 STAPHYLOCOCCUS AUREUS

4-5-1 Sample preparation and pre-incubation

Prepare a sample using a 1 in 10 dilution of not less than 1 g of the product to be examined as described in general chapter *2.6.12*, and use 10 mL or the quantity corresponding to 1 g or 1 mL to inoculate a suitable amount (determined as described under 3-4) of casein soya bean digest broth and mix. When testing transdermal patches ◊or orodispersible films◊, filter the volume of sample corresponding to 1 patch ◊or 1 film◊ of the preparation described under 4-5-1 in general chapter *2.6.12* through a sterile filter membrane and place in 100 mL of casein soya bean digest broth. Incubate at 30-35 °C for 18-24 h.

4-5-2 Selection and subculture

Subculture on a plate of mannitol salt agar and incubate at 30-35 °C for 18-72 h.

4-5-3 Interpretation

The possible presence of *S. aureus* is indicated by the growth of yellow/white colonies surrounded by a yellow zone. This is confirmed by identification tests.

The product complies with the test if colonies of the types described are not present or if the confirmatory identification tests are negative.

4-6 CLOSTRIDIA

4-6-1 Sample preparation and heat treatment

Prepare a sample using a 1 in 10 dilution (with a minimum total volume of 20 mL) of not less than 2 g or 2 mL of the product to be examined as described in general chapter *2.6.12*. Divide the sample into 2 portions of at least 10 mL. Heat 1 portion at 80 °C for 10 min and cool rapidly. Do not heat the other portion.

4-6-2 Selection and subculture

Use 10 mL or the quantity corresponding to 1 g or 1 mL of the product to be examined of both portions to inoculate suitable amounts (determined as described under 3-4) of reinforced medium for clostridia. Incubate under anaerobic conditions at 30-35 °C for 48 h. After incubation, make subcultures from each container on Columbia agar and incubate under anaerobic conditions at 30-35 °C for 48-72 h.

4-6-3 Interpretation

The occurrence of anaerobic growth of rods (with or without endospores) giving a negative catalase reaction indicates the presence of clostridia. This is confirmed by identification tests.

The product complies with the test if colonies of the types described are not present or if the confirmatory identification tests are negative.

4-7 CANDIDA ALBICANS

4-7-1 Sample preparation and pre-incubation

Prepare the product to be examined as described in general chapter *2.6.12*, and use 10 mL or the quantity corresponding to not less than 1 g or 1 mL to inoculate 100 mL of Sabouraud-dextrose broth and mix. Incubate at 30-35 °C for 3-5 days.

4-7-2 Selection and subculture

Subculture on a plate of Sabouraud-dextrose agar and incubate at 30-35 °C for 24-48 h.

4-7-3 Interpretation

Growth of white colonies may indicate the presence of *C. albicans*. This is confirmed by identification tests.

The product complies with the test if such colonies are not present or if the confirmatory identification tests are negative.

The following section is given for information.

5 RECOMMENDED SOLUTIONS AND CULTURE MEDIA

The following solutions and culture media have been found to be satisfactory for the purposes for which they are prescribed in the test for microbial contamination in the Pharmacopoeia. Other media may be used provided that their suitability can be demonstrated.

Stock buffer solution Place 34 g of potassium dihydrogen phosphate in a 1000 mL volumetric flask, dissolve in 500 mL of purified water, adjust to pH 7.2 ± 0.2 with sodium hydroxide, dilute to 1000.0 mL with purified water and mix. Dispense into containers and sterilise. Store at 2-8 °C.

Phosphate buffer solution pH 7.2 Prepare a mixture of stock buffer solution and purified water (1:800 *V/V*) and sterilise.

Buffered sodium chloride-peptone solution pH 7.0

Potassium dihydrogen phosphate	3.6 g
Disodium hydrogen phosphate dihydrate	7.2 g, equivalent to 0.067 M phosphate
Sodium chloride	4.3 g
Peptone (meat or casein)	1.0 g
Purified water	1000 mL

Sterilise in an autoclave using a validated cycle.

Casein soya bean digest broth

Pancreatic digest of casein	17.0 g
Papaic digest of soya bean	3.0 g
Sodium chloride	5.0 g
Dipotassium hydrogen phosphate	2.5 g
Glucose monohydrate	2.5 g
Purified water	1000 mL

Adjust the pH so that after sterilisation it is 7.3 ± 0.2 at 25 °C. Sterilise in an autoclave using a validated cycle.

Casein soya bean digest agar

Pancreatic digest of casein	15.0 g
Papaic digest of soya bean	5.0 g
Sodium chloride	5.0 g
Agar	15.0 g
Purified water	1000 mL

Adjust the pH so that after sterilisation it is 7.3 ± 0.2 at 25 °C. Sterilise in an autoclave using a validated cycle.

Sabouraud-dextrose agar

Dextrose	40.0 g
Mixture of peptic digest of animal tissue and pancreatic digest of casein (1:1)	10.0 g
Agar	15.0 g
Purified water	1000 mL

Adjust the pH so that after sterilisation it is 5.6 ± 0.2 at 25 °C. Sterilise in an autoclave using a validated cycle.

Potato dextrose agar

Infusion from potatoes	200 g
Dextrose	20.0 g
Agar	15.0 g
Purified water	1000 mL

Adjust the pH so that after sterilisation it is 5.6 ± 0.2 at 25 °C. Sterilise in an autoclave using a validated cycle.

Sabouraud-dextrose broth

Dextrose	20.0 g
Mixture of peptic digest of animal tissue and pancreatic digest of casein (1:1)	10.0 g
Purified water	1000 mL

Adjust the pH so that after sterilisation it is 5.6 ± 0.2 at 25 °C. Sterilise in an autoclave using a validated cycle.

Enterobacteria enrichment broth-Mossel

Pancreatic digest of gelatin	10.0 g
Glucose monohydrate	5.0 g
Dehydrated ox bile	20.0 g
Potassium dihydrogen phosphate	2.0 g
Disodium hydrogen phosphate dihydrate	8.0 g
Brilliant green	15 mg
Purified water	1000 mL

Adjust the pH so that after heating it is 7.2 ± 0.2 at 25 °C. Heat at 100 °C for 30 min and cool immediately.

Violet red bile glucose agar

Yeast extract	3.0 g
Pancreatic digest of gelatin	7.0 g
Bile salts	1.5 g
Sodium chloride	5.0 g
Glucose monohydrate	10.0 g
Agar	15.0 g
Neutral red	30 mg
Crystal violet	2 mg
Purified water	1000 mL

Adjust the pH so that after heating it is 7.4 ± 0.2 at 25 °C. Heat to boiling; do not heat in an autoclave.

MacConkey broth

Pancreatic digest of gelatin	20.0 g
Lactose monohydrate	10.0 g
Dehydrated ox bile	5.0 g
Bromocresol purple	10 mg
Purified water	1000 mL

Adjust the pH so that after sterilisation it is 7.3 ± 0.2 at 25 °C. Sterilise in an autoclave using a validated cycle.

MacConkey agar

Pancreatic digest of gelatin	17.0 g
Peptones (meat and casein)	3.0 g
Lactose monohydrate	10.0 g
Sodium chloride	5.0 g
Bile salts	1.5 g
Agar	13.5 g
Neutral red	30.0 mg
Crystal violet	1 mg
Purified water	1000 mL

Adjust the pH so that after sterilisation it is 7.1 ± 0.2 at 25 °C. Boil for 1 min with constant shaking then sterilise in an autoclave using a validated cycle.

Rappaport Vassiliadis *Salmonella* enrichment broth

Soya peptone	4.5 g
Magnesium chloride hexahydrate	29.0 g
Sodium chloride	8.0 g
Dipotassium phosphate	0.4 g
Potassium dihydrogen phosphate	0.6 g
Malachite green	0.036 g
Purified water	1000 mL

Dissolve, warming gently. Sterilise in an autoclave using a validated cycle, at a temperature not exceeding 115 °C. The pH is to be 5.2 ± 0.2 at 25 °C after heating and autoclaving.

Xylose, lysine, deoxycholate agar

Xylose	3.5 g
L-Lysine	5.0 g
Lactose monohydrate	7.5 g
Sucrose	7.5 g
Sodium chloride	5.0 g
Yeast extract	3.0 g
Phenol red	80 mg
Agar	13.5 g
Sodium deoxycholate	2.5 g
Sodium thiosulfate	6.8 g
Ferric ammonium citrate	0.8 g
Purified water	1000 mL

Adjust the pH so that after heating it is 7.4 ± 0.2 at 25 °C. Heat to boiling, cool to 50 °C and pour into Petri dishes. Do not heat in an autoclave.

Cetrimide agar

Pancreatic digest of gelatin	20.0 g
Magnesium chloride	1.4 g
Dipotassium sulfate	10.0 g
Cetrimide	0.3 g
Agar	13.6 g
Purified water	1000 mL
Glycerol	10.0 mL

Heat to boiling for 1 min with shaking. Adjust the pH so that after sterilisation it is 7.2 ± 0.2 at 25 °C. Sterilise in an autoclave using a validated cycle.

Mannitol salt agar

Pancreatic digest of casein	5.0 g
Peptic digest of animal tissue	5.0 g
Beef extract	1.0 g
D-Mannitol	10.0 g
Sodium chloride	75.0 g
Agar	15.0 g
Phenol red	0.025 g
Purified water	1000 mL

Heat to boiling for 1 min with shaking. Adjust the pH so that after sterilisation it is 7.4 ± 0.2 at 25 °C. Sterilise in an autoclave using a validated cycle.

Reinforced medium for clostridia

Beef extract	10.0 g
Peptone	10.0 g
Yeast extract	3.0 g
Soluble starch	1.0 g
Glucose monohydrate	5.0 g
Cysteine hydrochloride	0.5 g
Sodium chloride	5.0 g
Sodium acetate	3.0 g
Agar	0.5 g
Purified water	1000 mL

Hydrate the agar, dissolve by heating to boiling with continuous stirring. If necessary, adjust the pH so that after sterilisation it is 6.8 ± 0.2 at 25 °C. Sterilise in an autoclave using a validated cycle.

Columbia agar

Pancreatic digest of casein	10.0 g
Meat peptic digest	5.0 g
Heart pancreatic digest	3.0 g
Yeast extract	5.0 g
Maize starch	1.0 g
Sodium chloride	5.0 g
Agar, according to gelling power	10.0-15.0 g
Purified water	1000 mL

Hydrate the agar, dissolve by heating to boiling with continuous stirring. If necessary, adjust the pH so that after sterilisation it is 7.3 ± 0.2 at 25 °C. Sterilise in an autoclave using a validated cycle. Allow to cool to 45-50 °C; add, where necessary, gentamicin sulfate corresponding to 20 mg of gentamicin base and pour into Petri dishes.

2. Microbial Enumeration Tests[2]

(Ph. Eur. method 2.6.12)

1 INTRODUCTION

The tests described hereafter will allow quantitative enumeration of mesophilic bacteria and fungi that may grow under aerobic conditions.

The tests are designed primarily to determine whether a substance or preparation complies with an established specification for microbiological quality. When used for such purposes follow the instructions given below, including the number of samples to be taken, and interpret the results as stated below.

The methods are not applicable to products containing viable micro-organisms as active ingredients.

Alternative microbiological procedures, including automated methods, may be used, provided that their equivalence to the Pharmacopoeia method has been demonstrated.

2 GENERAL PROCEDURES

Carry out the determination under conditions designed to avoid extrinsic microbial contamination of the product to be examined. The precautions taken to avoid contamination must be such that they do not affect any micro-organisms that are to be revealed in the test.

If the product to be examined has antimicrobial activity, this is insofar as possible removed or neutralised. If inactivators are used for this purpose, their efficacy and their absence of toxicity for micro-organisms must be demonstrated.

If surface-active substances are used for sample preparation, their absence of toxicity for micro-organisms and their compatibility with inactivators used must be demonstrated.

3 ENUMERATION METHODS

Use the membrane filtration method or the plate-count methods, as prescribed. The most-probable-number (MPN) method is generally the least accurate method for microbial counts, however, for certain product groups with a very low bioburden, it may be the most appropriate method.

The choice of method is based on factors such as the nature of the product and the required limit of micro-organisms. The chosen method must allow testing of a sufficient sample size to judge compliance with the specification.
The suitability of the method chosen must be established.

4 GROWTH PROMOTION TEST, SUITABILITY OF THE COUNTING METHOD AND NEGATIVE CONTROLS

4-1 GENERAL CONSIDERATIONS

The ability of the test to detect micro-organisms in the presence of product to be tested must be established.

Suitability must be confirmed if a change in testing performance, or the product, which may affect the outcome of the test is introduced.

4-2 PREPARATION OF TEST STRAINS

Use standardised stable suspensions of test strains or prepare them as stated below. Seed lot culture maintenance

[2] *This chapter has undergone pharmacopoeial harmonisation. See chapter 5.8 Pharmacopoeial harmonisation.*

Table 2.6.12.-1. – *Preparation and use of test micro-organisms*

Micro-organism	Preparation of test strain	Growth promotion		Suitability of counting method in the presence of the product	
		Total aerobic microbial count	Total yeasts and moulds count	Total aerobic microbial count	Total yeasts and moulds count
Staphylococcus aureus such as: ATCC 6538 NCIMB 9518 CIP 4.83 NBRC 13276	Casein soya bean digest agar or casein soya bean digest broth 30-35 °C 18-24 h	Casein soya bean digest agar and casein soya bean digest broth ≤ 100 CFU 30-35 °C ≤ 3 days	-	Casein soya bean digest agar/MPN casein soya bean digest broth ≤ 100 CFU 30-35 °C ≤ 3 days	-
Pseudomonas aeruginosa such as: ATCC 9027 NCIMB 8626 CIP 82.118 NBRC 13275	Casein soya bean digest agar or casein soya bean digest broth 30-35 °C 18-24 h	Casein soya bean digest agar and casein soya bean digest broth ≤ 100 CFU 30-35 °C ≤ 3 days	-	Casein soya bean digest agar/MPN casein soya bean digest broth ≤ 100 CFU 30-35 °C ≤ 3 days	-
Bacillus subtilis such as: ATCC 6633 NCIMB 8054 CIP 52.62 NBRC 3134	Casein soya bean digest agar or casein soya bean digest broth 30-35 °C 18-24 h	Casein soya bean digest agar and casein soya bean digest broth ≤ 100 CFU 30-35 °C ≤ 3 days	-	Casein soya bean digest agar/MPN casein soya bean digest broth ≤ 100 CFU 30-35 °C ≤ 3 days	-
Candida albicans such as: ATCC 10231 NCPF 3179 IP 48.72 NBRC 1594	Sabouraud-dextrose agar or Sabouraud-dextrose broth 20-25 °C 2-3 days	Casein soya bean digest agar ≤ 100 CFU 30-35 °C ≤ 5 days	Sabouraud-dextrose agar ≤ 100 CFU 20-25 °C ≤ 5 days	Casein soya bean digest agar ≤ 100 CFU 30-35 °C ≤ 5 days MPN: not applicable	Sabouraud-dextrose agar ≤ 100 CFU 20-25 °C ≤ 5 days
Aspergillus brasiliensis such as: ATCC 16404 IMI 149007 IP 1431.83 NBRC 9455	Sabouraud-dextrose agar or potato-dextrose agar 20-25 °C 5-7 days, or until good sporulation is achieved	Casein soya bean digest agar ≤ 100 CFU 30-35 °C ≤ 5 days	Sabouraud-dextrose agar ≤ 100 CFU 20-25 °C ≤ 5 days	Casein soya bean digest agar ≤ 100 CFU 30-35 °C ≤ 5 days MPN: not applicable	Sabouraud-dextrose agar ≤ 100 CFU 20-25 °C ≤ 5 days

techniques (seed-lot systems) are used so that the viable micro-organisms used for inoculation are not more than 5 passages removed from the original master seed-lot. Grow each of the bacterial and fungal test strains separately as described in Table 2.6.12.-1.

Use buffered sodium chloride-peptone solution pH 7.0 or phosphate buffer solution pH 7.2 to make test suspensions; to suspend *A. brasiliensis* spores, 0.05 per cent of polysorbate 80 may be added to the buffer. Use the suspensions within 2 h or within 24 h if stored at 2-8 °C. As an alternative to preparing and then diluting a fresh suspension of vegetative cells of *A. brasiliensis* or *B. subtilis*, a stable spore suspension is prepared and then an appropriate volume of the spore suspension is used for test inoculation. The stable spore suspension may be maintained at 2-8 °C for a validated period of time.

4-3 NEGATIVE CONTROL

To verify testing conditions, a negative control is performed using the chosen diluent in place of the test preparation. There must be no growth of micro-organisms. A negative control is also performed when testing the products as described in section 5. A failed negative control requires an investigation.

4-4 GROWTH PROMOTION OF THE MEDIA

Test each batch of ready-prepared medium and each batch of medium, prepared either from dehydrated medium or from the ingredients described.

Inoculate portions/plates of casein soya bean digest broth and casein soya bean digest agar with a small number (not more than 100 CFU) of the micro-organisms indicated in Table 2.6.12.-1, using a separate portion/plate of medium for each. Inoculate plates of Sabouraud-dextrose agar with a small number (not more than 100 CFU) of the micro-organisms indicated in Table 2.6.12.-1, using a separate plate of medium for each. Incubate in the conditions described in Table 2.6.12.-1.

For solid media, growth obtained must not differ by a factor greater than 2 from the calculated value for a standardised inoculum. For a freshly prepared inoculum, growth of the micro-organisms comparable to that previously obtained with a previously tested and approved batch of medium occurs. Liquid media are suitable if clearly visible growth of the micro-organisms comparable to that previously obtained with a previously tested and approved batch of medium occurs.

4-5 SUITABILITY OF THE COUNTING METHOD IN THE PRESENCE OF PRODUCT

4-5-1 Preparation of the sample

The method for sample preparation depends upon the physical characteristics of the product to be tested. If none of the procedures described below can be demonstrated to be satisfactory, an alternative procedure must be developed.

Water-soluble products Dissolve or dilute (usually a 1 in 10 dilution is prepared) the product to be examined in buffered sodium chloride-peptone solution pH 7.0, phosphate buffer solution pH 7.2 or casein soya bean digest broth. If necessary, adjust to pH 6-8. Further dilutions, where necessary, are prepared with the same diluent.

Non-fatty products insoluble in water Suspend the product to be examined (usually a 1 in 10 dilution is prepared) in buffered sodium chloride-peptone solution

pH 7.0, phosphate buffer solution pH 7.2 or casein soya bean digest broth. A surface-active agent such as 1 g/L of polysorbate 80 may be added to assist the suspension of poorly wettable substances. If necessary, adjust to pH 6-8. Further dilutions, where necessary, are prepared with the same diluent.

Fatty products Dissolve in isopropyl myristate, sterilised by filtration or mix the product to be examined with the minimum necessary quantity of sterile polysorbate 80 or another non-inhibitory sterile surface-active agent, heated if necessary to not more than 40 °C, or in exceptional cases to not more than 45 °C. Mix carefully and if necessary maintain the temperature in a water-bath. Add sufficient of the pre-warmed chosen diluent to make a 1 in 10 dilution of the original product. Mix carefully whilst maintaining the temperature for the shortest time necessary for the formation of an emulsion. Further serial tenfold dilutions may be prepared using the chosen diluent containing a suitable concentration of sterile polysorbate 80 or another non-inhibitory sterile surface-active agent.

Fluids or solids in aerosol form Aseptically transfer the product into a membrane filter apparatus or a sterile container for further sampling. Use either the total contents or a defined number of metered doses from each of the containers tested.

Transdermal patches Remove the protective cover sheets ('release liners') of the transdermal patches and place them, adhesive side upwards, on sterile glass or plastic trays. Cover the adhesive surface with a sterile porous material, for example sterile gauze, to prevent the patches from sticking together, and transfer the patches to a suitable volume of the chosen diluent containing inactivators such as polysorbate 80 and/or lecithin. Shake the preparation vigorously for at least 30 min.

◊*Orodispersible films.* Dissolve 10 films to be examined in buffered sodium chloride-peptone solution pH 7.0, phosphate buffer solution pH 7.2 or casein soya bean digest broth. It may be necessary to heat the preparation to not more than 40 °C, or in exceptional cases to not more than 45 °C, with or without shaking, to achieve dissolution. If necessary, adjust to pH 6-8. Further dilutions, where necessary, are prepared with the same diluent.◊

4-5-2 Inoculation and dilution

Add to the sample prepared as described above (4-5-1) and to a control (with no test material included) a sufficient volume of the microbial suspension to obtain an inoculum of not more than 100 CFU. The volume of the suspension of the inoculum should not exceed 1 per cent of the volume of diluted product.

To demonstrate acceptable microbial recovery from the product, the lowest possible dilution factor of the prepared sample must be used for the test. Where this is not possible due to antimicrobial activity or poor solubility, further appropriate protocols must be developed. If inhibition of growth by the sample cannot otherwise be avoided, the aliquot of the microbial suspension may be added after neutralisation, dilution or filtration.

4-5-3 Neutralisation/removal of antimicrobial activity

The number of micro-organisms recovered from the prepared sample diluted as described in 4-5-2 and incubated following the procedure described in 4-5-4, is compared to the number of micro-organisms recovered from the control preparation.

If growth is inhibited (reduction by a factor greater than 2), then modify the procedure for the particular enumeration test to ensure the validity of the results. Modification of the procedure may include, for example, (1) an increase in the volume of the diluent or culture medium, (2) incorporation of specific or general neutralising agents into the diluent, (3) membrane filtration, or (4) a combination of the above measures.

Neutralising agents Neutralising agents may be used to neutralise the activity of antimicrobial agents (Table 2.6.12.-2). They may be added to the chosen diluent or the medium preferably before sterilisation. If used, their efficacy and their absence of toxicity for micro-organisms must be demonstrated by carrying out a blank with neutraliser and without product.

If no suitable neutralising method can be found, it can be assumed that the failure to isolate the inoculated organism is attributable to the microbicidal activity of the product. This information serves to indicate that the product is not likely to be contaminated with the given species of the micro-organism. However, it is possible that the product only inhibits some of the micro-organisms specified herein, but does not inhibit others not included amongst the test strains or for which the latter are not representative. Then, perform the test with the highest dilution factor compatible with microbial growth and the specific acceptance criterion.

Table 2.6.12.-2. – *Common neutralising agents for interfering substances*

Interfering substance	Potential neutralising method
Glutaraldehyde, mercurials	Sodium hydrogensulfite (sodium bisulfite)
Phenolics, alcohol, aldehydes, sorbate	Dilution
Aldehydes	Glycine
Quaternary Ammonium Compounds (QACs), parahydroxybenzoates (parabens), bis-biguanides	Lecithin
QACs, iodine, parabens	Polysorbate
Mercurials	Thioglycollate
Mercurials, halogens, aldehydes	Thiosulfate
EDTA (edetate)	Mg^{2+} or Ca^{2+} ions

4-5-4 Recovery of micro-organism in the presence of product

For each of the micro-organisms listed, separate tests are performed. Only micro-organisms of the added test strain are counted.

4-5-4-1 Membrane filtration. Use membrane filters having a nominal pore size not greater than 0.45 µm. The type of filter material is chosen such that the bacteria-retaining efficiency is not affected by the components of the sample to be investigated. For each of the micro-organisms listed, one membrane filter is used.

Transfer a suitable amount of the sample prepared as described under 4-5-1 to 4-5-3 (preferably representing 1 g of the product, or less if large numbers of CFU are expected) to the membrane filter, filter immediately and rinse the membrane filter with an appropriate volume of diluent.

For the determination of total aerobic microbial count (TAMC), transfer the membrane filter to the surface of casein soya bean digest agar. For the determination of total combined yeasts/moulds count (TYMC), transfer the membrane to the surface of Sabouraud-dextrose agar.

Incubate the plates as indicated in Table 2.6.12.-1. Perform the counting.

4-5-4-2 Plate-count methods. Perform plate-count methods at least in duplicate for each medium and use the mean count of the result.

4-5-4-2-1 Pour-plate method

For Petri dishes 9 cm in diameter, add to the dish 1 mL of the sample prepared as described under 4-5-1 to 4-5-3 and 15-20 mL of casein soya bean digest agar or Sabouraud-dextrose agar, both media being at not more than 45 °C. If larger Petri dishes are used, the amount of agar medium is increased accordingly. For each of the micro-organisms listed in Table 2.6.12.-1, at least 2 Petri dishes are used. Incubate the plates as indicated in Table 2.6.12.-1. Take the arithmetic mean of the counts per medium and calculate the number of CFU in the original inoculum.

4-5-4-2-2 Surface-spread method

For Petri dishes 9 cm in diameter, add 15-20 mL of casein soya bean digest agar or Sabouraud-dextrose agar at about 45 °C to each Petri dish and allow to solidify. If larger Petri dishes are used, the volume of the agar is increased accordingly. Dry the plates, for example in a laminar-air-flow cabinet or an incubator. For each of the micro-organisms listed in Table 2.6.12.-1, at least 2 Petri dishes are used. Spread a measured volume of not less than 0.1 mL of the sample prepared as described under 4-5-1 to 4-5-3 over the surface of the medium. Incubate and count as prescribed under 4-5-4-2-1

4-5-4-3 Most-probable-number (MPN) method.

The precision and accuracy of the MPN method is less than that of the membrane filtration method or the plate-count method. Unreliable results are obtained particularly for the enumeration of moulds. For these reasons the MPN method is reserved for the enumeration of TAMC in situations where no other method is available. If the use of the method is justified, proceed as follows.

Prepare a series of at least 3 serial tenfold dilutions of the product as described under 4-5-1 to 4-5-3. From each level of dilution, 3 aliquots of 1 g or 1 mL are used to inoculate 3 tubes with 9-10 mL of casein soya bean digest broth. If necessary, a surface-active agent such as polysorbate 80 or an inactivator of antimicrobial agents may be added to the medium. Thus, if 3 levels of dilution are prepared, 9 tubes are inoculated.

Incubate all tubes at 30-35 °C for not more than 3 days. If reading of the results is difficult or uncertain owing to the nature of the product to be examined, subculture in the same broth, or in casein soya bean digest agar, for 1-2 days at the same temperature and use these results. Determine the most probable number of micro-organisms per gram or millilitre of the product to be examined from Table 2.6.12.-3.

4-6 RESULTS AND INTERPRETATION

When verifying the suitability of the membrane filtration method or the plate-count method, a mean count of any of the test organisms not differing by a factor greater than 2 from the value of the control defined in 4-5-2 in the absence of the product must be obtained. When verifying the suitability of the MPN method the calculated value from the inoculum must be within 95 per cent confidence limits of the results obtained with the control.

If the above criteria cannot be met for one or more of the organisms tested with any of the described methods, the method and test conditions that come closest to the criteria are used to test the product.

Table 2.6.12.-3. – *Most-probable-number values of micro-organisms*

Observed combinations of numbers of tubes showing growth in each set			MPN per gram or per millilitre of product	95 per cent confidence limits
Number of grams or millilitres of product per tube				
0.1	0.01	0.001		
0	0	0	< 3	0-9.4
0	0	1	3	0.1-9.5
0	1	0	3	0.1-10
0	1	1	6.1	1.2-17
0	2	0	6.2	1.2-17
0	3	0	9.4	3.5-35
1	0	0	3.6	0.2-17
1	0	1	7.2	1.2-17
1	0	2	11	4-35
1	1	0	7.4	1.3-20
1	1	1	11	4-35
1	2	0	11	4-35
1	2	1	15	5-38
1	3	0	16	5-38
2	0	0	9.2	1.5-35
2	0	1	14	4-35
2	0	2	20	5-38
2	1	0	15	4-38
2	1	1	20	5-38
2	1	2	27	9-94
2	2	0	21	5-40
2	2	1	28	9-94
2	2	2	35	9-94
2	3	0	29	9-94
2	3	1	36	9-94
3	0	0	23	5-94
3	0	1	38	9-104
3	0	2	64	16-181
3	1	0	43	9-181
3	1	1	75	17-199
3	1	2	120	30-360
3	1	3	160	30-380
3	2	0	93	18-360
3	2	1	150	30-380
3	2	2	210	30-400
3	2	3	290	90-990
3	3	0	240	40-990
3	3	1	460	90-1980
3	3	2	1100	200-4000
3	3	3	> 1100	

5 TESTING OF PRODUCTS

5-1 AMOUNT USED FOR THE TEST

Unless otherwise prescribed, use 10 g or 10 mL of the product to be examined taken with the precautions referred to above. For fluids or solids in aerosol form, sample 10 containers. For transdermal patches, sample 10 patches. ◊For orodispersible films, sample 10 films.◊

The amount to be tested may be reduced for active substances that will be formulated in the following conditions: the amount per dosage unit (e.g. tablet, capsule, injection) is less than or equal to 1 mg or the amount per gram or millilitre (for preparations not presented in dose units) is less than 1 mg. In these cases, the amount to be tested is not less than the amount present in 10 dosage units or 10 g or 10 mL of the product.

For materials used as active substances where sample quantity is limited or batch size is extremely small (i.e. less than 1000 mL or 1000 g), the amount tested shall be 1 per cent of the batch unless a lesser amount is prescribed or justified and authorised.

For products where the total number of entities in a batch is less than 200 (e.g. samples used in clinical trials), the sample size may be reduced to 2 units, or 1 unit if the size is less than 100.

Select the sample(s) at random from the bulk material or from the available containers of the preparation. To obtain the required quantity, mix the contents of a sufficient number of containers to provide the sample.

5-2 EXAMINATION OF THE PRODUCT

5-2-1 Membrane filtration

Use a filtration apparatus designed to allow the transfer of the filter to the medium. Prepare the sample using a method that has been shown suitable as described in section 4 and transfer the appropriate amount to each of 2 membrane filters and filter immediately. Wash each filter following the procedure shown to be suitable.

For the determination of TAMC, transfer one of the membrane filters to the surface of casein soya bean digest agar. For the determination of TYMC, transfer the other membrane to the surface of Sabouraud-dextrose agar. Incubate the plate of casein soya bean digest agar at 30-35 °C for 3-5 days and the plate of Sabouraud-dextrose agar at 20-25 °C for 5-7 days. Calculate the number of CFU per gram or per millilitre of product.

When examining transdermal patches ◊or orodispersible films◊, filter 10 per cent of the volume of the preparation described under 4-5-1 separately through each of 2 sterile filter membranes. Transfer one membrane to casein soya bean digest agar for TAMC and the other membrane to Sabouraud-dextrose agar for TYMC.

5-2-2 Plate-count methods

5-2-2-1 Pour-plate method

Prepare the sample using a method that has been shown to be suitable as described in section 4. Prepare for each medium at least 2 Petri dishes for each level of dilution. Incubate the plates of casein soya bean digest agar at 30-35 °C for 3-5 days and the plates of Sabouraud-dextrose agar at 20-25 °C for 5-7 days. Select the plates corresponding to a given dilution and showing the highest number of colonies less than 250 for TAMC and 50 for TYMC. Take the arithmetic mean per culture medium of the counts and calculate the number of CFU per gram or per millilitre of product.

5-2-2-2 Surface-spread method

Prepare the sample using a method that has been shown to be suitable as described in section 4. Prepare at least 2 Petri dishes for each medium and each level of dilution. For incubation and calculation of the number of CFU proceed as described for the pour-plate method.

5-2-3 Most-probable-number method

Prepare and dilute the sample using a method that has been shown to be suitable as described in section 4. Incubate all tubes at 30-35 °C for 3-5 days. Subculture if necessary, using the procedure shown to be suitable. Record for each level of dilution the number of tubes showing microbial growth. Determine the most probable number of micro-organisms per gram or millilitre of the product to be examined from Table 2.6.12.-3.

5-3 INTERPRETATION OF THE RESULTS

The total aerobic microbial count (TAMC) is considered to be equal to the number of CFU found using casein soya bean digest agar; if colonies of fungi are detected on this medium, they are counted as part of the TAMC. The total combined yeasts/mould count (TYMC) is considered to be equal to the number of CFU found using Sabouraud-dextrose agar; if colonies of bacteria are detected on this medium, they are counted as part of the TYMC. When the TYMC is expected to exceed the acceptance criterion due to the bacterial growth, Sabouraud-dextrose agar containing antibiotics may be used. If the count is carried out by the MPN method the calculated value is the TAMC.

When an acceptance criterion for microbiological quality is prescribed it is interpreted as follows:

- 10^1 CFU: maximum acceptable count = 20;
- 10^2 CFU: maximum acceptable count = 200;
- 10^3 CFU: maximum acceptable count = 2000, and so forth.

The recommended solutions and media are described in general chapter *2.6.13.*

3. Test for Absence of Mycoplasmas

(Ph. Eur. method 2.6.7 as applied to vaccines for human use)

Where the test for mycoplasmas is prescribed for a master cell bank, for a working cell bank, for a virus seed lot or for control cells, both the culture method and the indicator cell culture method are used. Where the test for mycoplasmas is prescribed for a virus harvest, for a bulk vaccine or for the final lot (batch), the culture method is used. The indicator cell culture method may also be used, where necessary, for screening of media.

Nucleic acid amplification techniques (NAT) may be used as an alternative to one or both of the other methods after suitable validation.

CULTURE METHOD

CHOICE OF CULTURE MEDIA

The test is carried out using a sufficient number of both solid and liquid media to ensure growth in the chosen incubation conditions of small numbers of mycoplasmas that may be present in the product to be examined. Liquid media must contain phenol red. The range of media chosen is shown to have satisfactory nutritive properties for at least the micro-organisms shown below. The nutritive properties of each new batch of medium are verified for the appropriate micro-organisms in the list. When testing for mycoplasmas in the product to be examined, at least 1 of the following species will be included as a positive control:

- *Acholeplasma laidlawii* (vaccines for human and veterinary use where an antibiotic has been used during production);

— *Mycoplasma gallisepticum* (where avian material has been used during production or where the vaccine is intended for use in poultry);
— *Mycoplasma hyorhinis* (non-avian veterinary vaccines);
— *Mycoplasma orale* (vaccines for human and veterinary use);
— *Mycoplasma pneumoniae* (vaccines for human use) or other suitable species of D-glucose fermenter such as *Mycoplasma fermentans*;
— *Mycoplasma synoviae* (where avian material has been used during production or where the vaccine is intended for use in poultry).

The test strains are field isolates having undergone a limited number of subcultures (not more than 15), and are stored frozen or freeze-dried. After cloning, the strains are identified as being of the required species by comparison with type cultures, for example:

A. laidlawii	NCTC 10116	CIP 75.27	ATCC 23206
M. gallisepticum	NCTC 10115	CIP 104967	ATCC 19610
M. fermentans	NCTC 10117	CIP 105680	ATCC 19989
M. hyorhinis	NCTC 10130	CIP 104968	ATCC 17981
M. orale	NCTC 10112	CIP 104969	ATCC 23714
M. pneumoniae	NCTC 10119	CIP 103766	ATCC 15531
M. synoviae	NCTC 10124	CIP 104970	ATCC 25204

Acholeplasma laidlawii BRP, Mycoplasma fermentans BRP, Mycoplasma hyorhinis BRP, Mycoplasma orale BRP and *Mycoplasma synoviae BRP* are suitable for use as low-passage reference strains.

INCUBATION CONDITIONS

Incubate liquid media in tightly stoppered containers at 35-38 °C. Incubate solid media in microaerophilic conditions (nitrogen containing 5-10 per cent of carbon dioxide and sufficient humidity to prevent desiccation of the agar surface) at 35-38 °C.

NUTRITIVE PROPERTIES

Carry out the test for nutritive properties for each new batch of medium Inoculate the chosen media with the appropriate test micro-organisms; use not more than 100 CFU per 60 mm diameter plate containing 9 mL of solid medium and per 100 mL container of liquid medium; use a separate plate and container for each species of micro-organism. Incubate the media and make subcultures from 0.2 mL of liquid medium to solid medium at the specified intervals (see below under Test for mycoplasmas in the product to be examined). The solid medium complies with the test if adequate growth is found for each test micro-organism (growth obtained does not differ by a factor greater than 5 from the value calculated with respect to the inoculum). The liquid medium complies with the test if growth on agar plates subcultured from the broth is found for at least 1 subculture for each test micro-organism.

INHIBITORY SUBSTANCES

The test for inhibitory substances is carried out once for a given product and is repeated whenever there is a change in production method that may affect the detection of mycoplasmas.

To demonstrate absence of inhibitory substances, carry out the test for nutritive properties in the presence and absence of the product to be examined. If growth of a test micro-organism occurs more than 1 subculture sooner in the absence of the product to be examined than in its presence, or if plates directly inoculated with the product to be examined have fewer than 1/5 of the number of colonies of those inoculated without the product to be examined, inhibitory substances are present and they must be neutralised or their effect otherwise countered, for example by passage in substrates not containing inhibitors or dilution in a larger volume of medium before the test. If dilution is used, larger medium volumes may be used or the inoculum volume may be divided among several 100 mL flasks. The effectiveness of the neutralisation or other process is checked by repeating the test for inhibitory substances after neutralisation.

TEST FOR MYCOPLASMAS IN THE PRODUCT TO BE EXAMINED

Inoculate 10 mL of the product to be examined per 100 mL of each liquid medium. If it has been found that a significant pH change occurs upon the addition of the product to be examined, the liquid medium is restored to its original pH value by the addition of a solution of either sodium hydroxide or hydrochloric acid. Inoculate 0.2 mL of the product to be examined on each plate of each solid medium. Incubate liquid media for 20-21 days. Incubate solid media for not less than 14 days, except those corresponding to the 20-21 day subculture, which are incubated for 7 days. At the same time incubate an uninoculated 100 mL portion of each liquid medium and agar plates, as a negative control. On days 2-4 after inoculation, subculture each liquid medium by inoculating 0.2 mL on at least 1 plate of each solid medium. Repeat the procedure between the 6th and 8th days, again between the 13th and 15th days and again between the 19th and 21st days of the test. Observe the liquid media every 2 or 3 days and if a colour change occurs, subculture. If a liquid medium shows bacterial or fungal contamination, the test is invalid. The test is valid if at least 1 plate per medium and per inoculation day can be read. Include in the test positive controls prepared by inoculation of not more than 100 CFU of at least 1 test micro-organism on agar medium or into broth medium. Where the test for mycoplasmas is carried out regularly and where possible, it is recommended to use the test micro-organisms in regular rotation. The test micro-organisms used are those listed under Choice of culture media.

INTERPRETATION OF RESULTS

At the end of the prescribed incubation period, examine all inoculated solid media microscopically for the presence of mycoplasma colonies. The product complies with the test if growth of typical mycoplasma colonies has not occurred. The product does not comply with the test if growth of typical mycoplasma colonies has occurred on any of the solid media. The test is invalid if 1 or more of the positive controls do not show growth of mycoplasmas on at least 1 subculture plate. The test is invalid if 1 or more of the negative controls show growth of mycoplasmas. If suspect colonies are observed, a suitable validated method may be used to determine whether they are due to mycoplasmas.

The following section is published for information.

RECOMMENDED MEDIA FOR THE CULTURE METHOD

The following media are recommended. Other media may be used, provided that their ability to sustain the growth of mycoplasmas has been demonstrated on each batch in the presence and absence of the product to be examined.

HAYFLICK MEDIA (RECOMMENDED FOR THE GENERAL DETECTION OF MYCOPLASMAS)

Liquid medium

Beef heart infusion broth (1)	90.0 mL
Horse serum (unheated)	20.0 mL
Yeast extract (250 g/L)	10.0 mL
Phenol red (0.6 g/L solution)	5.0 mL
Penicillin (20 000 IU/mL)	0.25 mL
Deoxyribonucleic acid (2 g/L solution)	1.2 mL

Adjust to pH 7.8.

Solid medium

Prepare as described above replacing beef heart infusion broth by beef heart infusion agar containing 15 g/L of agar.

FREY MEDIA (RECOMMENDED FOR THE DETECTION OF M. SYNOVIAE)

Liquid medium

Beef heart infusion broth (1)	90.0 mL
Essential vitamins (2)	0.025 mL
Glucose monohydrate (500 g/L solution)	2.0 mL
Swine serum (inactivated at 56 °C for 30 min)	12.0 mL
β-Nicotinamide adenine dinucleotide (10 g/L solution)	1.0 mL
Cysteine hydrochloride (10 g/L solution)	1.0 mL
Phenol red (0.6 g/L solution)	5.0 mL
Penicillin (20 000 IU/mL)	0.25 mL

Mix the solutions of β-nicotinamide adenine dinucleotide and cysteine hydrochloride and after 10 min add to the other ingredients. Adjust to pH 7.8.

Solid medium

Beef heart infusion broth (1)	90.0 mL
Agar, purified (3)	1.4 g

Adjust to pH 7.8, sterilise by autoclaving then add:

Essential vitamins (2)	0.025 mL
Glucose monohydrate (500 g/L solution)	2.0 mL
Swine serum (unheated)	12.0 mL
β-Nicotinamide adenine dinucleotide (10 g/L solution)	1.0 mL
Cysteine hydrochloride (10 g/L solution)	1.0 mL
Phenol red (0.6 g/L solution)	5.0 mL
Penicillin (20 000 IU/mL)	0.25 mL

FRIIS MEDIA (RECOMMENDED FOR THE DETECTION OF NON-AVIAN MYCOPLASMAS)

Liquid medium

Hanks' balanced salt solution (modified) (4)	800 mL
Distilled water	67 mL
Brain heart infusion (5)	135 mL
PPLO Broth (6)	248 mL
Yeast extract (170 g/L)	60 mL
Bacitracin	250 mg
Meticillin	250 mg
Phenol red (5 g/L)	4.5 mL
Horse serum	165 mL
Swine serum	165 mL

Adjust to pH 7.40-7.45.

Solid medium

Hanks' balanced salt solution (modified) (4)	200 mL
DEAE-dextran	200 mg
Agar, purified (3)	15.65 g

Mix well and sterilise by autoclaving. Cool to 100 °C. Add to 1740 mL of liquid medium as described above.

(1) *Beef heart infusion broth*

Beef heart (for preparation of the infusion)	500 g
Peptone	10 g
Sodium chloride	5 g
Distilled water	to 1000 mL

Sterilise by autoclaving.

(2) *Essential vitamins*

Biotin	100 mg
Calcium pantothenate	100 mg
Choline chloride	100 mg
Folic acid	100 mg
i-Inositol	200 mg
Nicotinamide	100 mg
Pyridoxal hydrochloride	100 mg
Riboflavine	10 mg
Thiamine hydrochloride	100 mg
Distilled water	to 1000 mL

(3) *Agar, purified*

A highly refined agar for use in microbiology and immunology, prepared by an ion-exchange procedure that results in a product having superior purity, clarity and gel strength. It contains about:

Water	12.2 per cent
Ash	1.5 per cent
Acid-insoluble ash	0.2 per cent
Chlorine	0
Phosphate (calculated as P_2O_5)	0.3 per cent
Total nitrogen	0.3 per cent
Copper	8 ppm
Iron	170 ppm
Calcium	0.28 per cent
Magnesium	0.32 per cent

(4) *Hanks' balanced salt solution (modified)*

Sodium chloride	6.4 g
Potassium chloride	0.32 g
Magnesium sulfate heptahydrate	0.08 g
Magnesium chloride hexahydrate	0.08 g
Calcium chloride, anhydrous	0.112 g
Disodium hydrogen phosphate dihydrate	0.0596 g
Potassium dihydrogen phosphate, anhydrous	0.048 g
Distilled water	to 800 mL

(5) *Brain heart infusion*

Calf-brain infusion	200 g
Beef-heart infusion	250 g
Proteose peptone	10 g
Glucose monohydrate	2 g
Sodium chloride	5 g
Disodium hydrogen phosphate, anhydrous	2.5 g
Distilled water	to 1000 mL

(6) *PPLO broth*

Beef-heart infusion	50 g
Peptone	10 g
Sodium chloride	5 g
Distilled water	to 1000 mL

INDICATOR CELL CULTURE METHOD

Cell cultures are stained with a fluorescent dye that binds to DNA. Mycoplasmas are detected by their characteristic particulate or filamentous pattern of fluorescence on the cell surface and, if contamination is heavy, in surrounding areas. Mitochondria in the cytoplasm may be stained but are readily distinguished from mycoplasmas.

If for viral suspensions the interpretation of results is affected by marked cytopathic effects, the virus may be neutralised using a specific antiserum that has no inhibitory effects on mycoplasmas or a cell culture substrate that does not allow growth of the virus may be used. To demonstrate the absence of inhibitory effects of serum, carry out the positive control tests in the presence and absence of the antiserum.

VERIFICATION OF THE SUBSTRATE

Use Vero cells or another cell culture (for example, the production cell line) that is equivalent in effectiveness for detecting mycoplasmas. Test the effectiveness of the cells to be used by applying the procedure shown below and inoculating not more than 100 CFU or CFU-like micro-organisms of suitable reference strains of *M. hyorhinis* and *M. orale*. The following strains have been found to be suitable:

M. hyorhinis			ATCC 29052
M. orale	NCTC 10112	CIP 104969	ATCC 23714

The cells are suitable if both reference strains are detected.

The indicator cells must be subcultured without an antibiotic before use in the test.

TEST METHOD

1. Seed the indicator cell culture at a suitable density (for example, 2×10^4 to 2×10^5 cells/mL, 4×10^3 to 2.5×10^4 cells/cm^2) that will yield confluence after 3 days of growth. Inoculate 1 mL of the product to be examined into the cell culture vessel and incubate at 35-38 °C.

2. After at least 3 days of incubation, when the cells have grown to confluence, make a subculture on cover slips in suitable containers or on some other surface (for example, chambered slides) suitable for the test procedure. Seed the cells at low density so that they reach 50 per cent confluence after 3-5 days of incubation. Complete confluence impairs visualisation of mycoplasmas after staining and must be avoided.

3. Remove the medium and rinse the indicator cells with *phosphate buffered saline pH 7.4 R*, then add a suitable fixing solution (a freshly prepared mixture of 1 volume of *glacial acetic acid R* and 3 volumes of *methanol R* is suitable when *bisbenzimide R* is used for staining).

4. Remove the fixing solution and wash the cells with sterile *water R*. Dry the slides completely if they are to be stained more than 1 h later (particular care is needed for staining of slides after drying owing to artefacts that may be produced).

5. Add a suitable DNA stain and allow to stand for a suitable time (*bisbenzimide working solution R* and a standing time of 10 min are suitable).

6. Remove the stain and rinse the monolayer with *water R*.

7. Mount each coverslip, where applicable (a mixture of equal volumes of *glycerol R* and *phosphate-citrate buffer solution pH 5.5 R* is suitable for mounting). Examine by fluorescence (for bisbenzimide stain a 330 nm/380 nm excitation filter and an LP 440 nm barrier filter are suitable) at 400 × magnification or greater.

8. Compare the microscopic appearance of the test cultures with that of the negative and positive controls, examining for extranuclear fluorescence. Mycoplasmas produce pinpoints or filaments over the indicator cell cytoplasm. They may also produce pinpoints and filaments in the intercellular spaces. Multiple microscopic fields are examined according to the protocol established during validation.

INTERPRETATION OF RESULTS

The product to be examined complies with the test if fluorescence typical of mycoplasmas is not present. The test is invalid if the positive controls do not show fluorescence typical of mycoplasmas. The test is invalid if the negative controls show fluorescence typical of mycoplasmas.

NUCLEIC ACID AMPLIFICATION TECHNIQUES (NAT)

NAT (*2.6.21*) may be used for detection of mycoplasmas by amplification of nucleic acids extracted from a test sample with specific primers that reveal the presence of the target nucleic acid. NAT indicate the presence of a particular nucleic acid sequence and not necessarily the presence of viable mycoplasmas. A number of different techniques are available. This general chapter does not prescribe a particular method for the test. The procedure applied must be validated as described, taking account of the guidelines presented at the end of this section. Where a commercial kit is used, certain elements of the validation may be carried out by the manufacturer and information provided to the user but it must be remembered that full information on the primers may not be available and that production of the kit may be modified or discontinued.

NAT are applied where prescribed in a monograph. They may also be used instead of the culture method and the indicator cell culture method after suitable validation.

Direct NAT Can be applied in the presence of cytotoxic material and where a rapid method is needed.

Cell-culture enrichment followed by NAT The test sample and a suitable cell substrate (as described under the indicator cell-culture method) are cultured together for a suitable period; the nucleic acids are then extracted from cells and supernatant and used for detection by NAT.

VALIDATION

Reference standards are required at various stages during validation and for use as controls during routine application of the test. The reference standards may be mycoplasmas or nucleic acids.

For validation of the limit of detection, the following species represent an optimal selection in terms of the frequency of occurrence as contaminants and phylogenetic relationships:

— *A. laidlawii*;
— *M. fermentans*;
— *M. hyorhinis* (where cell-culture enrichment is used, a fastidious strain such as ATCC 29052 is included);
— *M. orale*;
— *M. pneumoniae* or *M. gallisepticum*;
— *M. synoviae* (where there is use of or exposure to avian material during production);
— *Mycoplasma arginini*;
— *Spiroplasma citri* (where there is use of or exposure to insect or plant material during production).

Demonstration of specificity requires the use of a suitable range of bacterial species other than mycoplasmas. Bacterial genera with close phylogenetic relation to mycoplasmas are most appropriate for this validation; these include *Clostridium*, *Lactobacillus* and *Streptococcus*.

Comparability studies for use of NAT as an alternative method For each mycoplasma test species:

— as an alternative to the culture method: the NAT test system must be shown to detect 10 CFU/mL;
— as an alternative to the indicator cell culture method: the NAT test system must be shown to detect 100 CFU/mL;

or an equivalent limit of detection in terms of the number of copies of mycoplasma nucleic acid in the test sample (using suitable reference standards of mycoplasma nucleic acid).

CONTROLS

Internal controls

Internal controls are necessary for routine verification of absence of inhibition. The internal control may contain the primer binding-site, or some other suitable sequence may be used. It is preferably added to the test material before isolating the nucleic acid and therefore acts as an overall control (extraction, reverse transcription, amplification, detection).

External controls

The external positive control contains a defined number of target-sequence copies or CFUs from 1 or more suitable species of mycoplasma chosen from those used during validation of the test conditions. 1 of the positive controls is set close to the positive cut-off point to demonstrate that the expected sensitivity is achieved. The external negative control contains no target sequence but does not necessarily represent the same matrix as the test article.

INTERPRETATION OF RESULTS

The primers used may also amplify non-mycoplasmal bacterial nucleic acid, leading to false positive results. Procedures are established at the time of validation for dealing with confirmation of positive results, where necessary.

The following section is published for information.

VALIDATION OF NUCLEIC ACID AMPLIFICATION TECHNIQUES (NAT) FOR THE DETECTION OF MYCOPLASMAS: GUIDELINES

1 SCOPE

Nucleic acid amplification techniques (NAT) are either qualitative or quantitative tests for the presence of nucleic acid. For the detection of mycoplasma contamination of various samples such as vaccines and cell substrates, qualitative tests are adequate and may be considered to be limit tests.

These guidelines describe methods to validate qualitative nucleic acid amplification analytical procedures for assessing mycoplasma contamination. They may also be applicable for real-time NAT used as limit tests for the control of contaminants.

The 2 characteristics regarded as the most important for validation of the analytical procedure are the specificity and the detection limit. In addition, the robustness of the analytical procedure should be evaluated.

For the purpose of this document, an analytical procedure is defined as the complete procedure from extraction of nucleic acid to detection of the amplified products.

Where commercial kits are used for part or all of the analytical procedure, documented validation points already covered by the kit manufacturer can replace validation by the user. Nevertheless, the performance of the kit with respect to its intended use has to be demonstrated by the user (e.g. detection limit, robustness, cross-detection of other classes of bacteria).

NAT may be used as:

— a complementary test (for example, for cytotoxic viral suspensions) or for in-process control purposes;

— an alternative method to replace an official method (indicator cell culture method or culture method).

These guidelines will thus separate these 2 objectives by presenting first a guideline for the validation of the NAT themselves, and second, a guideline for a comparability study between NAT and official methods.

2 GUIDELINE FOR MYCOPLASMA NAT VALIDATION

3 parameters should be evaluated: specificity, detection limit and robustness.

2-1 Specificity

Specificity is the ability to unequivocally assess target nucleic acid in the presence of components that may be expected to be present.

The specificity of NAT is dependent on the choice of primers, the choice of probe (for analysis of the final product) and the stringency of the test conditions (for both the amplification and detection steps).

The ability of the NAT to detect a large panel of mycoplasma species will depend on the choice of primers, probes and method parameters. This ability should be demonstrated using characterised reference panels (e.g. reference strains provided by the EDQM). Since NAT systems are usually based on a mix of primers, the theoretical analysis of primers and probes by comparison with databases is not recommended, because interpretation of the results may be quite complex and may not reflect the experimental results.

Moreover, as it is likely that the primers will detect other bacterial species, the potential cross-detection should be documented in the validation study. Bacterial genera such as gram-positive bacteria with close phylogenetic relation to mycoplasmas are most appropriate for this validation; these include *Clostridium*, *Lactobacillus* and *Streptococcus*. However, this is not an exhaustive list and species to be tested will depend on the theoretical ability (based on primers/probes sequences) of the NAT system to detect such other species.

Based on the results from this validation of the specificity, if a gap in the specificity of the method is identified (such as detection of non-mycoplasmal bacterial nucleic acid), an appropriate strategy must be proposed in the validation study to allow interpretation of positive results on a routine basis. For example, a second test may be performed using an alternative method without this specificity gap or using an official method.

2-2 Detection limit

The detection limit of an individual analytical procedure is the lowest amount of target nucleic acid in a sample that can be detected but not necessarily quantitated as an exact value.

For establishment of the detection limit, a positive cut-off point should be determined for the nucleic acid amplification analytical procedure. The positive cut-off point (as defined in general chapter *2.6.21*) is the minimum number of target sequence copies per volume of sample that can be detected in 95 per cent of test runs. This positive cut-off point is influenced by the distribution of mycoplasmal genomes in the individual samples being tested and by factors such as enzyme efficiency, and can result in different 95 per cent cut-off values for individual analytical test runs.

To determine the positive cut-off point, a dilution series of characterised and calibrated (either in CFUs or nucleic acid copies) in-house working strains or EDQM standards should be tested on different days to examine variation between test runs.

For validation of the limit of detection, the following species represent an optimal selection in terms of the frequency of occurrence as contaminants and phylogenetic relationships:
— *A. laidlawii*;
— *M. fermentans*;
— *M. hyorhinis*;
— *M. orale*;
— *M. pneumoniae* or *M. gallisepticum*;
— *M. synoviae* (where there is use of or exposure to avian material during production);
— *M. arginini*;
— *S. citri* (where there is use of or exposure to insect or plant material during production).

For each strain, at least 3 independent 10-fold dilution series should be tested, with a sufficient number of replicates at each dilution to give a total number of 24 test results for each dilution, to enable a statistical analysis of the results.

For example, a laboratory may test 3 dilution series on different days with 8 replicates for each dilution, 4 dilution series on different days with 6 replicates for each dilution, or 6 dilution series on different days with 4 replicates for each dilution. In order to keep the number of dilutions at a manageable level, a preliminary test should be performed to obtain a preliminary value for the positive cut-off point (i.e. the highest dilution giving a positive signal). The range of dilutions can then be chosen around the predetermined preliminary cut-off point. The concentration of mycoplasmas (CFUs or copies) that can be detected in 95 per cent of test runs can then be calculated using an appropriate statistical evaluation.

These results may also serve to evaluate the variability of the analytical procedure.

2-3 Robustness

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small but deliberate variations in method parameters, and provides an indication of its reliability during normal usage.

The evaluation of robustness should be considered during the development phase. It should show the reliability of the analytical procedure with respect to deliberate variations in method parameters. For NAT, small variations in the method parameters can be crucial. However, the robustness of the method can be demonstrated during its development when small variations in the concentrations of reagents (e.g. $MgCl_2$, primers or deoxyribonucleotides) are tested. Modifications of extraction kits or extraction procedures as well as different thermal cycler types may also be evaluated.

Finally, robustness of the method can be evaluated through collaborative studies.

3 GUIDELINE FOR COMPARABILITY STUDY

NAT may be used instead of official methods (indicator cell culture method and/or culture method). In this case a comparability study should be carried out. This comparability study should include mainly a comparison of the respective detection limits of the alternative method and official methods. However, specificity (mycoplasma panel detected, putative false positive results) should also be considered.

For the detection limit, acceptability criteria are defined as follows:
— if the alternative method is proposed to replace the culture method, the NAT system must be shown to detect 10 CFU/mL for each mycoplasma test species described in paragraph 2-2;
— if the alternative method is proposed to replace the indicator cell culture method, the NAT system must be shown to detect 100 CFU/mL for each mycoplasma test species described in paragraph 2-2

For both cases, suitable standards calibrated for the number of nucleic acid copies and the number of CFUs may be used for establishing that these acceptability criteria are reached. The relation between CFUs and nucleic acid copies for the reference preparations should be previously established to compare the performance of the alternative NAT method with the performance of the official methods.

1 of the following 2 strategies can be used to perform this comparability study:
— perform the NAT alternative method in parallel with the official method(s) to evaluate simultaneously the detection limit of both methods using the same samples of calibrated strains;
— compare the performance of the NAT alternative method using previously obtained data from official method validation. In this case, calibration of standards used for both validations as well as their stabilities should be documented carefully.

Comparability study reports should describe all the validation elements described in section 2 (specificity, limit of detection and variability, as well as robustness) in order to assess all the advantages and/or disadvantages of the alternative NAT method compared to official methods.

4. Mycobacteria

(Ph. Eur. method 2.6.2)

If the sample to be examined may be contaminated by micro-organisms other than mycobacteria, treat it with a suitable decontamination solution, such as acetylcysteine-sodium hydroxide solution or sodium laurilsulfate solution.

Inoculate 0.2 mL of the sample in triplicate onto each of 2 suitable solid media (Löwenstein-Jensen medium and Middlebrook 7H10 medium are considered suitable). Inoculate 0.5 mL in triplicate into a suitable liquid medium. Incubate all media at 37 °C for 56 days.

Establish the fertility of the media in the presence of the preparation to be examined by inoculation of a suitable strain of a *Mycobacterium* sp. such as BCG and if necessary use a suitable neutralising substance.

If contaminating micro-organisms develop during the first 8 days of incubation, repeat the test and carry out at the same time a bacteriological sterility test.

If at the end of the incubation time no growth of mycobacteria occurs in any of the test media, the preparation complies with the test.

5. Extraneous Agents in Viral Vaccines

(Ph. Eur. method 2.6.16)

INTRODUCTION

A strategy for testing extraneous agents in viral vaccines must be developed based on a risk assessment following the principles of viral contamination risk detailed in general chapter *5.1.7. Viral safety*. This strategy includes a full package of suitable tests that are able to detect different families of extraneous agents that may infect the source of virus strains including cell substrates and raw material of animal or plant origin. It also takes into account the capacity of the manufacturing process to remove or inactivate viruses. The list of tests summarised in Table 2.6.16.-1 must be adapted depending on the extraneous agents that have the potential to contaminate the product: for *in vitro* tests, the risk assessment may allow, with the agreement of the competent authority, the use of other permissive cell lines or

molecular biology methods depending on the manufacturing process and the incubation temperature for the growth of particular viruses. If *in vivo* tests are more relevant than *in vitro* tests for the detection of some adventitious viruses (e.g. suckling mice for the vesicular stomatitis virus and fertilised SPF eggs for the influenza virus) the decision to maintain or to introduce such *in vivo* assays in a testing strategy must be justified by the risk assessment.

New, sensitive molecular methods with broad detection capabilities are available. These new approaches include high-throughput sequencing (HTS) methods, nucleic acid amplification techniques (NAT) (e.g. polymerase chain reaction (PCR), reverse transcriptase PCR (RT-PCR), product-enhanced reverse transcriptase (PERT) assays) for whole virus families or random-priming methods (associated or not with sequencing), hybridisation to oligonucleotide arrays, and mass spectrometry with broad-spectrum PCR. These methods may be used either as an alternative to *in vivo* tests and specific NAT or as a supplement/alternative to *in vitro* culture tests based on the risk assessment and with the agreement of the competent authority.

In tests that require prior neutralisation of the virus, use specific antibodies of non-human, non-simian origin; if the virus has been propagated in avian tissues, the antibodies must also be of non-avian origin. To prepare antiserum, use an immunising antigen produced in cell cultures from a species different from that used for the production of the vaccine and free from extraneous agents. Where the use of SPF eggs is prescribed, the eggs are obtained from a flock free from specified pathogens (*5.2.2*).

TEST METHODS

Relevant tests for extraneous agents to be carried out at various production stages are indicated in Table 2.6.16.-1 using the methods described below, based on the risk assessment.

Take samples at the time of harvesting, and if not tested immediately, keep at a temperature below −40 °C.

Bacterial and fungal contamination

Each virus seed lot and virus harvest complies with the test for sterility (*2.6.1*).

Mycoplasmas (*2.6.7*)

Each virus seed lot and virus harvest complies with the test for mycoplasmas.

Spiroplasmas

Spiroplasmas may be introduced into virus seed lots through contamination of raw materials of plant origin or when insect cell lines are used for virus propagation. When appropriate, virus seed lots are demonstrated to be free of spiroplasmas using a validated method approved by the competent authority. NAT methods for detection of mycoplasmas (*2.6.7*) may be used to detect spiroplasmas after validation and agreement from the competent authority.

Mycobacteria (*2.6.2*)

A 2.7 mL sample of each virus seed lot and each virus harvest is tested for the presence of *Mycobacterium* spp. by culture methods known to be sensitive for the detection of these organisms. NAT (*2.6.21*) may be used as an alternative, provided such an assay is validated and shown to be comparable to the culture method.

Test in suckling mice

Each virus seed lot is tested in suckling mice if the risk assessment indicates that this test provides a risk mitigation, taking into account the overall testing package. Inoculate no fewer than 20 suckling mice, each less than 24 h old, intracerebrally with 0.01 mL and intraperitoneally with at least 0.1 mL of the virus seed lot. Observe the suckling mice daily for at least 4 weeks. Carry out an autopsy of all suckling mice that die after the first 24 h of the test or that show signs of illness, and examine for evidence of viral infection by direct macroscopical observation. The virus seed lot passes the test if no suckling mice show evidence of infection attributable to the seed lot. The test is not valid unless at least 80 per cent of the original inoculated suckling mice survive the observation period.

Table 2.6.16.-1. – *Relevant tests for extraneous agents at various production stages*

	Virus seed lots	Virus harvests	Production of culture substrates	
			Control cells	Control eggs
Bacterial and fungal contamination	+	+	-	-
Mycoplasmas	+	+	-	-
Spiroplasmas(1)	+	-	-	-
Mycobacteria	+	+	-	-
Test in suckling mice(2)	+	-	-	-
Avian viruses(3)	+	+	-	-
Test for extraneous agents in cell cultures(4)	+	+	+	+
Insect viruses(5)	+	+	-	-
Test on control cells (microscopic examination)	-	-	+	-
Haemadsorbing viruses	-	-	+	-
Test on control eggs (haemagglutinating agents)	-	-	-	+
Avian leucosis viruses(6)	-	-	+	+
Test for specific viruses by NAT(7)	+	+	-	-
Test for viruses using broad molecular methods(8)	+	+	-	-

(1) If insect cells or raw materials of plant origin are used.
(2) If the risk assessment indicates that this test provides a risk mitigation taking into account the overall testing package.
(3) If the virus is propagated in avian or primary avian tissues. If the risk assessment indicates that this test provides a risk mitigation taking into account the overall testing package.
(4) Test performed in suitable permissive cell cultures. Based on a risk assessment.
(5) If the virus is propagated in insect cells.
(6) If the virus is propagated in primary avian tissues or in eggs.
(7) Based on a risk assessment.
(8) These methods may be used either as an alternative to *in vivo* tests and specific NAT or as a supplement/alternative to *in vitro* culture tests based on the risk assessment and in agreement with the competent authority.

Avian viruses

Each virus seed lot propagated in avian tissues and each virus harvest propagated in primary avian tissues is tested for avian viruses if the risk assessment indicates that this test provides a risk mitigation taking into account the overall testing package. Neutralise a sample equivalent to 100 human doses of vaccine or 10 mL, whichever is the greater. Using 0.5 mL per egg, inoculate a group of fertilised SPF eggs, 9-11 days

old, by the allantoic route and a second group, 5-7 days old, into the yolk sac. Incubate for 7 days. The virus seed lot or harvest complies with the test if the allantoic and yolk sac fluids show no sign of the presence of any haemagglutinating agent and if all embryos and chorio-allantoic membranes examined for gross pathology, are normal. The test is not valid unless at least 80 per cent of the inoculated eggs survive for 7 days.

Test for extraneous agents in cell cultures

For each virus seed lot, each virus harvest and each production cell culture (control cells or control eggs), tests for other extraneous agents must be carried out based on a risk assessment. The origin of the cell substrate and virus strain, as well as the potential extraneous agents that may be inadvertently introduced during production processes or through the use of animal- or plant-derived raw materials, must be taken into account when choosing suitable permissive cells.

For each virus seed lot and virus harvest, neutralised samples, equivalent (unless otherwise prescribed) to 500 human doses of vaccine or 50 mL, whichever is the greater, are tested for the presence of extraneous agents by inoculation into continuous simian and human cell cultures. If the virus is grown in simian or human cells, the neutralised virus harvest is tested on a separate culture of these cells. If the virus is grown in a mammalian cell system other than simian or human, or in avian cells, cells of that species, but from a separate batch, are also inoculated. The cells are incubated at 36 ± 1 °C and observed for a period of 14 days. If the production cell culture is maintained at a temperature other than 36 ± 1 °C, a supplementary test for extraneous agents is carried out at the production temperature using the same type of cells used for growth of the virus. A subculture of 14 days is carried out followed by a haemadsorbing test. The virus seed lot or harvest passes the tests if none of the cell cultures show evidence of the presence of any extraneous agents after 14 and 28 days of incubation, and no evidence of any haemadsorbing viruses after 28 days. The test is not valid unless at least 80 per cent of the cell cultures remain viable.

Insect viruses

Each virus seed lot and virus harvest propagated in insect cells is tested for insect viruses. Neutralised samples, equivalent (unless otherwise prescribed) to 500 human doses of vaccine or 50 mL, whichever is the greater, are tested for the presence of extraneous agents by inoculation into at least 1 cell culture different from that used in production and permissible to insect viruses, and that also allows detection of human arboviruses (e.g. BHK-21). The choice of cells is approved by the competent authority and takes into account the origin of the production cells and the likely contaminants that may be detected by the chosen cells. The cells are incubated at an appropriate temperature and observed for a period of 14 days. A subculture of 14 days is carried out followed by a haemadsorbing test. The virus seed lot or harvest passes the tests if none of the cell cultures show evidence of the presence of any extraneous agents after 14 and 28 days of incubation, and no evidence of any haemadsorbing virus after 28 days. The test is not valid unless at least 80 per cent of the cell cultures remain viable.

Tests on control cells

Where cell cultures are used for virus production, examine the control cells microscopically for the absence of any virus causing cytopathic degeneration throughout the incubation time of the inoculated production cell cultures or for no less than 14 days beyond the time of inoculation of the production vessels, whichever is the longer. The test is not valid unless at least 80 per cent of the control cell cultures survive to the end of the observation period.

At 14 days or at the time of the last virus harvest, whichever is the longer, pool the supernatant fluids from the control cells and examine for the presence of extraneous agents over a period of 14 days as described above for the virus seed lot and the virus harvest by inoculation of relevant cell cultures depending on the type of cells used for virus growth.

Haemadsorbing viruses

Where cell cultures are used for virus production, a microscopic examination of the control cells is carried out as described above for the test for extraneous agents in cell cultures. At 14 days or at the time of the last virus harvest, whichever is the longer, examine no fewer than 25 per cent of the control cultures for the presence of haemadsorbing viruses by the addition of guinea-pig red blood cells. If the test for haemadsorbing viruses is not feasible, carry out a test for haemagglutinating viruses. If the guinea-pig red blood cells have been stored, they shall have been stored at 5 ± 3 °C for not more than 7 days. Read half of the cultures after incubation at 5 ± 3 °C for 30 min and the other half after incubation at 20-25 °C for 30 min. No evidence of haemadsorbing agents is found.

Tests on control eggs

Where eggs are used for virus production, examine 0.25 mL of the allantoic fluid from each control egg for haemagglutinating agents by mixing directly with chicken red blood cells and after a passage in SPF eggs carried out as follows: inoculate a 5 mL sample of the pooled amniotic fluids from the control eggs in 0.5 mL volumes into the allantoic cavity and into the amniotic cavity of SPF eggs. The control eggs comply with the test if no evidence of the presence of haemagglutinating agents is found in either test.

In addition, inoculate 5 mL samples of the pooled amniotic fluids from the control eggs into suitable permissive cells including human, simian and avian cells. Observe the cell cultures for 14 days at a suitable incubation temperature. The control eggs comply with the test if no evidence of the presence of extraneous agents is found. The test is not valid unless 80 per cent of the inoculated cultures survive to the end of the observation period.

Avian leucosis viruses

For each virus propagated in primary avian cell tissues or in eggs, the production cell culture (control cells or control eggs) is tested for avian leucosis viruses. When cell cultures are used for virus production, a microscopic examination of the control cells is carried out as described above for the test for extraneous agents prior to the test for avian leucosis viruses. At 14 days or at the time of the last virus harvest, whichever is the longer, carry out the test for avian leucosis viruses on DF-1 cells or leucosis-free chick-embryo cell cultures with amplification through 5 passages using at least 5 mL of the supernatant fluid from the control cells or at least 10 mL of a sample of the pooled amniotic fluids from the control eggs. PERT assay end-point can be used after DF-1 amplification for detection of exogenous avian retroviruses (including avian leucosis virus). For specific detection of avian leucosis virus, several end-points can be used such as immunostaining, enzyme-linked immunosorbent assay (ELISA) or complement fixation for avian leucosis (COFAL). Control cells or control eggs comply with the test if there is no evidence of the presence of any avian leucosis virus.

Tests for specific viruses by NAT

Based on a risk assessment related to the manufacturing process, each virus seed lot and each virus harvest may be tested by NAT (*2.6.21*) for specific viruses that are not detected by conventional *in vivo* or cell culture assays.

Test for viruses using broad molecular methods

With the agreement of the competent authority, broad molecular methods (e.g. HTS) may be used either as an alternative to *in vivo* tests and specific NAT, or as a supplement/alternative to *in vitro* culture tests based on the risk assessment.

Both NAT (*2.6.21*) and broad molecular methods are carried out with or without prior amplification in suitable permissive cells. In cases of positive results with either broad molecular methods or NAT, a follow-up investigation must be conducted to determine whether detected nucleic acids are due to the presence of infectious extraneous agents and/or are known to constitute a risk to human health.

C. Efficacy of Antimicrobial Preservation

(Ph. Eur. general texts 5.1.3)

If a pharmaceutical preparation does not itself have adequate antimicrobial activity, antimicrobial preservatives may be added, particularly to aqueous preparations, to prevent proliferation or to limit microbial contamination which, during normal conditions of storage and use, particularly for multidose containers, could occur in a product and present a hazard to the patient from infection and spoilage of the preparation. Antimicrobial preservatives must not be used as a substitute for good manufacturing practice.

The efficacy of an antimicrobial preservative may be enhanced or diminished by the active constituent of the preparation or by the formulation in which it is incorporated or by the container and closure used. The antimicrobial activity of the preparation in its final container is investigated over the period of validity to ensure that such activity has not been impaired by storage. Such investigations may be carried out on samples removed from the final container immediately prior to testing.

During development of a pharmaceutical preparation, it shall be demonstrated that the antimicrobial activity of the preparation as such or, if necessary, with the addition of a suitable preservative or preservatives provides adequate protection from adverse effects that may arise from microbial contamination or proliferation during storage and use of the preparation.

The efficacy of the antimicrobial activity may be demonstrated by the test described below. The test is not intended to be used for routine control purposes.

TEST FOR EFFICACY OF ANTIMICROBIAL PRESERVATION

The test consists of challenging the preparation, wherever possible in its final container, with a prescribed inoculum of suitable micro-organisms, storing the inoculated preparation at a prescribed temperature, withdrawing samples from the container at specified intervals of time and counting the organisms in the samples so removed.

The preservative properties of the preparation are adequate if, in the conditions of the test, there is a significant fall or no increase, as appropriate, in the number of micro-organisms in the inoculated preparation after the times and at the temperatures prescribed. The acceptance criteria, in terms of decrease in the number of micro-organisms with time, vary for different types of preparations according to the degree of protection intended (see Tables 5.1.3.-1/2/3).

Test micro-organisms

Pseudomonas aeruginosa	ATCC 9027; NCIMB 8626; CIP 82.118.
Staphylococcus aureus	ATCC 6538; NCTC 10788; NCIMB 9518; CIP 4.83.
Candida albicans	ATCC 10231; NCPF 3179; IP 48.72.
Aspergillus brasiliensis	ATCC 16404; IMI 149007; IP 1431.83.

Single-strain challenges are used and the designated micro-organisms are supplemented, where appropriate, by other strains or species that may represent likely contaminants to the preparation. It is recommended, for example, that *Escherichia coli* (ATCC 8739; NCIMB 8545; CIP 53.126) is used for all oral preparations and *Zygosaccharomyces rouxii* (NCYC 381; IP 2021.92) for oral preparations containing a high concentration of sugar.

Preparation of inoculum

Preparatory to the test, inoculate the surface of casein soya bean digest agar (*2.6.12*) for bacteria or Sabouraud-dextrose agar without the addition of antibiotics (*2.6.12*) for fungi, with the recently grown stock culture of each of the specified micro-organisms. Incubate the bacterial cultures at 30-35 °C for 18-24 h, the culture of *C. albicans* at 20-25 °C for 48 h, and the culture of *A. brasiliensis* at 20-25 °C for 1 week or until good sporulation is obtained. Subcultures may be needed after revival before the micro-organism is in its optimal state, but it is recommended that their number be kept to a minimum.

To harvest the bacterial and *C. albicans* cultures, use a sterile suspending fluid, containing 9 g/L of *sodium chloride R*, for dispersal and transfer of the surface growth into a suitable vessel. Add sufficient suspending fluid to reduce the microbial count to about 10^8 micro-organisms per millilitre. To harvest the *A. brasiliensis* culture, use a sterile suspending fluid containing 9 g/L of *sodium chloride R* and 0.5 g/L of *polysorbate 80 R* and adjust the spore count to about 10^8 per millilitre by adding the same solution.

Remove immediately a suitable sample from each suspension and determine the number of colony-forming units per millilitre in each suspension by plate count or membrane filtration (*2.6.12*). This value serves to determine the inoculum and the baseline to use in the test. The suspensions shall be used immediately.

METHOD

To count the viable micro-organisms in the inoculated products, use the agar medium used for the initial cultivation of the respective micro-organisms.

Inoculate a series of containers of the product to be examined, each with a suspension of one of the test organisms to give an inoculum of 10^5 to 10^6 micro-organisms per millilitre or per gram of the preparation. The volume of the suspension of inoculum does not exceed 1 per cent of the volume of the product. Mix thoroughly to ensure homogeneous distribution.

Maintain the inoculated product at 20-25 °C, protected from light. Remove a suitable sample from each container, typically 1 mL or 1 g, at zero hour and at appropriate intervals according to the type of the product and determine the number of viable micro-organisms by plate count or membrane filtration (*2.6.12*). Ensure that any residual antimicrobial activity of the product is eliminated by dilution, by filtration or by the use of a specific inactivator. When dilution procedures are used, due allowance is made for the

reduced sensitivity in the recovery of small numbers of viable micro-organisms. When a specific inactivator is used, the ability of the system to support the growth of the test organisms is confirmed by the use of appropriate controls.

The procedure is validated to verify its ability to demonstrate the required reduction in count of viable micro-organisms.

ACCEPTANCE CRITERIA

The criteria for evaluation of antimicrobial activity are given in Tables 5.1.3.-1/2/3 in terms of the log_{10} reduction in the number of viable micro-organisms against the value obtained for the inoculum.

Table 5.1.3.-1. - *Parenteral preparations, eye preparations, intrauterine preparations and intramammary preparations*

		Log_{10} reduction				
		6 h	24 h	7 d	14 d	28 d
Bacteria	A	2	3	-	-	NR
	B	-	1	3	-	NI
Fungi	A	-	-	2	-	NI
	B	-	-	-	1	NI

NR: no recovery.
NI: no increase in number of viable micro-organisms compared to the previous reading.

The A criteria express the recommended efficacy to be achieved. In justified cases where the A criteria cannot be attained, for example for reasons of an increased risk of adverse reactions, the B criteria must be satisfied.

Table 5.1.3.-2. - *Ear preparations, nasal preparations, preparations for cutaneous application and preparations for inhalation*

		Log_{10} reduction			
		2 d	7 d	14 d	28 d
Bacteria	A	2	3	-	NI
	B	-	-	3	NI
Fungi	A	-	-	2	NI
	B	-	-	1	NI

NI: no increase in number of viable micro-organisms compared to the previous reading.

The A criteria express the recommended efficacy to be achieved. In justified cases where the A criteria cannot be attained, for example for reasons of an increased risk of adverse reactions, the B criteria must be satisfied.

Table 5.1.3.-3. - *Oral preparations, oromucosal preparations and rectal preparations*

	Log_{10} reduction	
	14 d	28 d
Bacteria	3	NI
Fungi	1	NI

NI: no increase in number of viable micro-organisms compared to the previous reading.

The above criteria express the recommended efficacy to be achieved.

D. Microbiological Quality of Non-sterile Pharmaceutical Preparations and Substances for Pharmaceutical Use[1]

(Ph. Eur. general texts 5.1.4)

◊This chapter does not apply to products containing viable micro-organisms as active ingredients.◊

The presence of certain micro-organisms in non-sterile preparations may have the potential to reduce or even inactivate the therapeutic activity of the product and has a potential to adversely affect the health of the patient. Manufacturers therefore have to ensure a low bioburden of finished dosage forms by implementing current guidelines on Good Manufacturing Practice during the manufacture, storage and distribution of pharmaceutical preparations.

Microbial examination of non-sterile products is performed according to the methods given in general chapters *2.6.12* and *2.6.13*. Acceptance criteria for non-sterile pharmaceutical products based upon the total aerobic microbial count (TAMC) and the total combined yeasts/moulds count (TYMC) are given in Tables 5.1.4.-1 and 5.1.4.-2. Acceptance criteria are based on individual results or on the average of replicate counts when replicate counts are performed (e.g. direct plating methods).

When an acceptance criterion for microbiological quality is prescribed it is interpreted as follows:
- 10^1 CFU: maximum acceptable count = 20;
- 10^2 CFU: maximum acceptable count = 200;
- 10^3 CFU: maximum acceptable count = 2000, and so forth.

Table 5.1.4.-1 includes a list of specified micro-organisms for which acceptance criteria are set. The list is not necessarily exhaustive and for a given preparation it may be necessary to test for other micro-organisms depending on the nature of the starting materials and the manufacturing process.

If it has been shown that none of the prescribed tests will allow valid enumeration of micro-organisms at the level prescribed, a validated method with a limit of detection as close as possible to the indicated acceptance criterion is used.

In addition to the micro-organisms listed in Table 5.1.4.-1, the significance of other micro-organisms recovered is evaluated in terms of:
- use of the product: hazard varies according to the route of administration (eye, nose, respiratory tract);
- nature of the product: its ability to support growth, the presence of adequate antimicrobial preservation;
- method of application;
- intended recipient: risk may differ for neonates, infants, the debilitated;
- use of immunosuppressive agents, corticosteroids;
- presence of disease, wounds, organ damage.

Where warranted, a risk-based assessment of the relevant factors is conducted by personnel with specialised training in microbiology and the interpretation of microbiological data. For raw materials, the assessment takes account of processing to which the product is subjected, the current technology of testing and the availability of materials of the desired quality.

[1] *This chapter has undergone pharmacopoeial harmonisation. See chapter 5.8 Pharmacopoeial harmonisation.*

Table 5.1.4.-1. – *Acceptance criteria for microbiological quality of non-sterile dosage forms*

Route of administration	TAMC (CFU/g or CFU/mL)	TYMC (CFU/g or CFU/mL)	Specified micro-organisms
Non-aqueous preparations for oral use	10^3	10^2	Absence of *Escherichia coli* (1 g or 1 mL)
Aqueous preparations for oral use	10^2	10^1	Absence of *Escherichia coli* (1 g or 1 mL)
Rectal use	10^3	10^2	-
Oromucosal use Gingival use Cutaneous use Nasal use Auricular use	10^2	10^1	Absence of *Staphylococcus aureus* (1 g or 1 mL) Absence of *Pseudomonas aeruginosa* (1 g or 1 mL)
Vaginal use	10^2	10^1	Absence of *Pseudomonas aeruginosa* (1 g or 1 mL) Absence of *Staphylococcus aureus* (1 g or 1 mL) Absence of *Candida albicans* (1 g or 1 mL)
Transdermal patches (limits for 1 patch including adhesive layer and backing)	10^2	10^1	Absence of *Staphylococcus aureus* (1 patch) Absence of *Pseudomonas aeruginosa* (1 patch)
◊Orodispersible films (limits for 1 film)	10^2	10^1	Absence of *Escherichia coli* (1 film) Absence of *Staphylococcus aureus* (1 film) Absence of *Pseudomonas aeruginosa* (1 film)◊
Inhalation use (special requirements apply to liquid preparations for nebulisation)	10^2	10^1	Absence of *Staphylococcus aureus* (1 g or 1 mL) Absence of *Pseudomonas aeruginosa* (1 g or 1 mL) Absence of bile-tolerant gram-negative bacteria (1 g or 1 mL)
♦Special Ph. Eur. provision for oral dosage forms containing raw materials of natural (animal, vegetal or mineral) origin for which antimicrobial pretreatment is not feasible and for which the competent authority accepts TAMC of the raw material exceeding 10^3 CFU/g or CFU/mL.	10^4	10^2	Not more than 10^2 CFU of bile-tolerant gram-negative bacteria (1 g or 1 mL) Absence of *Salmonella* (10 g or 10 mL) Absence of *Escherichia coli* (1 g or 1 mL) Absence of *Staphylococcus aureus* (1 g or 1 mL)♦
♦Special Ph. Eur. provision for premixes for medicated feeding stuffs for veterinary use using excipients of plant origin for which antimicrobial treatment is not feasible.	10^5	10^4	Not more than 10^4 CFU of bile-tolerant gram-negative bacteria (1 g or 1 mL) Absence of *Escherichia coli* (1 g or 1 mL) Absence of *Salmonella* (25 g or 25 mL)♦

Table 5.1.4.-2. – *Acceptance criteria for microbiological quality of non-sterile substances for pharmaceutical use*

	TAMC (CFU/g or CFU/mL)	TYMC (CFU/g or CFU/mL)
Substances for pharmaceutical use	10^3	10^2

♦*Recommended acceptance criteria for microbiological quality of herbal medicinal products for oral use and extracts used in their preparation are given in general chapter 5.1.8.*♦

E. Microbiological Examination of Cell-based Preparations

(Ph. Eur. method 2.6.27)

This chapter does not concern the examination of human blood or blood components, which is covered by Directive 2002/98/EC of the European Parliament and of the Council of 27 January 2003 and Commission Directive 2004/33/EC of 22 March 2004 implementing Directive 2002/98/EC.

1 INTRODUCTION

The approaches to microbiological examination of cell-based preparations outlined in this general chapter take into account the characteristics and limitations of these preparations, in particular their shelf-life, which may not always allow for completion of conventional microbiological examination tests before administration to the patient, as well as the amounts available for testing and sampling-related issues. These approaches may be applied when the test for sterility, described in general chapter *2.6.1. Sterility*, is required but cannot be performed for technical reasons or due to the characteristics of the specific cell-based preparation.

1-1 SHELF-LIFE

The shelf-life of cell-based preparations is dependent on the cell characteristics and on the preservation conditions.
For non-cryopreserved cell-based preparations, the shelf-life usually does not exceed 3-4 days and sometimes not more than a few hours. In such cases the microbiological status of the final preparation cannot be determined, according to general chapter *2.6.1*, before the time of administration.

1-2 SAMPLE COMPOSITION

Microbial contaminants may be found either inside or on the surface of cells or other components of the cell-based preparation and may not be detected if only supernatants, such as culture or transport media, are analysed. The sample tested must be representative of all of the components of the cell-based preparation, unless otherwise justified.

1-3 SAMPLE SIZE

Due to constraints surrounding the use of a single donor or manufacturing-related capacities, the sample volume available for testing at the end of the production process may be limited. Nevertheless, with regard to the sampling error, which may lead to microbial contamination not being

detected, the sample size must be sufficient to ensure suitable sensitivity and specificity of the chosen test method.

1-4 RATIONALE FOR METHOD SELECTION

Method selection must be based on the characteristics of the final preparation and the manufacturing process. To ensure safety for the intended use, the choice of method or combination of methods could be supported by a risk analysis of the potential exposure to microbiological contaminants, and the characteristics and intended use of the cell-based preparation. The media and incubation times used in these methods must be chosen taking into account the properties of the source material and the conditions during the manufacturing process that may support growth of specific micro-organisms (e.g. psychrophilic, thermophilic or fastidious bacteria or fungi). The composition of the cell-based preparation may impede certain test methods for physical reasons such as initial turbidity of the culture media after addition of the test sample.

The following approaches to microbiological examination may be applied:

— automated growth-based methods;
— a combination of preculturing and detection by alternative methods (*5.1.6*);
— direct detection by alternative methods (*5.1.6*);
— methods based on the sterility test prescribed in general chapter *2.6.1*.

2 GENERAL CONSIDERATIONS

2-1 GENERAL PRECAUTIONS

The test is carried out under aseptic conditions according to current regulations for potentially infective material.
The precautions taken to avoid contamination are such that they do not affect any micro-organisms that are to be revealed in the test. The test is performed under working conditions that are monitored regularly by appropriate sampling of the working area and by carrying out appropriate controls. Testing shall take account of the potential for the presence of inhibitory substances in the sample that may affect the outcome of the test.

2-2 HANDLING CONSTRAINTS

2-2-1 Shelf-life

'Negative-to-date' is understood as an intermediate reading of a test method (*2.6.1* or an automated growth-based method) that has not yet been completed. Where cell-based preparations have limiting shelf-lives, 'negative-to-date' results may be used as the readout, where justified. Based on the risk analysis of the characteristics and intended use of the cell-based preparation, results from additional microbiological in-process control may be needed at the time of use.

2-2-2 Sampling

The test sample must be representative of all of the components of the cell-based preparation and be taken from the final preparation. Where this is not possible, surrogate testing may be performed, for example on the liquids last in contact with the cells being processed.

3 METHODS FOR MICROBIOLOGICAL EXAMINATION OF CELL-BASED PREPARATIONS

3-1 AUTOMATED GROWTH-BASED METHOD

3-1-1 Growth promotion test

This section outlines the conditions for confirming the suitability of the culture media used for microbiological examination.

At least 2 suitable culture media intended for detection of fungi and aerobic and anaerobic bacteria are used. Each batch of sterile medium is tested for its growth-promoting capacities by inoculating duplicate test containers of each medium with not more than 100 CFU of each of the strains listed in Table 2.6.27.-1, and incubating for a maximum of 7 days for detection of microbial growth at the temperature defined for testing (see Table 2.6.27.-3). The test media are satisfactory if there is clear evidence of growth in all inoculated media containers within this incubation period.

Table 2.6.27.-1. – *Micro-organisms used for growth promotion test*

Aerobic medium	
Staphylococcus aureus	for example, ATCC 6538, CIP 4.83, NCTC 10788, NCIMB 9518
Bacillus subtilis	for example, ATCC 6633, CIP 52.62, NCIMB 8054
Pseudomonas aeruginosa	for example, ATCC 9027, NCIMB 8626, CIP 82.118
Candida albicans	for example, ATCC 10231, IP 48.72, NCPF 3179
Aspergillus brasiliensis	for example, ATCC 16404, IP 1431.83, IMI 149007
Anaerobic medium	
Clostridium sporogenes	for example, ATCC 19404, CIP 79.3, NCTC 532 or ATCC 11437
Bacteroides fragilis	for example, ATCC 25285, CIP 77.16, NCTC 9343

3-1-2 Method suitability

For a validated automated growth-based method only a confirmation of the suitability of the method for the given cell-based preparation must be performed with respect to specificity (absence of false positive results), sensitivity, reproducibility and robustness. Regardless of the type of cell-based preparation, the manufacturing process, the sample volume analysed or the type of test system, the suitability of the method is to be confirmed in the presence of the test sample. During the confirmation of the suitability of the method, particularly to determine sensitivity, the test is carried out using the micro-organisms listed in Table 2.6.27.-2. Sensitivity is meant as the capacity to detect 100 CFU or less. Not more than 100 CFU of the chosen micro-organisms are inoculated into the medium in the presence of the test sample, using at least 3 replicates.
The microbial count in the micro-organism suspensions used for inoculation is determined by streaking an appropriate sample on agar plates. If between 1 and 100 CFU are detected for each strain within the duration of the assay, the method is suitable for the intended test sample.

It may be necessary to modify the list of micro-organisms in Table 2.6.27.-2 depending on the origin of the cells and any micro-organisms previously found or associated with the particular type of cells.

In some cases, the cell-based preparation itself can inactivate contaminating micro-organisms. Appropriate measures must be taken to ensure the suitability of any additional microbial strains used for confirmation of method suitability.

Table 2.6.27.-2. – *Micro-organisms used for method suitability*

Aerobic medium	
Aspergillus brasiliensis	for example, ATCC 16404, IP 1431.83, IMI 149007
Bacillus subtilis	for example, ATCC 6633, CIP 52.62, NCIMB 8054
Candida albicans	for example, ATCC 10231, IP 48.72, NCPF 3179
Pseudomonas aeruginosa	for example, ATCC 9027, NCIMB 8626, CIP 82.118
Staphylococcus aureus	for example, ATCC 6538, CIP 4.83, NCTC 10788, NCIMB 9518
Streptococcus pyogenes	for example, ATCC 19615, CIP 1042.26, NCIMB 13285
Micrococcus sp.	for example, ATCC 700405
Anaerobic medium	
Clostridium sporogenes	for example, ATCC 19404, CIP 79.3, NCTC 532 or ATCC 11437
Cutibacterium acnes	for example, ATCC 11827

3-1-3 Testing of the preparation to be examined

Sample A sample that is representative of the characteristics of the cell-based preparation is tested. The sample is added to the culture medium as soon as possible. If it is necessary to store samples, the impact of the storage on potential contaminants is evaluated.

For cell-based preparations where the total volume (*V*) of the batch is between 1 mL and 1 L in a single container, the following table indicates the inoculation volume to be used.

Total cell-based preparation volume (mL)	Total inoculum volume (divided between aerobic and anaerobic bottles)
$10 \leq V \leq 1000$	1 per cent of total volume of preparation to be tested
$1 \leq V < 10$	100 µL
$V < 1$	Not applicable

For other volumes or multiple containers, alternative approaches should be used and have to be justified (see section 2-2-2). Enlargement of the total volume by means of dilution may be envisaged to assure complete inoculation of sample volumes of 100 µL. For preparation volumes less than 1 mL, where final sampling is not possible, surrogate testing, in-process testing or other appropriate testing should be used and has to be justified.

Analysis Samples are inoculated into containers of culture medium as soon as possible and incubated for not less than 7 days. Depending on the results obtained during method suitability testing and considering relevant micro-organisms, the incubation period may be extended up to 14 days. Selection of incubation temperatures should enable detection of a broad range of micro-organisms. This is typically in the range of 30-37 °C; however, for cell-based preparations with a very short shelf-life, a growth-accelerating temperature of not less than 35 °C may be more appropriate to obtain a relevant 'negative-to-date' readout of the test. In addition, for preparations where there is a significant risk of contamination from the environment, 2 temperature ranges of, for example, 20-25 °C (for aerobic) and 30-37 °C (for anaerobic) are used, in order to cover both environmental and clinical micro-organisms.

Table 2.6.27.-3 lists possible alternative approaches for the choice of incubation temperatures. The temperature and time for incubation are based on the results of the suitability study for the specific cell-based preparation.

Table 2.6.27.-3. – *Possible temperature settings in automated culturing systems used alone or in combination with manual testing*

	Aerobic incubation	Anaerobic incubation
Option 1	20-25 °C (automated system), if necessary 30-35 °C (automated system)	30-35 °C (automated system)
Option 2	35-37 °C (automated system); where relevant, additional incubation at a lower temperature (manual method)*	35-37 °C (automated system)
Option 3	30-32 °C (automated system)	30-32 °C (automated system)
Option 4	30-32 °C (automated system)	35-37 °C (automated system)

* Where relevant, incubate in addition at a temperature between 20 °C and 30 °C. Incubation can be performed using commercially available microbiological media, either aerobic bottles intended for automated systems or casein soya bean digest broth.

3-1-4 Observation and interpretation of results

Media are examined, visually or with automated systems, at least daily and at the end of the observation period for evidence of microbial growth. If no growth is observed during or at the end of the observation period, the product is 'culture negative'. If growth is observed in a valid test, the product is 'culture positive'.

If the inoculated bottles are stored for more than 12 h before being placed into the automated culturing system, a subculture of each incubated bottle is performed to check for false negatives. This addresses cases in which, where a micro-organism is fast-growing and the conditions are optimal, micro-organisms may start to proliferate during storage. As a consequence, there may not be a significant increase in the relevant parameters at the time of testing and microbial contaminants may not be recognised by the system (false negative result).

3-2 ALTERNATIVE METHODS

3-2-1 Combination of preculturing and detection by alternative methods

The samples to be tested are incubated in both aerobic and anaerobic liquid cultivation media or equivalent solid media for a short period of time (e.g. 12-24 h depending on the sensitivity of the alternative approach used). An alternative method suitable for rapid detection of micro-organisms is then performed (e.g. nucleic acid amplification techniques (*2.6.21*), flow cytometry (*2.7.24*), bioluminescence (*5.1.6*)).

3-2-2 Direct detection by alternative methods (*5.1.6*)

Where a cell-based preparation has a very short shelf-life (e.g. a few hours) or where standard methods do not provide satisfactory detection of micro-organisms, non-growth-based, direct detection methods may be carried out for microbiological examination (e.g. nucleic acid amplification techniques (*2.6.21*), flow cytometry (*2.7.24*), bioluminescence (*5.1.6*)).

This approach enables a result to be obtained within a very short time, although at the expense of lower sensitivity, in comparison to growth-based methods. Depending on the approach used, both viable and non-viable micro-organisms may be detected.

3-2-3 Method validation

Validation is carried out according to the general recommendations of general chapter *5.1.6* and according to the recommendations specific to cell-based preparations in section 3-1-2 for automated growth-based methods.

The sensitivity of these approaches must be validated considering the doubling times of potentially contaminating micro-organisms during pre-incubation.

F. Microbiological Examination of Herbal Medicinal Products for Oral Use and Extracts used in their Preparation

(Ph. Eur. method 2.6.31)

1 MICROBIAL ENUMERATION TESTS

Total aerobic microbial count (TAMC)

Perform as described in general chapter *2.6.12.*

Total combined yeasts/moulds count (TYMC)

Perform as described in general chapter *2.6.12.* Due to the natural high bioburden in the products covered by general chapter *5.1.8*, use of Sabouraud-dextrose agar containing antibiotics is suitable.

2 TEST FOR SPECIFIED MICRO-ORGANISMS

2-1 INTRODUCTION

The tests described hereafter will allow determination of the absence or limited occurrence of specified micro-organisms that may be detected under the conditions described.

The tests are designed primarily to determine whether a product, substance or preparation (hereinafter referred to as 'the product') complies with an established specification for microbiological quality. When used for such purposes, follow the instructions given below, including the number of samples to be taken, and interpret the results as stated below.

Alternative microbiological procedures, including automated methods, may be used, provided that their equivalence to the Pharmacopoeia method has been demonstrated.

2-2 GENERAL PROCEDURES

The preparation of samples is carried out as described in general chapter *2.6.12.*

If the product to be examined has antimicrobial activity, this is as far as possible removed or neutralised as described in general chapter *2.6.12.*

If surface-active substances are used for sample preparation, their absence of toxicity for micro-organisms and their compatibility with inactivators used must be demonstrated as described in general chapter *2.6.12.*

2-3 GROWTH-PROMOTING AND INHIBITORY PROPERTIES OF THE MEDIA, SUITABILITY OF THE TEST AND NEGATIVE CONTROLS

The ability of the test to detect micro-organisms in the presence of the product to be examined must be established. Suitability must be confirmed if a change in testing performance, or the product, which may affect the outcome of the test is introduced.

2-3-1 PREPARATION OF TEST STRAINS

Use standardised stable suspensions of test strains or prepare them as stated below. Seed lot culture maintenance techniques (seed-lot systems) are used so that the viable micro-organisms used for inoculation are not more than 5 passages removed from the original master seed lot.

2-3-1-1 Aerobic micro-organisms

Grow each of the bacterial test strains separately in casein soya bean digest broth or on casein soya bean digest agar at 30-35 °C for 18-24 h.

- *Staphylococcus aureus* such as ATCC 6538, NCIMB 9518, CIP 4.83 or NBRC 13276;
- *Pseudomonas aeruginosa* such as ATCC 9027, NCIMB 8626, CIP 82.118 or NBRC 13275;
- *Escherichia coli* such as ATCC 8739, NCIMB 8545, CIP 53.126 or NBRC 3972;
- *Salmonella enterica* subsp. *enterica* serovar Typhimurium such as ATCC 14028 or, as an alternative, *S. enterica* subsp. *enterica* serovar Abony such as NBRC 100797, NCTC 6017 or CIP 80.39;
- *Bacillus subtilis* such as ATCC 6633, NCIMB 8054, CIP 52.62 or NBRC 3134.

Use buffered sodium chloride-peptone solution pH 7.0 or phosphate buffer solution pH 7.2 to make test suspensions. Use the suspensions within 2 h, or within 24 h if stored at 2-8 °C. As an alternative to preparing and then diluting a fresh suspension of vegetative cells of *B. subtilis*, a stable spore suspension is prepared and then an appropriate volume is used for test inoculation. The stable spore suspension may be maintained at 2-8 °C for a validated period of time.

2-3-2 NEGATIVE CONTROL

To verify testing conditions, a negative control is performed using the chosen diluent in place of the test preparation. There must be no growth of micro-organisms. A negative control is also performed when testing the products as described in section 2-4. A failed negative control requires an investigation.

2-3-3 GROWTH-PROMOTING AND INHIBITORY PROPERTIES OF THE MEDIA

Test each batch of ready-prepared medium and each batch of medium prepared either from dehydrated medium or from the ingredients described.

Verify suitable properties of relevant media as described in Table 2.6.31.-1.

Test for growth-promoting properties, liquid media

Inoculate a portion of the appropriate medium with a small number (not more than 100 CFU) of the appropriate micro-organism. Incubate at the specified temperature for not more than the shortest period of time specified in the test. Incubate the casein soya bean digest broth at 30-35 °C for not more than 3 days. Clearly visible growth of the micro-organism comparable to that obtained with a previously tested and approved batch of medium occurs.

Test for growth-promoting properties, solid media

Perform the surface-spread method, inoculating each plate with a small number (not more than 100 CFU) of the appropriate micro-organism. Incubate at the specified temperature for not more than the shortest period of time specified in the test. Growth of the micro-organism comparable to that obtained with a previously tested and approved batch of medium occurs.

Test for inhibitory properties, liquid or solid media

Inoculate the appropriate medium with at least 100 CFU of the appropriate micro-organism. Incubate at the specified temperature for not less than the longest period of time specified in the test. No growth of the test micro-organism occurs.

Test for indicative properties

Perform the surface-spread method, inoculating each plate with a small number (not more than 100 CFU) of the appropriate micro-organism. Incubate at the specified temperature for a period of time within the range specified in the test. Colonies are comparable in appearance and indication reactions to those obtained with a previously tested and approved batch of medium.

2-3-4 SUITABILITY OF THE TEST METHOD

For each product to be examined, perform the sample preparation as described in the relevant paragraph in section 2-4. Add each test strain at the time of mixing, in the prescribed growth medium (casein soya bean digest broth or

Table 2.6.31.-1. – *Growth-promoting, inhibitory and indicative properties of media*

	Medium	Property	Test strains
Test for bile-tolerant gram-negative bacteria	Casein soya bean digest broth	Growth promoting	*S. aureus* *P. aeruginosa* *B. subtilis*
	Enterobacteria enrichment broth-Mossel	Growth promoting	*E. coli* *P. aeruginosa*
		Inhibitory	*S. aureus*
	Violet red bile glucose agar	Growth promoting + indicative	*E. coli* *P. aeruginosa*
Test for *Escherichia coli*	Casein soya bean digest broth	Growth promoting	*S. aureus* *P. aeruginosa* *B. subtilis*
	MacConkey broth	Growth promoting	*E. coli*
		Inhibitory	*S. aureus*
	MacConkey agar	Growth promoting + indicative	*E. coli*
Test for *Salmonella*	Buffered peptone medium	Growth promoting	*S. enterica* subsp. *enterica* serovar Typhimurium or *S. enterica* subsp. *enterica* serovar Abony
	Rappaport Vassiliadis *Salmonella* enrichment broth	Growth promoting	*S. enterica* subsp. *enterica* serovar Typhimurium or *S. enterica* subsp. *enterica* serovar Abony
		Inhibitory	*S. aureus*
	Xylose, lysine, deoxycholate agar	Growth promoting + indicative	*S. enterica* subsp. *enterica* serovar Typhimurium or *S. enterica* subsp. *enterica* serovar Abony

buffered peptone medium). For the enumeration method for bile-tolerant gram-negative bacteria, inoculate *E. coli* and *P. aeruginosa* individually. For the tests for *E. coli* and *Salmonella*, inoculate the specified micro-organism individually.

Any antimicrobial activity of the product necessitates a modification of the test procedure (see section 4-5-3 of general chapter *2.6.12*).

If for a given product the antimicrobial activity with respect to a micro-organism for which testing is prescribed cannot be neutralised, then it is to be assumed that the inhibited micro-organism will not be present in the product.

2-3-4-1 Test for absence

Use a number of micro-organisms equivalent to not more than 100 CFU in the inoculated test preparation. Perform the test as described in the relevant paragraph in section 2-4 using the shortest incubation period prescribed. The specified micro-organisms must be detected with the indication reactions as described in section 2-4.

2-3-4-2 Enumeration test

Semi-quantitative test (probable-number method).

Use a number of micro-organisms equivalent to not more than 100 CFU per gram or millilitre of product. Perform the test as described in the relevant paragraph in section 2-4 using the shortest incubation period prescribed. The dilution corresponding to 0.1 g or 0.1 mL of product must be positive.

2-4 TESTING OF PRODUCTS

2-4-1 BILE-TOLERANT GRAM-NEGATIVE BACTERIA

2-4-1-1 Enumeration test

Semi-quantitative test (probable-number method).

2-4-1-1-1 Sample preparation and pre-incubation. Prepare a sample using a 10-fold dilution of not less than 1 g of the product to be examined as described in general chapter *2.6.12*, but using casein soya bean digest broth as the chosen diluent, mix and incubate at 20-25 °C for a time sufficient to resuscitate the bacteria but not sufficient to encourage multiplication of the organisms (2-3 h).

2-4-1-1-2 Selection and subculture. Inoculate suitable quantities of enterobacteria enrichment broth-Mossel with the preparation as described above and/or, depending on the limit applied for the particular product, with 3 of the 4 dilutions of the preparation, which contain respectively 0.1 g, 0.01 g, 0.001 g and 0.0001 g (or 0.1 mL, 0.01 mL, 0.001 mL and 0.0001 mL) of the product to be examined. Incubate at 30-35 °C for 24-48 h. Subculture each of the cultures on a plate of violet red bile glucose agar. Incubate at 30-35 °C for 18-24 h.

2-4-1-1-3 Interpretation. Growth of colonies constitutes a positive result. Note the smallest quantity of the product that gives a positive result and the largest quantity that gives a negative result.

Determine from Table 2.6.31.-2 the probable number of bacteria.

Table 2.6.31.-2. – *Interpretation of results*

Results for each quantity of product				Probable number of bacteria per gram or millilitre of product
0.1 g or 0.1 mL	0.01 g or 0.01 mL	0.001 g or 0.001 mL	0.0001 g or 0.0001 mL	
+	+	+	+	$> 10^4$
+	+	+	-	$< 10^4$ and $> 10^3$
+	+	-	-	$< 10^3$ and $> 10^2$
+	-	-	-	$< 10^2$ and > 10
-	-	-	-	< 10

2-4-2 ESCHERICHIA COLI

2-4-2-1 Test for absence

2-4-2-1-1 Sample preparation and pre-incubation. Prepare a sample using a 10-fold dilution of not less than 1 g of the product to be examined as described in general chapter *2.6.12*, and use 10 mL or the quantity corresponding to 1 g or 1 mL to inoculate a suitable amount (determined as described in section 2-3-4) of casein soya bean digest broth, mix and incubate at 30-35 °C for 18-24 h.

2-4-2-1-2 Selection and subculture. Shake the container, transfer 1 mL of casein soya bean digest broth to 100 mL of MacConkey broth and incubate at 42-44 °C for 24-48 h.

Subculture on a plate of MacConkey agar at 30-35 °C for 18-72 h.

2-4-2-1-3 Interpretation. Growth of colonies indicates the possible presence of *E. coli*. This is confirmed by identification tests.

The product complies with the test if no colonies are present or if the identification tests are negative.

2-4-2-2 Enumeration test

Semi-quantitative test (probable-number method).

2-4-2-2-1 Sample preparation and pre-incubation. Prepare a sample using a 10-fold dilution of not less than 1 g of the product to be examined as described in general chapter *2.6.12*, and use the quantities corresponding respectively to 0.1 g, 0.01 g and 0.001 g (or 0.1 mL, 0.01 mL and 0.001 mL) to inoculate a suitable amount (determined as described in section 2-3-4) of casein soya bean digest broth, mix and incubate at 30-35 °C for 18-24 h.

2-4-2-2-2 Selection and subculture. Shake the container, transfer 1 mL of casein soya bean digest broth to 100 mL of MacConkey broth and incubate at 42-44 °C for 24-48 h. Subculture on a plate of MacConkey agar at 30-35 °C for 18-72 h.

2-4-2-2-3 Interpretation. Growth of colonies indicates the possible presence of *E. coli*. This is confirmed by identification tests.

Note the smallest quantity of the product that gives a positive result and the largest quantity that gives a negative result.

Determine from Table 2.6.31.-3 the probable number of bacteria.

Table 2.6.31.-3. – *Interpretation of results*

Results for each quantity of product			Probable number of bacteria per gram or millilitre of product
0.1 g or 0.1 mL	0.01 g or 0.01 mL	0.001 g or 0.001 mL	
+	+	+	$> 10^3$
+	+	-	$< 10^3$ and $> 10^2$
+	-	-	$< 10^2$ and > 10
-	-	-	< 10

2-4-3 SALMONELLA

2-4-3-1 Test for absence

2-4-3-1-1 Sample preparation and pre-incubation. Use 25 g or 25 mL of the product to be examined to inoculate 225 mL of buffered peptone medium and mix (e.g. homogenise in a filter bag by using a blender). Incubate at 30-35 °C for 18-24 h.

2-4-3-1-2 Selection and subculture. Transfer 0.1 mL of buffered peptone medium to 10 mL of Rappaport Vassiliadis *Salmonella* enrichment broth and incubate at 30-35 °C for 18-24 h. Subculture on plates of xylose, lysine and deoxycholate agar. Incubate at 30-35 °C for 18-48 h.

2-4-3-1-3 Interpretation. The possible presence of *Salmonella* is indicated by the growth of well-developed, red colonies, with or without black centres. This is confirmed by identification tests.

The product complies with the test if colonies of the types described are not present or if the identification tests are negative.

The following section is given for information.

RECOMMENDED SOLUTIONS AND CULTURE MEDIA

The solutions and culture media mentioned in this chapter and described in general chapter *2.6.13* and the following buffered peptone medium have been found to be satisfactory for the purposes for which they are prescribed in this chapter. Other media may be used provided that their suitability can be demonstrated.

Buffered peptone medium

Potassium dihydrogen phosphate	1.5 g
Disodium hydrogen phosphate dodecahydrate	9.0 g
Sodium chloride	5.0 g
Peptone	10.0 g
Purified water	1000 mL

Adjust the pH so that after sterilisation it is 7.0 ± 0.2 at 25 °C. Sterilise in an autoclave using a validated cycle.

G. Microbiological Quality of Herbal Medicinal Products for Oral Use and Extracts Used in their Preparation

(Ph. Eur. general texts 5.1.8)

This general chapter presents recommended acceptance criteria for the microbiological quality of both herbal medicinal products for oral use and the extracts that are used in their preparation.

Microbial examination of non-sterile products is performed according to the methods given in general chapters *2.6.12*, *2.6.13* and *2.6.31*. Acceptance criteria based upon the total aerobic microbial count (TAMC) and the total combined yeasts/moulds count (TYMC) are given below.

Acceptance criteria are based on individual results or on the average of replicate counts when replicate counts are performed (e.g. direct plating methods).

A list of specified micro-organisms for which acceptance criteria are set can be found below. The list is not necessarily exhaustive and for a given preparation it may be necessary to test for other micro-organisms depending on the nature of the starting materials, the manufacturing process and the intended use.

Medicinal products containing live yeasts (live biotherapeutic products) are not within the scope of this general chapter.

HERBAL MEDICINAL PRODUCTS

A. Herbal medicinal products containing herbal drugs, with or without excipients, intended for the preparation of infusions and decoctions using boiling water (for example herbal teas, with or without added flavourings)

TAMC (*2.6.12*)	Acceptance criterion: 10^7 CFU/g Maximum acceptable count: 50 000 000 CFU/g
TYMC (*2.6.12*)	Acceptance criterion: 10^5 CFU/g Maximum acceptable count: 500 000 CFU/g
Escherichia coli (*2.6.31*)	Acceptance criterion: 10^3 CFU/g
Salmonella (*2.6.31*)	Absence (25 g)

B. Herbal medicinal products containing, for example, extracts and/or herbal drugs, with or without excipients, where the method of processing (for example, extraction) or, where appropriate, in the case of herbal drugs, of pre-treatment reduces the levels of organisms to below those stated for this category

TAMC (*2.6.12*)	Acceptance criterion: 10^4 CFU/g or CFU/mL Maximum acceptable count: 50 000 CFU/g or CFU/mL
TYMC (*2.6.12*)	Acceptance criterion: 10^2 CFU/g or CFU/mL Maximum acceptable count: 500 CFU/g or CFU/mL
Bile-tolerant gram-negative bacteria (*2.6.31*)	Acceptance criterion: 10^2 CFU/g or CFU/mL
Escherichia coli (*2.6.31*)	Absence (1 g or 1 mL)
Salmonella (*2.6.31*)	Absence (25 g or 25 mL)

C. Herbal medicinal products containing, for example, extracts and/or herbal drugs, with or without excipients, where it can be demonstrated that the method of processing (for example, extraction with low-strength ethanol or water that is not boiling, or low-temperature concentration) or, in the case of herbal drugs, of pre-treatment, would not reduce the level of organisms sufficiently to reach the criteria required under B

TAMC (*2.6.12*)	Acceptance criterion: 10^5 CFU/g or CFU/mL Maximum acceptable count: 500 000 CFU/g or CFU/mL
TYMC (*2.6.12*)	Acceptance criterion: 10^4 CFU/g or CFU/mL Maximum acceptable count: 50 000 CFU/g or CFU/mL
Bile-tolerant gram-negative bacteria (*2.6.31*)	Acceptance criterion: 10^4 CFU/g or CFU/mL
Escherichia coli (*2.6.31*)	Absence (1 g or 1 mL)
Salmonella (*2.6.31*)	Absence (25 g or 25 mL)

EXTRACTS

Extracts should fulfill the acceptance criteria for category B herbal medicinal products. However, where it can be demonstrated that the method of processing would not reduce the level of micro-organisms sufficiently to reach the category B criteria, the extracts shall meet the requirements for category C herbal medicinal products.

The recommended acceptance criteria apply to extracts that are to be incorporated into herbal medicinal products for oral use. More-stringent acceptance criteria may be required for extracts that are to be incorporated into pharmaceutical preparations to be administered by other routes in order to satisfy the acceptance criteria for the intended route of administration (*5.1.4*).

It is recognised that for some herbal medicinal products and extracts used in their preparation the criteria given above for TAMC, TYMC and bile-tolerant gram-negative bacteria cannot be met because of the typical level of microbial contamination. Less-stringent acceptance criteria may be applied on the basis of a risk assessment that takes account of qualitative and quantitative characterisation of the microbial contamination and the intended use of the herbal medicinal product or extract.

If it has been shown that none of the prescribed tests for a herbal medicinal product or extract will allow valid enumeration of micro-organisms at the level prescribed, a validated method with a limit of detection as close as possible to the indicated acceptance criterion is used.

H. Microbiological Examination of Live Biotherapeutic Products

Microbiological Examination of Live Biotherapeutic Products: Tests for Enumeration of Microbial Contaminants

(Ph. Eur. method 2.6.36)

1. INTRODUCTION

The tests described hereafter will allow enumeration of live biotherapeutic product (LBP) contaminants: mesophilic bacteria and yeasts/moulds that may grow under aerobic conditions.

The tests are designed primarily to determine whether an LBP substance or preparation complies with the established specifications for microbial contamination.

Alternative microbiological procedures (*5.1.6*), including automated methods, may be used, provided that their equivalence to the Pharmacopoeia method has been demonstrated.

2. GENERAL PROCEDURES

Carry out the determination under conditions designed to avoid extrinsic microbial contamination of the LBP to be tested. The precautions taken to avoid contamination must be such that they do not affect any contaminant micro-organisms that are to be revealed in the test.

If the LBP to be tested prevents the enumeration of contaminant micro-organisms, apply the decision tree shown in Figure 2.6.36.-1.

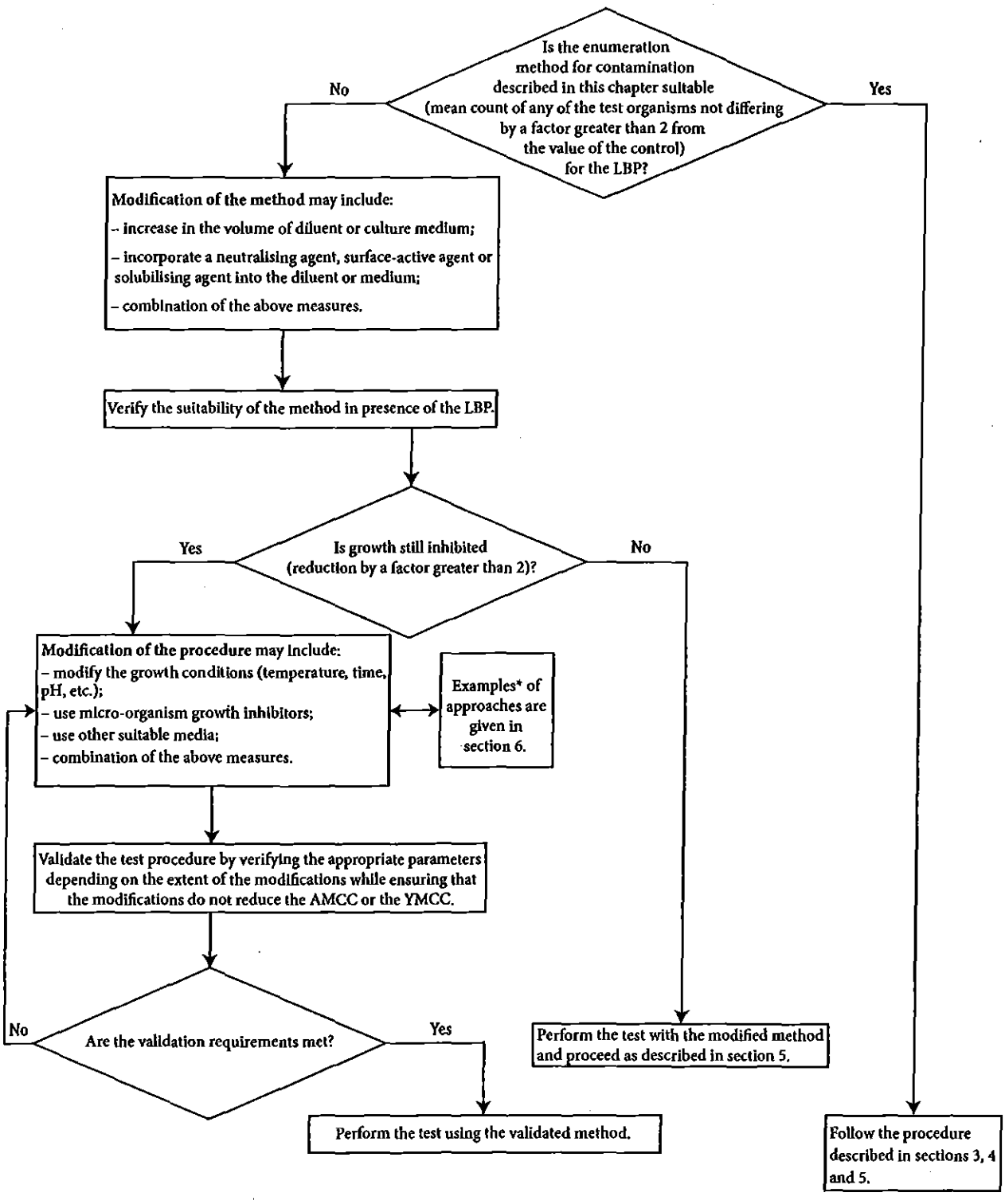
If components other than the active substance (i.e. the micro-organism) of the LBP to be tested have inhibitory activity, this is insofar as possible removed or neutralised as described in general chapter *2.6.12*. If inactivators are used for this purpose, their efficacy and their absence of toxicity for the target contaminant micro-organisms must be demonstrated.

If surface-active substances are used for sample preparation, their absence of toxicity for the target contaminant micro-organisms and their compatibility with inactivators used must be demonstrated.

3. ENUMERATION METHODS

Use the plate-count or the most-probable-number (MPN) method. The MPN method is generally the least accurate method for microbial counts.

The choice of method is based on factors such as the nature of the LBP and the required limit of microbial contamination. The chosen method must allow testing of a sufficient sample size to judge compliance with the specification. The suitability of the method chosen must be established.



* This section should be understood as informative suggestion. Other procedures are possible as far as they are justified.

Figure 2.6.36.-1. – *Decision tree for the microbial contamination enumeration methods*

4. GROWTH PROMOTION TEST, SUITABILITY OF THE COUNTING METHOD AND NEGATIVE CONTROLS

4-1. GENERAL CONSIDERATIONS

The ability of the test to detect microbial contamination in the presence of the LBP to be tested must be established.

Suitability must be confirmed if a change in testing performance, or in the LBP, which may affect the outcome of the test is introduced.

4-2. PREPARATION OF TEST STRAINS

Use standardised stable suspensions of test strains or prepare them as stated below. Seed lot culture maintenance techniques (seed-lot systems) are used so that the viable micro-organisms used for inoculation are not more than 5 passages removed from the original master seed-lot. Grow each of the bacterial and fungal test strains separately as described in Table 2.6.36.-1.

Use buffered sodium chloride-peptone solution pH 7.0 or phosphate buffer solution pH 7.2 to make test suspensions; to suspend *A. brasiliensis* spores, 0.05 per cent of polysorbate 80 may be added to the buffer. Use the suspensions within 2 h or within 24 h if stored at 2-8 °C. As an alternative to preparing and then diluting a fresh suspension of vegetative cells of *A. brasiliensis* or *B. subtilis*, a stable spore suspension is prepared and then an appropriate volume of the spore suspension is used for test inoculation. The stable spore suspension may be maintained at 2-8 °C for a validated period of time.

4-3. NEGATIVE CONTROL

To verify testing conditions, a negative control is performed using the chosen diluent in place of the test preparation. There must be no growth of micro-organisms. A negative control is also performed when testing the LBP as described in section 5. A failed negative control requires an investigation.

4-4. GROWTH PROMOTION OF THE MEDIA

Test each batch of ready-prepared medium and each batch of medium, prepared either from dehydrated medium or from the ingredients described.

Inoculate portions/plates of casein soya bean digest broth and casein soya bean digest agar with a small number (not more than 100 CFU) of the micro-organisms indicated in Table 2.6.36.-1, using a separate portion/plate of medium for each. Inoculate plates of Sabouraud-dextrose agar with a small number (not more than 100 CFU) of the micro-organisms indicated in Table 2.6.36.-1, using a separate plate of medium for each. Incubate in the conditions described in Table 2.6.36.-1.

For solid media, growth obtained must not differ by a factor greater than 2 from the calculated value for a standardised inoculum. For a freshly prepared inoculum, growth of the micro-organisms comparable to that previously obtained with a previously tested and approved batch of medium occurs. Liquid media are suitable if clearly visible growth of the micro-organisms comparable to that previously obtained with a previously tested and approved batch of medium occurs.

4-5. SUITABILITY OF THE COUNTING METHOD IN THE PRESENCE OF THE LBP TO BE TESTED

4-5-1. Preparation of the sample

The method for sample preparation depends upon the physical characteristics of the LBP to be tested. If none of the procedures described below can be demonstrated to be satisfactory, an alternative procedure must be developed.

Prepare a homogenous suspension of the LBP to be tested (usually a 1 in 10 dilution is prepared) in buffered sodium chloride-peptone solution pH 7.0, phosphate buffer solution pH 7.2 or casein soya bean digest broth. A surface-active agent such as a 1 g/L solution of polysorbate 80 may be added to assist the suspension of poorly wettable substances. If necessary, adjust to pH 6-8. Further dilutions, where necessary, are prepared with the same diluent.

4-5-2. Inoculation and dilution

Add to the sample prepared as described above (4-5-1) and to a control (with no test material included) a sufficient volume of the microbial suspension to obtain an inoculum of not more than 100 CFU. The volume of the suspension of the inoculum should not exceed 1 per cent of the volume of diluted LBP.

To demonstrate acceptable recovery of the test micro-organisms from the LBP, the lowest possible dilution factor of the prepared sample must be used for the test. Where this is not possible due to inhibitory activity of the LBP, further appropriate protocols must be developed (see decision tree shown in Figure 2.6.36.-1). If inhibition of growth by the sample cannot otherwise be avoided, the aliquot of the test micro-organisms may be added after neutralisation or dilution.

Inhibitory activity The number of micro-organisms recovered from the prepared sample diluted as described in 4-5-2 and incubated following the procedure described in 4-5-3, is compared to the number of micro-organisms recovered from the control preparation.

If growth is inhibited (reduction by a factor greater than 2), follow the decision tree shown in Figure 2.6.36.-1 and modify the procedure for the particular enumeration test to ensure the validity of the results. Modification of the procedure may include, for example, (1) an increase in the volume of the diluent or culture medium, (2) incorporation of specific or general neutralising agents into the diluent, (3) membrane filtration, or (4) a combination of the above measures.

If growth is still inhibited (reduction by a factor greater than 2) continue to follow the decision tree shown in Figure 2.6.36.-1.

A new approach needs to be validated using the appropriate parameters depending on the extent of modification, while ensuring that the modifications do not reduce the AMCC or the YMCC.

4-5-3. Recovery of the test micro-organisms in the presence of an LBP

For each of the test micro-organisms listed, separate tests are performed. Only micro-organisms of the added test strain are counted.

4-5-3-1. Plate-count methods Perform plate-count methods at least in duplicate for each medium and use the mean count of the result.

4-5-3-1-1. Pour-plate method

For Petri dishes 9 cm in diameter, add to the dish 1 mL of the sample prepared as described under 4-5-1 and 4-5-2, and 15-20 mL of casein soya bean digest agar or Sabouraud-dextrose agar, both media being at not more than 45 °C. If larger Petri dishes are used, the amount of agar medium is increased accordingly. For each of the micro-organisms listed in Table 2.6.36.-1, at least 2 Petri dishes are used. Incubate the plates as indicated in Table 2.6.36.-1. Take the arithmetic mean of the counts per medium and calculate the number of CFU in the original inoculum.

4-5-3-1-2. Surface-spread method

For Petri dishes 9 cm in diameter, add 15-20 mL of casein soya bean digest agar or Sabouraud-dextrose agar at about 45 °C to each Petri dish and allow to solidify. If larger Petri dishes are used, the volume of the agar is increased accordingly. Dry the plates, for example in a laminar-air-flow cabinet or an incubator. For each of the micro-organisms listed in Table 2.6.36.-1, at least 2 Petri dishes are used. Spread a measured volume of not less than 0.1 mL of the sample prepared as described under 4-5-1 and 4-5-2 over the surface of the medium. Incubate and count as prescribed under 4-5-3-1-1.

4-5-3-2. Most-probable-number (MPN) method The precision and accuracy of the MPN method is less than the plate-count method. Unreliable results are obtained particularly for the enumeration of moulds. For these reasons the MPN method is reserved for the enumeration of AMCC

Table 2.6.36.-1. – *Preparation and use of test micro-organisms*

Micro-organism	Preparation of test strain	Growth promotion		Suitability of counting method in the presence of the LBP	
		Aerobic microbial contamination count (AMCC)	Yeasts and moulds contamination count (YMCC)	Aerobic microbial contamination count (AMCC)	Yeasts and moulds contamination count (YMCC)
Staphylococcus aureus such as: ATCC 6538 NCIMB 9518 CIP 4.83 NBRC 13276	Casein soya bean digest agar or casein soya bean digest broth 30-35 °C 18-24 h	Casein soya bean digest agar and casein soya bean digest broth ≤ 100 CFU 30-35 °C ≤ 3 days	-	Casein soya bean digest agar/MPN: casein soya bean digest broth ≤ 100 CFU 30-35 °C ≤ 3 days	-
Pseudomonas aeruginosa such as: ATCC 9027 NCIMB 8626 CIP 82.118 NBRC 13275	Casein soya bean digest agar or casein soya bean digest broth 30-35 °C 18-24 h	Casein soya bean digest agar and casein soya bean digest broth ≤ 100 CFU 30-35 °C ≤ 3 days	-	Casein soya bean digest agar/MPN: casein soya bean digest broth ≤ 100 CFU 30-35 °C ≤ 3 days	-
Bacillus subtilis such as: ATCC 6633 NCIMB 8054 CIP 52.62 NBRC 3134	Casein soya bean digest agar or casein soya bean digest broth 30-35 °C 18-24 h	Casein soya bean digest agar and casein soya bean digest broth ≤ 100 CFU 30-35 °C ≤ 3 days	-	Casein soya bean digest agar/MPN: casein soya bean digest broth ≤ 100 CFU 30-35 °C ≤ 3 days	-
Candida albicans such as: ATCC 10231 NCPF 3179 IP 48.72 NBRC 1594	Sabouraud- dextrose agar or Sabouraud-dextrose broth 20-25 °C 2-3 days	Casein soya bean digest agar ≤ 100 CFU 30-35 °C ≤ 5 days	Sabouraud- dextrose agar ≤ 100 CFU 20-25 °C ≤ 5 days	Casein soya bean digest agar ≤ 100 CFU 30-35 °C ≤ 5 days MPN: not applicable	Sabouraud- dextrose agar ≤ 100 CFU 20-25 °C ≤ 5 days
Aspergillus brasiliensis such as: ATCC 16404 IMI 149007 IP 1431.83 NBRC 9455	Sabouraud- dextrose agar or potato-dextrose agar 20-25 °C 5-7 days, or until good sporulation is achieved	Casein soya bean digest agar ≤ 100 CFU 30-35 °C ≤ 5 days	Sabouraud- dextrose agar ≤ 100 CFU 20-25 °C ≤ 5 days	Casein soya bean digest agar ≤ 100 CFU 30-35 °C ≤ 5 days MPN: not applicable	Sabouraud- dextrose agar ≤ 100 CFU 20-25 °C ≤ 5 days

in situations where no other method is available. If the use of the method is justified, proceed as follows.

Prepare a series of at least 3 serial tenfold dilutions of the LBP as described under 4-5-1 and 4-5-2. From each level of dilution, 3 aliquots of 1 g or 1 mL are used to inoculate 3 tubes with 9-10 mL of casein soya bean digest broth. If necessary, a surface-active agent such as polysorbate 80 may be added to the medium. Thus, if 3 levels of dilution are prepared, 9 tubes are inoculated.

Incubate all tubes at 30-35 °C for not more than 3 days. If reading of the results is difficult or uncertain owing to the nature of the LBP to be tested, subculture in the same broth, or in casein soya bean digest agar, for 1-2 days at the same temperature and use these results. Determine the most probable number of micro-organisms per gram or millilitre of the LBP to be tested from Table 2.6.36.-2.

4-6. RESULTS AND INTERPRETATION

When verifying the suitability of the plate-count method, a mean count of any of the test organisms not differing by a factor greater than 2 from the value of the control defined in 4-5-2 in the absence of the LBP must be obtained. When verifying the suitability of the MPN method the calculated value from the inoculum must be within 95 per cent confidence limits of the results obtained with the control.

If the above criteria cannot be met, follow the decision tree shown in Figure 2.6.36.-1 and modify the procedure for the particular enumeration test to ensure the validity of the results.

5. TESTING OF LIVE BIOTHERAPEUTIC PRODUCTS

5-1. AMOUNT USED FOR THE TEST

Unless otherwise prescribed, use 10 g or 10 mL of the LBP to be tested taken with the precautions referred to above.

For LBP where the total number of entities in a batch is less than 200 (e.g. samples used in clinical trials), the sample size may be reduced to 2 units, or 1 unit if the size is less than 100.

Select the sample(s) at random from the bulk material or from the available containers of the preparation. To obtain the required quantity, mix the contents of a sufficient number of containers to provide the sample.

Table 2.6.36.-2. – *Most-probable-number values of micro-organisms*

Observed combinations of numbers of tubes showing growth in each set			MPN per gram or per millilitre of LBP	95 per cent confidence limits
Number of grams or millilitres of LBP per tube				
0.1	0.01	0.001		
0	0	0	< 3	0 - 9.4
0	0	1	3	0.1 - 9.5
0	1	0	3	0.1 - 10
0	1	1	6.1	1.2 - 17
0	2	0	6.2	1.2 - 17

Observed combinations of numbers of tubes showing growth in each set			MPN per gram or per millilitre of LBP	95 per cent confidence limits
Number of grams or millilitres of LBP per tube				
0.1	0.01	0.001		
0	3	0	9.4	3.5 - 35
1	0	0	3.6	0.2 - 17
1	0	1	7.2	1.2 - 17
1	0	2	11	4 - 35
1	1	0	7.4	1.3 - 20
1	1	1	11	4 - 35
1	2	0	11	4 - 35
1	2	1	15	5 - 38
1	3	0	16	5 - 38
2	0	0	9.2	1.5 - 35
2	0	1	14	4 - 35
2	0	2	20	5 - 38
2	1	0	15	4 - 38
2	1	1	20	5 - 38
2	1	2	27	9 - 94
2	2	0	21	5 - 40
2	2	1	28	9 - 94
2	2	2	35	9 - 94
2	3	0	29	9 - 94
2	3	1	36	9 - 94
3	0	0	23	5 - 94
3	0	1	38	9 - 104
3	0	2	64	16 - 181
3	1	0	43	9 - 181
3	1	1	75	17 - 199
3	1	2	120	30 - 360
3	1	3	160	30 - 380
3	2	0	93	18 - 360
3	2	1	150	30 - 380
3	2	2	210	30 - 400
3	2	3	290	90 - 990
3	3	0	240	40 - 990
3	3	1	460	90 - 1980
3	3	2	1100	200 - 4000
3	3	3	> 1100	

5-2. EXAMINATION OF THE LBP

5-2-1. Plate-count methods

5-2-1-1. Pour-plate method Prepare the sample using a method that has been shown to be suitable as described in section 4. Prepare for each medium at least 2 Petri dishes for each level of dilution. Incubate the plates of casein soya bean digest agar at 30-35 °C for 3-5 days and the plates of Sabouraud-dextrose agar at 20-25 °C for 5-7 days. Select the plates corresponding to a given dilution and showing the highest number of contaminating colonies less than 250 for AMCC and 50 for YMCC. Take the arithmetic mean per culture medium of the counts and calculate the number of CFU per gram or per millilitre of LBP.

5-2-1-2. Surface-spread method Prepare the sample using a method that has been shown to be suitable as described in section 4. Prepare at least 2 Petri dishes for each medium and each level of dilution. For incubation and calculation of the number of contaminant CFU proceed as described for the pour-plate method.

5-2-2. Most-probable-number method

Prepare and dilute the sample using a method that has been shown to be suitable as described in section 4. Incubate all tubes at 30-35 °C for 3-5 days. Subculture if necessary, using the procedure shown to be suitable. Record for each level of dilution the number of tubes showing microbial growth. Determine the most probable number of contaminant micro-organisms per gram or millilitre of the LBP to be tested from Table 2.6.36.-2.

5-3. INTERPRETATION OF THE RESULTS

The aerobic microbial contamination count (AMCC) is considered to be equal to the number of contaminating CFU found using casein soya bean digest agar; if colonies of contaminating yeasts/moulds are detected on this medium, they are counted as part of the AMCC. The combined yeasts/moulds contaminants count (YMCC) is considered to be equal to the number of CFU found using Sabouraud-dextrose agar; if colonies of contaminating bacteria are detected on this medium, they may be excluded from the YMCC. When the YMCC is expected to exceed the acceptance criterion due to bacterial growth, Sabouraud-dextrose agar containing antibiotics can be used (see decision tree shown in Figure 2.6.36.-1). If the count is carried out by the MPN method the calculated value is the AMCC.

When an acceptance criterion for microbial contamination is prescribed it is interpreted as follows:

— 10^1 CFU: maximum acceptable count = 20;
— 10^2 CFU: maximum acceptable count = 200;
— 10^3 CFU: maximum acceptable count = 2000.

The recommended solutions and media are described in general chapter *2.6.13.*

6. APPROACHES FOR ENUMERATION OF MICROBIAL CONTAMINANTS IN THE PRESENCE OF INHIBITION DUE TO THE LBP

This section should be understood as informative suggestion. Other procedures are possible as far as they are justified. Examples of approaches for enumeration of microbial contaminants for LBP inhibiting the microbial contamination enumeration are indicated below.

Each approach needs to be validated using the appropriate parameters depending on the extent of modification, while ensuring that the modifications do not reduce the AMCC or the YMCC.

The test strains indicated in Table 2.6.36.-1 are used. Preparation of test strains is performed as described in 4-2. Negative control is performed as described in 4-3. Growth promotion and suitability of new culture media chosen is carried out by inoculating each batch of the medium with a small number (not more than 100 CFU) of the micro-organisms. Growth obtained does not differ by a factor greater than 2 from the calculated value for a standardised

inoculum counted on casein soya bean digest agar (AMCC) or Sabouraud-dextrose agar (YMCC).

6-1. ENUMERATION OF AEROBIC MICROBIAL CONTAMINANTS

LBP containing lactic acid bacteria may be tested for aerobic microbial contamination on sugar-free agar plates incubated aerobically at 30-35 °C for 72 h. Lactic acid bacteria grow slowly as pinpoint colonies, while contaminants can easily be detected as larger colonies and are fast growing.

Alternatively, the aerobic microbial contamination may be tested on casein soya bean digest agar plates supplemented with 5 per cent of sheep blood at 30-35 °C for 44-48 h. The addition of blood enhances growth of contaminants and formation of distinctive colony morphology that are better discriminated in the presence of lactic acid bacteria.

For LBP containing *Bacillus clausii* spores, the aerobic microbial contamination may be enumerated on sporulating agar. The medium, by promoting the sporulation of *Bacillus clausii*, inhibits the vegetative growth of the LBP micro-organism, making the detection of contaminants possible. Plates are incubated at 33-37 °C for 48 h.

LBP containing *Saccharomyces cerevisiae*, var. *boulardii* may be tested for aerobic microbial contamination on casein soya bean digest agar containing cycloheximide, a suitable inhibitor of *Saccharomyces*. Plates are incubated at 30-35 °C for 3-5 days.

For LBP for which suitable media and growth conditions to enumerate aerobic microbial contamination are not available, not only the absence of specific contaminants (*Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Salmonella* spp. and bile-tolerant Gram-negative bacteria) is verified using the method described in general chapter *2.6.38*, and tests for other contaminating micro-organisms are carried out based on a risk assessment (e.g. specific environmental contaminants).

6-2. ENUMERATION OF YEAST AND MOULD CONTAMINANTS

For LBP containing bacteria, the test may be carried out with Sabouraud-dextrose agar containing antimicrobials (e.g. chloramphenicol) incubated at 20-25 °C for 5-7 days.

For LBP containing *Saccharomyces cerevisae*, var. *boulardii*, yeasts and moulds may be determined using several media (Sabouraud-dextrose agar supplemented with chloramphenicol and cycloheximide, Czapek-Dox agar, potato dextrose agar).

Microbiological Examination of Live Biotherapeutic Products: Tests for Specified Micro-organisms

(Ph. Eur. method 2.6.38)

1. INTRODUCTION

The tests described hereafter are intended for determination of the absence or limited occurrence of specified micro-organisms that may be detected under the conditions described. The tests are designed primarily to determine whether a live biotherapeutic product (LBP) complies with the established specification for microbiological quality.

Alternative microbiological procedures, including automated methods, may be used, provided that their equivalence to the Pharmacopoeia method has been demonstrated.

2. GENERAL PROCEDURES

The preparation of samples is carried out as described in general chapter *2.6.36*.

If the LBP to be tested prevents the detection of specified micro-organisms apply the decision tree shown in Figure 2.6.38.-1.

If components other than the active substance (i.e. the micro-organism) of the LBP to be tested have inhibitory activity against the target contaminant micro-organisms, this is insofar as possible removed or neutralised as described in general chapter *2.6.36*.

If surface-active agents are used for sample preparation, their absence of toxicity for the target contaminant micro-organisms and their compatibility with inactivators used must be demonstrated as described in general chapter *2.6.36*.

3. GROWTH-PROMOTING AND INHIBITORY PROPERTIES OF THE MEDIA, SUITABILITY OF THE TEST AND NEGATIVE CONTROLS

The ability of the test to detect the target micro-organisms in the presence of the LBP to be tested must be established. Suitability must be confirmed if a change is introduced in the testing procedure or the LBP, which may affect the outcome of the test.

3-1. PREPARATION OF TEST STRAINS

Use standardised stable suspensions of test strains or prepare them as stated below. Seed lot culture maintenance techniques (seed-lot systems) are used so that the viable micro-organisms used for inoculation are not more than 5 passages removed from the original master seed-lot.

3-1-1. Aerobic micro-organisms

Grow each of the bacterial test strains separately in casein soya bean digest broth or on casein soya bean digest agar at 30-35 °C for 18-24 h. Grow the test strain for *Candida albicans* separately on Sabouraud-dextrose agar or in Sabouraud-dextrose broth at 20-25 °C for 2-3 days.

- *Staphylococcus aureus* such as ATCC 6538, NCIMB 9518, CIP 4.83 or NBRC 13276;
- *Pseudomonas aeruginosa* such as ATCC 9027, NCIMB 8626, CIP 82.118 or NBRC 13275;
- *Escherichia coli* such as ATCC 8739, NCIMB 8545, CIP 53.126 or NBRC 3972;
- *Salmonella enterica* subsp. *enterica* serovar Typhimurium, such as ATCC 14028 or, as an alternative, *Salmonella enterica* subsp. *enterica* serovar Abony such as NBRC 100797, NCTC 6017 or CIP 80.39;
- *Candida albicans* such as ATCC 10231, NCPF 3179, IP 48.72 or NBRC 1594.

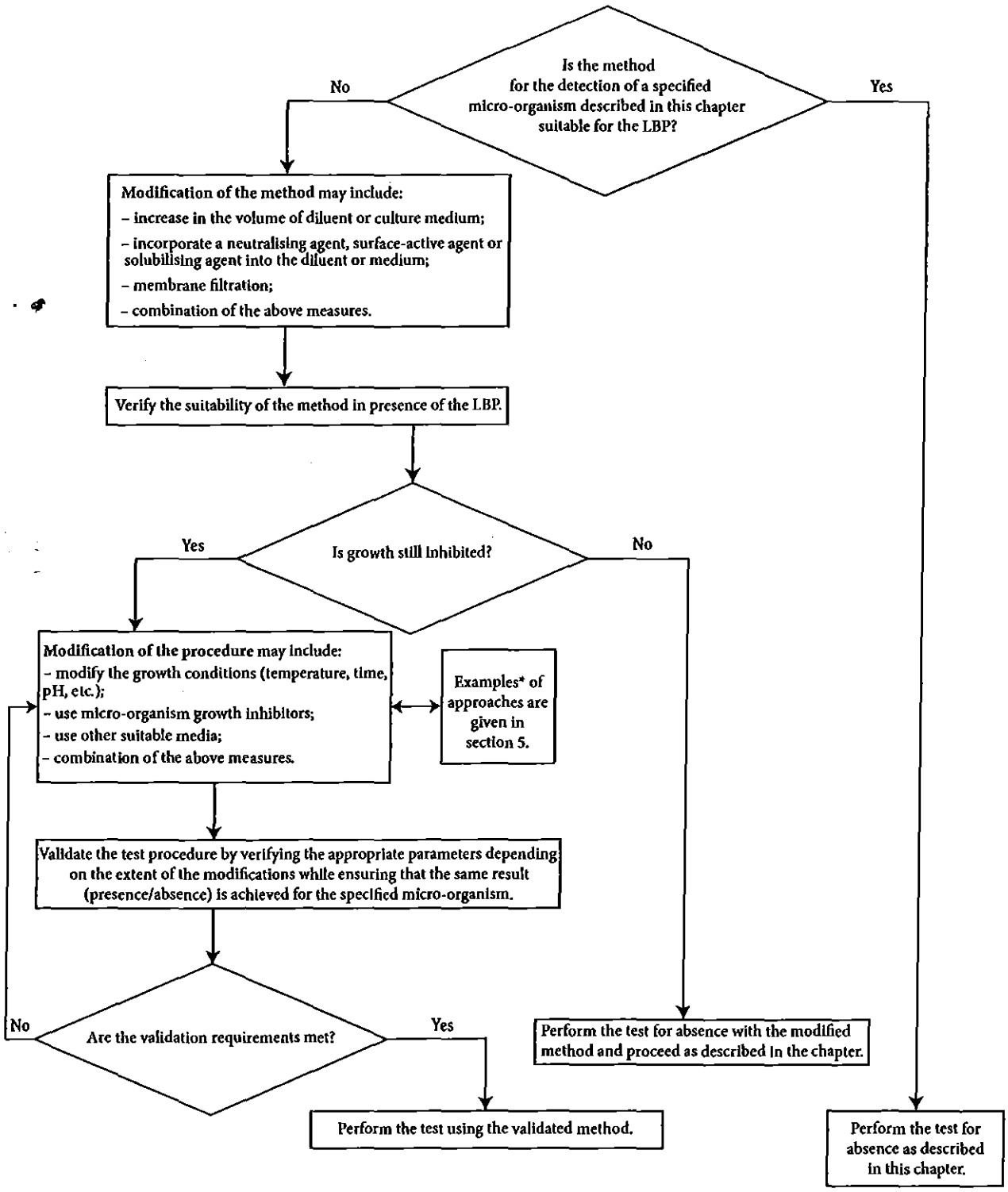
Use buffered sodium chloride-peptone solution pH 7.0 or phosphate buffer solution pH 7.2 to make test suspensions. Use the suspensions within 2 h or within 24 h if stored at 2-8 °C.

3-1-2. Anaerobic micro-organisms

Clostridia. Use a *Clostridium sporogenes* strain such as ATCC 11437 (NBRC 14293, NCIMB 12343, CIP 100651) or ATCC 19404 (NCTC 532 or CIP 79.03) or NBRC 14293. Grow the clostridial test strain under anaerobic conditions in reinforced medium for Clostridia at 30-35 °C for 24-48 h. As an alternative to preparing and then diluting down a fresh suspension of vegetative cells of *C. sporogenes*, a stable spore suspension is used for test inoculation. The stable spore suspension may be maintained at 2-8 °C for a validated period.

3-2. NEGATIVE CONTROL

To verify testing conditions, a negative control is performed using the chosen diluent in place of the test preparation. There must be no growth of micro-organisms. A negative control is also performed when testing the LBP as described in section 4. A failed negative control requires an investigation.



* This section should be understood as informative suggestion. Other procedures are possible as far as they are justified.

Figure 2.6.38.-1. – *Decision tree for the test for specified micro-organisms*

3-3. GROWTH PROMOTION AND INHIBITORY PROPERTIES OF THE MEDIA

Test each batch of ready-prepared medium and each batch of medium prepared either from dehydrated medium or from ingredients.

Verify suitable properties of relevant media as described in Table 2.6.38.-1.

3-3-1. Test for growth promoting properties, liquid media

Inoculate a portion of the appropriate medium of the same volume to be used in the test (see sections 3-4 and 4) with a small number (not more than 100 CFU) of the appropriate micro-organism. Incubate at the specified temperature for not more than the shortest period of time specified in the test. Clearly visible growth of the micro-organism comparable to

that previously obtained with a previously tested and approved batch of medium occurs.

3-3-2. Test for growth promoting properties, solid media

Perform the surface-spread method, inoculating each plate with a small number (not more than 100 CFU) of the appropriate micro-organism. Incubate at the specified temperature for not more than the shortest period of time specified in the test. Growth of the micro-organism is comparable to that previously obtained with a previously tested and approved batch of medium and does not differ by a factor greater than 2.

3-3-3. Test for inhibitory properties, liquid or solid media

Inoculate the appropriate medium with at least 100 CFU of the appropriate micro-organism. Incubate at the specified temperature for not less than the longest period of time specified in the test. No growth of the test micro-organism occurs.

3-3-4. Test for indicative properties

Perform the surface-spread method, inoculating each plate with a small number (not more than 100 CFU) of the appropriate micro-organism. Incubate at the specified temperature for not more than the shortest validated period of time specified in the test. Colonies are comparable in appearance and indication reactions to those previously obtained with a previously tested and approved batch of medium.

3-4. SUITABILITY OF THE TEST METHOD

For each LBP to be tested, perform the sample preparation as described in the relevant paragraph in section 4. Inoculate the test strains individually. Add each test strain at the time of mixing, in the prescribed growth medium. Use a number of micro-organisms equivalent to not more than 100 CFU in the inoculated test preparation. The inoculum should not exceed 1 per cent of the volume of the growth medium. Perform the test as described in the relevant paragraph in section 4 using for each step of the test the shortest incubation time to be used in the test.

The specified micro-organisms must be detected with the appearance and indication reactions described in section 4. If non-characteristic colonies and reactions are obtained, the method may still be suitable provided that all colony types are identified when performing the test.

Any inhibitory activity of the LBP against the target micro-organisms necessitates a modification of the test procedure (see decision tree shown in Figure 2.6.38.-1) and then the suitability for the LBP must be confirmed.

4. TESTING OF LIVE BIOTHERAPEUTIC PRODUCTS

4-1. BILE-TOLERANT GRAM-NEGATIVE BACTERIA

4-1-1. Sample preparation and pre-incubation

Prepare a sample using a 1 in 10 dilution of not less than 1 g or 1 mL of the LBP to be tested as described in general chapter *2.6.36*, but using casein soya bean digest broth as the chosen diluent, mix and incubate at 20-25 °C for a time sufficient to resuscitate the bacteria but not sufficient to encourage multiplication of the micro-organisms (usually 2 h but not more than 5 h).

4-1-2. Test for absence

Unless otherwise prescribed, use the volume corresponding to 1 g of the LBP, as prepared in 4-1-1, to inoculate enterobacteria enrichment broth-Mossel. Incubate at 30-35 °C for 24-48 h. Subculture on violet red bile glucose agar. Incubate at 30-35 °C for 18-24 h.

Table 2.6.38.-1. – *Growth promoting, inhibitory and indicative properties of media*

	Medium	Property	Test strains
Test for bile-tolerant Gram-negative bacteria	Enterobacteria enrichment broth-Mossel	Growth promoting	*E. coli* *P. aeruginosa*
		Inhibitory	*S. aureus*
	Violet red bile glucose agar	Growth promoting + indicative	*E. coli* *P. aeruginosa*
Test for *Escherichia coli*	MacConkey broth	Growth promoting	*E. coli*
		Inhibitory	*S. aureus*
	MacConkey agar	Growth promoting + indicative	*E. coli*
Test for *Salmonella*	Rappaport Vassiliadis *Salmonella* enrichment broth	Growth promoting	*Salmonella enterica* subsp. *enterica* serovar Typhimurium or *Salmonella enterica* subsp. *enterica* serovar Abony
		Inhibitory	*S. aureus*
	Xylose, lysine, deoxycholate agar	Growth promoting + indicative	*Salmonella enterica* subsp. *enterica* serovar Typhimurium or *Salmonella enterica* subsp. *enterica* serovar Abony
Test for *Pseudomonas aeruginosa*	Cetrimide agar	Growth promoting	*P. aeruginosa*
		Inhibitory	*E. coli*
Test for *Staphylococcus aureus*	Mannitol salt agar	Growth promoting + indicative	*S. aureus*
		Inhibitory	*E. coli*
Test for Clostridia	Reinforced medium for Clostridia	Growth promoting	*C. sporogenes*
	Columbia agar	Growth promoting	*C. sporogenes*
Test for *Candida albicans*	Sabouraud-dextrose broth	Growth promoting	*C. albicans*
	Sabouraud-dextrose agar	Growth promoting + indicative	*C. albicans*

The LBP complies with the test if there is no growth of colonies.

4-2. ESCHERICHIA COLI

4-2-1. Sample preparation and pre-enrichment

Prepare a sample using a 1 in 10 dilution of not less than 1 g or 1 mL of the LBP to be tested as described in general chapter *2.6.36* and use 10 mL or the quantity corresponding to 1 g or 1 mL to inoculate a suitable amount (determined as

described under 3-4) of casein soya bean digest broth, mix and incubate at 30-35 °C for 18-24 h.

4-2-2. Selection and subculture
Shake the container, transfer 1 mL of the casein soya bean digest broth to 100 mL of MacConkey broth and incubate at 42-44 °C for 24-48 h. Subculture on MacConkey agar at 30-35 °C for 18-72 h.

4-2-3. Interpretation
Growth of colonies indicates the possible presence of *E. coli*. This is confirmed by identification tests.

The LBP complies with the test if colonies are not present or if the confirmatory identification tests are negative.

4-3. SALMONELLA

4-3-1. Sample preparation and pre-enrichment
Prepare the LBP to be tested as described in general chapter *2.6.36* and use the quantity corresponding to not less than 10 g or 10 mL to inoculate a suitable amount (determined as described under 3-4) of casein soya bean digest broth, mix and incubate at 30-35 °C for 18-24 h.

4-3-2. Selection and subculture
Transfer 0.1 mL of the casein soya bean digest broth to 10 mL of Rappaport Vassiliadis *Salmonella* enrichment broth and incubate at 30-35 °C for 18-24 h. Subculture on xylose, lysine, deoxycholate agar. Incubate at 30-35 °C for 18-48 h.

4-3-3. Interpretation
Growth of well-developed, red colonies, with or without black centres indicates the possible presence of *Salmonella*. This is confirmed by identification tests.

The LBP complies with the test if colonies of the types described are not present or if the confirmatory identification tests are negative.

4-4. PSEUDOMONAS AERUGINOSA

4-4-1. Sample preparation and pre-enrichment
Prepare a sample using a 1 in 10 dilution of not less than 1 g or 1 mL of the LBP to be tested as described in general chapter *2.6.36* and use 10 mL or the quantity corresponding to 1 g or 1 mL to inoculate a suitable amount (determined as described under 3-4) of casein soya bean digest broth and mix. Incubate at 30-35 °C for 18-24 h.

4-4-2. Selection and subculture
Subculture on cetrimide agar and incubate at 30-35 °C for 18-72 h.

4-4-3. Interpretation
Growth of colonies indicates the possible presence of *P. aeruginosa*. This is confirmed by identification tests.

The LBP complies with the test if colonies are not present or if the confirmatory identification tests are negative.

4-5. STAPHYLOCOCCUS AUREUS

4-5-1. Sample preparation and pre-enrichment
Prepare a sample using a 1 in 10 dilution of not less than 1 g or 1 mL of the LBP to be tested as described in general chapter *2.6.36* and use 10 mL or the quantity corresponding to 1 g or 1 mL to inoculate a suitable amount (determined as described under 3-4) of casein soya bean digest broth and mix. Incubate at 30-35 °C for 18-24 h.

4-5-2. Selection and subculture
Subculture on mannitol salt agar and incubate at 30-35 °C for 18-72 h.

4-5-3. Interpretation
Growth of yellow/white colonies surrounded by a yellow zone indicates the possible presence of *S. aureus*. This is confirmed by identification tests.

The LBP complies with the test if colonies of the types described are not present or if the confirmatory identification tests are negative.

4-6. CLOSTRIDIA

4-6-1. Sample preparation and heat treatment
Prepare a sample using a 1 in 10 dilution (with a minimum total volume of 20 mL) of not less than 2 g or 2 mL of the LBP to be tested as described in general chapter *2.6.36*. Divide the sample into 2 portions of at least 10 mL. Heat 1 portion at 80 °C for 10 min and cool rapidly. Do not heat the other portion.

4-6-2. Selection and subculture
Use 10 mL or the quantity corresponding to 1 g or 1 mL of the LBP to be tested of both portions to inoculate suitable amounts (determined as described under 3-4) of reinforced medium for Clostridia. Incubate under anaerobic conditions at 30-35 °C for 48 h. After incubation, make subcultures from each container on Columbia agar and incubate under anaerobic conditions at 30-35 °C for 48-72 h.

4-6-3. Interpretation
The occurrence of anaerobic growth of rods (with or without endospores) giving a negative catalase reaction may indicate the presence of Clostridia. This is confirmed by identification tests.

The LBP complies with the test if colonies of the types described are not present or if the confirmatory identification tests are negative.

4-7. CANDIDA ALBICANS

4-7-1. Sample preparation and pre-enrichment
Prepare the LBP to be tested as described in general chapter *2.6.36* and use 10 mL or the quantity corresponding to not less than 1 g or 1 mL to inoculate 100 mL of Sabouraud-dextrose broth and mix. Incubate at 30-35 °C for 3-5 days.

4-7-2. Selection and subculture
Subculture on Sabouraud-dextrose agar and incubate at 30-35 °C for 24-48 h.

4-7-3. Interpretation
Growth of white colonies may indicate the presence of *C. albicans*. This is confirmed by identification tests.

The LBP complies with the test if colonies of the types described are not present or if the confirmatory identification tests are negative.

5. APPROACHES FOR TESTING SPECIFIED MICRO-ORGANISMS AND ADDITIONAL TESTING

This section should be understood as informative suggestion. Other procedures are possible as far as they are justified.

If the detection of the specified micro-organism is inhibited by the LBP, its detection is carried out under conditions that neutralise the inhibition or limit the growth of the LBP micro-organisms. The modified test for specified micro-organisms is validated using the appropriate validation parameters depending on the extent of the modifications while ensuring that the same result (presence/absence) is achieved for the specified micro-organism.

A suitable combination of antibiotics may be used to inhibit the LBP and detect the specified micro-organism.
For example, for LBP samples containing *S. cerevisiae*, var. *boulardii*, pre-enrichment and selective media (Sabouraud-dextrose broth and agar) can be supplemented with chloramphenicol and cycloheximide to detect contaminant *C. albicans*.

Alternative pre-enrichment media (e.g. buffered peptone medium for *Salmonella* — see general chapter *2.6.31*), media and test/growth conditions may be used to support growth of the target micro-organisms while limiting growth of the micro-organisms of the LBP.

LBP containing *Bacillus clausii* spores are tested for contaminating *Bacillus cereus* on chromogenic selective agar media plates. Incubation time and temperature depends on the specific medium.

A proposed approach to detect *Bacillus cereus* is described below.

Prepare a sample using a 1 in 10 dilution of not less than 1 g or 1 mL of the LBP to be tested and use 10 mL or the quantity corresponding to 1 g or 1 mL to inoculate a suitable amount of casein soya bean digest broth, mix and incubate at 35-37 °C for 18-24 h. Subculture on selective media (e.g. *B. cereus* Mossel agar). For each LBP to be tested, validate incubation time and temperature. The appearance of *B. cereus* colonies depends on the medium used. The LBP complies with the test if no *B. cereus* colonies are present.

For LBP containing *E. coli*, use *Enterococcus faecalis* and *Enterococcus faecium* as microbial indicators of faecal contamination to replace the test for absence of *E. coli*. Use selective or differential chromogenic media and, for each LBP to be tested, validate incubation time and temperature. The LBP complies with the test when there is no growth of *Enterococcus* spp.

A search for pathogenic *E. coli* strains may be carried out by suitable methods (e.g. molecular methods for the detection of gene *stx* or *eae*) based on risk-assessment.

6. RECOMMENDED SOLUTIONS AND CULTURE MEDIA

The solutions and culture media mentioned in this chapter and described in chapter *2.6.13* have been found satisfactory for bile-tolerant Gram-negative bacteria, *E. coli*, *P. aeruginosa*, *S. aureus*, Clostridia, *Salmonella* and *C. albicans*. Other media may be used provided that their suitability can be demonstrated and the analytical method where they are used is validated with the appropriate validation parameters.

Appendix XVII

A. Particle Size of Powders

1. Particle Size Classification of Powders (Sieve Test)

(Ph. Eur. method 2.9.12)

The degree of fineness of a powder may be expressed by reference to sieves that comply with the specifications for non-analytical sieves (*2.1.4*).

Where the degree of fineness of powders is determined by sieving, it is defined in relation to the sieve number(s) used either by means of the following terms or, where such terms cannot be used, by expressing the fineness of the powder as a percentage *m/m* passing the sieve(s) used.

The following terms are used in the description of powders:

Coarse powder
Not less than 95 per cent by mass passes through a number 1400 sieve and not more than 40 per cent by mass passes through a number 355 sieve.

Moderately fine powder
Not less than 95 per cent by mass passes through a number 355 sieve and not more than 40 per cent by mass passes through a number 180 sieve.

Fine powder
Not less than 95 per cent by mass passes through a number 180 sieve and not more than 40 per cent by mass passes through a number 125 sieve.

Very fine powder
Not less than 95 per cent by mass passes through a number 125 sieve and not more than 40 per cent by mass passes through a number 90 sieve.

If a single sieve number is given, not less than 97 per cent of the powder passes through the sieve of that number, unless otherwise prescribed.

Assemble the sieves and operate in a suitable manner until sifting is practically complete. Weigh the separated fractions of the powder.

Additional points for monographs other than those of the European Pharmacopoeia

Within the monographs of the British Pharmacopoeia, the above terms may be used to specify the degree of coarseness or fineness of a medicinal or pharmaceutical substance in powder form that is to be incorporated into a formulated preparation. The following terms may also be used for such purposes.

When the use of sieves is inappropriate, the definition is expressed in terms of the particle size as determined by suitable microscopical examination.

Moderately coarse powder Not less than 95% by weight passes through a number 710 sieve and not more than 40% by weight passes through a number 250 sieve.

Microfine powder Not less than 90% by weight passes through a number 45 sieve.

Superfine powder Not less than 90% by number of the particles are less than 10 µm in size.

2. Powder Fineness[1]

(Ph. Eur. method 2.9.35)

Particle-size distribution is estimated by analytical sieving (*2.9.38*) or by application of other suitable methods where appropriate. A simple descriptive classification of powder fineness is provided in this chapter. For practical reasons, sieves are commonly used to measure powder fineness. Sieving is most suitable where a majority of the particles are larger than about 75 µm, although it can be used for some powders having smaller particle sizes where the method can be validated. Light diffraction is also a widely used technique for measuring the size of a wide range of particles.

Where the cumulative distribution has been determined by analytical sieving or by application of other methods, particle size may be characterised in the following manner:

x_{90} = particle size corresponding to 90 per cent of the cumulative undersize distribution;

x_{50} = median particle size (i.e. 50 per cent of the particles are smaller and 50 per cent of the particles are larger);

x_{10} = particle size corresponding to 10 per cent of the cumulative undersize distribution.

It is recognised that the symbol d is also widely used to designate these values. Therefore, the symbols d_{90}, d_{50}, d_{10} may be used.

The following parameters may be defined based on the cumulative distribution.

$Q_r(x)$ = cumulative distribution of particles with a dimension less than or equal to x where the subscript r reflects the distribution type.

r	Distribution type
0	Number
1	Length
2	Area
3	Volume

Therefore, by definition:

$Q_r(x) = 0.90$ when $x = x_{90}$

$Q_r(x) = 0.50$ when $x = x_{50}$

$Q_r(x) = 0.10$ when $x = x_{10}$

An alternative but less informative method of classifying powder fineness is by use of the descriptive terms in Table 2.9.35.-1.

Table 2.9.35.-1.

Classification of powders by fineness		
Descriptive term	x_{50} (µm)	Cumulative distribution by volume basis, $Q_3(x)$
Coarse	> 355	$Q_3(355) < 0.50$
Moderately fine	180 - 355	$Q_3(180) < 0.50$ and $Q_3(355) \geq 0.50$
Fine	125 - 180	$Q_3(125) < 0.50$ and $Q_3(180) \geq 0.50$
Very fine	≤ 125	$Q_3(125) \geq 0.50$

[1] *See the International Standard ISO 3310/1 (1975).*

B. Sieves and Filters

1. Sieves

(Ph. Eur. method 2.1.4)

Sieves are constructed of suitable materials with square meshes. For purposes other than analytical procedures, sieves with circular meshes may be used, the internal diameters of which are 1.25 times the aperture of the square mesh of the corresponding sieve size. There must be no reaction between the material of the sieve and the substance being sifted. Degree of comminution is prescribed in the monograph using the sieve number, which is the size of the mesh in micrometres, given in parenthesis after the name of the substance (Table 2.1.4.-1).

Maximum tolerance[1] for an aperture (+ X): no aperture size shall exceed the nominal size by more than X, where:

$$X = \frac{2(w^{0.75})}{3} + 4(w^{0.25})$$

w = width of aperture.

Tolerance for mean aperture (± Y): the average aperture size shall not depart from the nominal size by more than ± Y, where:

$$Y = \frac{w^{0.98}}{27} + 1.6$$

Intermediary tolerance (+ Z): not more than 6 per cent of the total number of apertures shall have sizes between "nominal + X" and "nominal + Z", where:

$$Z = \frac{X + Y}{2}$$

Wire diameter d: the wire diameters given in Table 2.1.4.-1 apply to woven metal wire cloth mounted in a frame. The nominal sizes of the wire diameters may depart from these values within the limits d_{max} and d_{min}. The limits define a permissible range of choice ± 15 per cent of the recommended nominal dimensions. The wires in a test sieve shall be of a similar diameter in warp and weft directions.

[1] *See the International Standard ISO 3310/1 (1975).*

Table 2.1.4.-1 *(values in micrometers)*

Sieve numbers (Nominal dimensions of apertures)	Tolerances for apertures			Wire diameters		
	Maximum tolerance for an aperture	Tolerance for mean aperture	Intermediary tolerance	Recommended nominal dimensions	Admissible limits	
	$+X$	$\pm Y$	$+Z$	d	d_{max}	d_{min}
11 200	770	350	560	2500	2900	2100
8000	600	250	430	2000	2300	1700
5600	470	180	320	1600	1900	1300
4000	370	130	250	1400	1700	1200
2800	290	90	190	1120	1300	950
2000	230	70	150	900	1040	770
1400	180	50	110	710	820	600
1000	140	30	90	560	640	480
710	112	25	69	450	520	380
500	89	18	54	315	360	270
355	72	13	43	224	260	190
250	58	9.9	34	160	190	130
180	47	7.6	27	125	150	106
125	38	5.8	22	90	104	77
90	32	4.6	18	63	72	54
63	26	3.7	15	45	52	38
45	22	3.1	13	32	37	27
38	–	–	–	30	35	24

2. Filters[2]

(Ph. Eur. method 2.1.2)

Table 2.1.2.-1

Porosity number (Ph. Eur.)[3]	Maximum diameter of pores in micrometres	Germany	France	United Kingdom
1.6	less than 1.6	5f	–	–
–	1 - 2.5	5	–	5
4	1.6 - 4	–	–	–
–	4 - 6	–	5	–
10	4 - 10	4f	–	4
16	10 - 16	4	4	–
40	16 - 40	3	3	3
–	40 - 50	–	–	2
100	40 - 100	2	2	–
–	100 - 120	–	–	1
160	100 - 160	1	1	–
–	150 - 200	0	0	–
250	160 - 250	–	–	–
–	200 - 500	–	00	–

Special Uses

Diameters in micrometres

< 2.5	Bacteriological filtration
4 - 10	Ultra-fine filtration, separation of micro-organisms of large diameter
10 - 40	Analytical filtration, very fine filtration of mercury, very fine dispersion of gases
40 - 100	Fine filtration, filtration of mercury, fine dispersion of gases
100 - 160	Filtration of coarse materials, dispersion and washing of gases, support for other filter materials
160 - 500	Filtration of very coarse materials, dispersion and washing of gases.

3. Particle-size Distribution Estimation by Analytical Sieving[4]

(Ph. Eur. method 2.9.38)

Sieving is one of the oldest methods of classifying powders and granules by particle-size distribution. When using a woven sieve cloth, the sieving will essentially sort the particles by their intermediate size dimension (i.e. breadth or width). Mechanical sieving is most suitable where the majority of the particles are larger than about 75 µm. For smaller particles, their light weight provides insufficient force during sieving to overcome the surface forces of cohesion and adhesion that cause the particles to stick to each other and to the sieve, and thus cause particles that would be expected to pass through the sieve to be retained. For such materials other means of agitation such as air-jet sieving or sonic-sifter sieving may be more appropriate. Nevertheless, sieving can sometimes be

[2] *The given limits are only approximate.*

[3] *The European Pharmacopoeia has adopted the system proposed by the International Organization for Standardization (ISO).*

[4] *This chapter has undergone pharmacopoeial harmonisation. See chapter 5.8 Pharmacopoeial harmonisation.*

used for some powders or granules having median particle sizes smaller than 75 µm where the method can be validated. In pharmaceutical terms, sieving is usually the method of choice for classification of the coarser grades of single powders or granules. It is a particularly attractive method in that powders and granules are classified only on the basis of particle size, and in most cases the analysis can be carried out in the dry state.

Among the limitations of the sieving method are the need for an appreciable amount of sample (normally at least 25 g, depending on the density of the powder or granule, and the diameter of the test sieves) and the difficulty in sieving oily or other cohesive powders or granules that tend to clog the sieve openings. The method is essentially a two-dimensional estimate of size because passage through the sieve aperture is frequently more dependent on maximum width and thickness than on length.

This method is intended for estimation of the total particle-size distribution of a single material. It is not intended for determination of the proportion of particles passing or retained on 1 or 2 sieves.

Estimate the particle-size distribution as described under Dry sieving method, unless otherwise specified in the individual monograph. Where difficulty is experienced in reaching the endpoint (i.e. material does not readily pass through the sieves) or when it is necessary to use the finer end of the sieving range (below 75 µm), serious consideration must be given to the use of an alternative particle-sizing method.

Sieving is carried out under conditions that do not cause the test sample to gain or lose moisture. The relative humidity of the environment in which the sieving is carried out must be controlled to prevent moisture uptake or loss by the sample. In the absence of evidence to the contrary, analytical test sieving is normally carried out at ambient humidity. Any special conditions that apply to a particular material must be detailed in the individual monograph.

Principles of analytical sieving

Analytical test sieves are constructed from a woven-wire mesh, which is of simple weave that is assumed to give nearly square apertures and is joined to the base of an open cylindrical container. The basic analytical method involves stacking the sieves on top of one another in ascending degrees of coarseness, and then placing the test powder on the top sieve. The nest of sieves is subjected to a standardised period of agitation, and then the mass of material retained on each sieve is accurately determined. The test gives the mass percentage of powder in each sieve size range.

This sieving process for estimating the particle-size distribution of a single pharmaceutical powder is generally intended for use where at least 80 per cent of the particles are larger than 75 µm. The size parameter involved in determining particle-size distribution by analytical sieving is the length of the side of the minimum square aperture through which the particle will pass.

TEST SIEVES

Test sieves suitable for pharmacopoeial tests conform to the current edition of *ISO 3310-1: Test sieves – Technical requirements and testing - Part 1: Test sieves of metal wire cloth* (see Table 2.9.38.-1). Unless otherwise specified in the monograph, use those ISO sieves listed as principal sizes in Table 2.9.38.-1 that are recommended in the particular region.

Sieves are selected to cover the entire range of particle sizes present in the test sample. A nest of sieves having a $\sqrt{2}$ progression of the area of the sieve openings is recommended. The nest of sieves is assembled with the coarsest screen at the top and the finest at the bottom. Use micrometres or millimetres in denoting test sieve openings.

Test sieves are made from stainless steel or, less preferably, from brass or another suitable non-reactive wire.

Calibration and recalibration of test sieves is in accordance with the current edition of ISO 3310-1. Sieves are carefully examined for gross distortions and fractures, especially at their screen frame joints, before use. Sieves may be calibrated optically to estimate the average opening size, and opening variability, of the sieve mesh. Alternatively, for the evaluation of the effective opening of test sieves in the size range of 212-850 µm, standard glass spheres are available. Unless otherwise specified in the individual monograph, perform the sieve analysis at controlled room temperature and at ambient relative humidity.

Cleaning test sieves Ideally, test sieves are cleaned using only a low-pressure air jet or a liquid stream. If some apertures remain blocked by test particles, careful gentle brushing may be used as a last resort.

Test sample

If the test sample mass is not given in the monograph for a particular material, use a test sample having a mass of 25-100 g, depending on the bulk density of the material, for test sieves having a 200 mm diameter. For 76 mm sieves, the amount of material that can be accommodated is approximately 1/7 that which can be accommodated by a 200 mm sieve. Determine the most appropriate mass for a given material by test sieving accurately weighed samples of different masses, such as 25 g, 50 g and 100 g, for the same time period on a mechanical shaker (note: if the test results are similar for the 25 g and 50 g samples, but the 100 g sample shows a lower percentage through the finest sieve, the 100 g sample size is too large). Where only a sample of 10-25 g is available, smaller diameter test sieves conforming to the same mesh specifications may be substituted, but the endpoint must be redetermined. The use of test samples having a smaller mass (e.g. down to 5 g) may be needed. For materials with low apparent particle density, or for materials mainly comprising particles with a highly iso-diametrical shape, sample masses below 5 g for a 200 mm screen may be necessary to avoid excessive blocking of the sieve. During validation of a particular sieve-analysis method, it is expected that the problem of sieve blocking will have been addressed.

If the test material is prone to absorbing or losing significant amounts of water with varying humidity, the test must be carried out in an appropriately controlled environment. Similarly, if the test material is known to develop an electrostatic charge, careful observation must be made to ensure that such charging does not influence the analysis. An antistatic agent, such as colloidal silicon dioxide and/or aluminum oxide, may be added at a 0.5 per cent (*m/m*) level to minimise this effect. If both of the above effects cannot be eliminated, an alternative particle-sizing technique must be selected.

Agitation methods

Several different sieve and powder-agitation devices are commercially available, all of which may be used to perform sieve analyses. However, the different methods of agitation may give different results for sieve analyses and endpoint determinations because of the different types and magnitudes of the forces acting on the individual particles under test.

Table 2.9.38.-1.

ISO Nominal Aperture			US Sieve No.	Recommended USP Sieves (µm)	European Sieve No.	Japanese Sieve No.
Principal sizes	Supplementary sizes					
R 20/3	R 20	R 40/3				
11.20 mm	11.20 mm	11.20 mm			11 200	
	10.00 mm					
		9.50 mm				
	9.00 mm					
8.00 mm	8.00 mm	8.00 mm				
	7.10 mm					
		6.70 mm				
	6.30 mm					
5.60 mm	5.60 mm	5.60 mm			5600	3.5
	5.00 mm					
		4.75 mm				4
	4.50 mm					
4.00 mm	4.00 mm	4.00 mm	5	4000	4000	4.7
	3.55 mm					
		3.35 mm	6			5.5
	3.15 mm					
2.80 mm	2.80 mm	2.80 mm	7	2800	2800	6.5
	2.50 mm					
		2.36 mm	8			7.5
	2.24 mm					
2.00 mm	2.00 mm	2.00 mm	10	2000	2000	8.6
	1.80 mm					
		1.70 mm	12			10
	1.60 mm					
1.40 mm	1.40 mm	1.40 mm	14	1400	1400	12
	1.25 mm					
		1.18 mm	16			14
	1.12 mm					
1.00 mm	1.00 mm	1.00 mm	18	1000	1000	16
	900 µm					
		850 µm	20			18
	800 µm					
710 µm	710 µm	710 µm	25	710	710	22
	630 µm					
		600 µm	30			26
	560 µm					
500 µm	500 µm	500 µm	35	500	500	30
	450 µm					
		425 µm	40			36
	400 µm					
355 µm	355 µm	355 µm	45	355	355	42
	315 µm					
		300 µm	50			50
	280 µm					
250 µm	250 µm	250 µm	60	250	250	60
	224 µm					
		212 µm	70			70
	200 µm					
180 µm	180 µm	180 µm	80	180	180	83
	160 µm					
		150 µm	100			100
	140 µm					
125 µm	125 µm	125 µm	120	125	125	119
	112 µm					
		106 µm	140			140
	100 µm					
90 µm	90 µm	90 µm	170	90	90	166
	80 µm					
		75 µm	200			200
	71 µm					
63 µm	63 µm	63 µm	230	63	63	235
	56 µm					
		53 µm	270			282
	50 µm					
45 µm	45 µm	45 µm	325	45	45	330
	40 µm					
		38 µm			38	391

Methods using mechanical agitation or electromagnetic agitation, and that can induce either a vertical oscillation or a horizontal circular motion, or tapping or a combination of both tapping and horizontal circular motion are available. Entrainment of the particles in an air stream may also be used. The results must indicate which agitation method was used and the agitation parameters used (if they can be varied), since changes in the agitation conditions will give different results for the sieve analysis and endpoint determination, and may be sufficiently different to give a failing result under some circumstances.

Endpoint determination

The test sieving analysis is complete when the mass on any of the test sieves does not change by more than 5 per cent or 0.1 g (10 per cent in the case of 76 mm sieves) of the previous mass on that sieve. If less than 5 per cent of the total sample mass is present on a given sieve, the endpoint for that sieve is increased to a mass change of not more than 20 per cent of the previous mass on that sieve.

If more than 50 per cent of the total sample mass is found on any one sieve, unless this is indicated in the monograph, the test is repeated, but with the addition to the sieve nest of a more coarse sieve intermediate between that carrying the excessive mass and the next coarsest sieve in the original nest, i.e. addition of the ISO series sieve omitted from the nest of sieves.

SIEVING METHODS

Mechanical agitation (Dry sieving method)

Tare each test sieve to the nearest 0.1 g. Place an accurately weighed quantity of test sample on the top (coarsest) sieve, and replace the lid. Agitate the nest of sieves for 5 min, then carefully remove each sieve from the nest without loss of material. Reweigh each sieve, and determine the mass of material on each one. Determine the mass of material in the collecting pan in a similar manner. Re-assemble the nest of sieves, and agitate for 5 min. Remove and weigh each sieve as previously described. Repeat these steps until the endpoint criteria are met (see Endpoint determination under Test sieves). Upon completion of the analysis, reconcile the masses of material. Total loss must not exceed 5 per cent of the mass of the original test sample.

Repeat the analysis with a fresh sample, but using a single sieving time equal to that of the combined times used above. Confirm that this sieving time conforms to the requirements for endpoint determination. When this endpoint has been validated for a specific material, then a single fixed time of sieving may be used for future analyses, providing the particle-size distribution falls within normal variation.

If there is evidence that the particles retained on any sieve are aggregates rather than single particles, the use of mechanical dry sieving is unlikely to give good reproducibility, and a different particle-size analysis method must be used.

Air-entrainment methods (Air-jet and sonic-sifter sieving)

Different types of commercial equipment that use a moving air current are available for sieving. A system that uses a single sieve at a time is referred to as air-jet sieving. It uses the same general sieving methodology as that described under Dry sieving method, but with a standardised air jet replacing the normal agitation mechanism. It requires sequential analyses on individual sieves starting with the finest sieve to obtain a particle-size distribution. Air-jet sieving often includes the use of finer test sieves than used in ordinary dry sieving. This technique is more suitable where only oversize or undersize fractions are needed.

In the sonic-sifter method, a nest of sieves is used, and the test sample is carried in a vertically oscillating column of air that lifts the sample and then carries it back against the mesh openings at a given number of pulses per minute. It may be necessary to lower the sample amount to 5 g when sonic sifting is employed.

The air-jet sieving and sonic-sifter sieving methods may be useful for powders or granules when the mechanical sieving techniques are incapable of giving a meaningful analysis.

These methods are highly dependent upon proper dispersion of the powder in the air current. This requirement may be hard to achieve if the method is used at the lower end of the sieving range (i.e. below 75 µm), when the particles tend to be more cohesive, and especially if there is any tendency for the material to develop an electrostatic charge. For the above reasons endpoint determination is particularly critical, and it is very important to confirm that the oversize material comprises single particles and is not composed of aggregates.

INTERPRETATION

The raw data must include the mass of the test sample, the total sieving time, the precise sieving methodology, and the set values for any variable parameters, in addition to the masses retained on the individual sieves and in the pan.

It may be convenient to convert the raw data into a cumulative mass distribution, and if it is desired to express the distribution in terms of a cumulative mass undersize, the range of sieves used must include a sieve through which all the material passes. If there is evidence on any of the test sieves that the material remaining on it is composed of aggregates formed during the sieving process, the analysis is invalid.

C. Specific Surface Area by Air Permeability

(Ph. Eur. method 2.9.14)

The test is intended for the determination of the specific surface area of dry powders expressed in square metres per gram in the sub-sieve region. The effect of molecular flow ("slip flow") which may be important when testing powders consisting of particles less than a few micrometres is not taken into account in the equation used to calculate the specific surface area.

APPARATUS

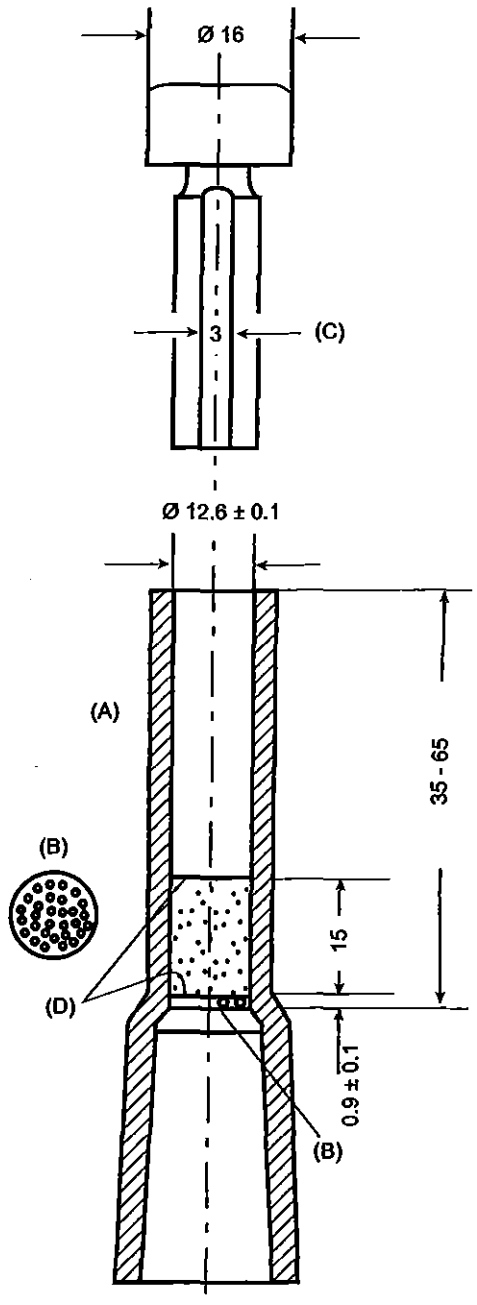


Figure 2.9.14.-1. – *Permeability cell*
Dimensions in millimetres

The apparatus consists of the following parts:

(a) a *permeability cell* (see Figure 2.9.14.-1), which consists of a cylinder with an inner diameter of 12.6 ± 0.1 mm (*A*), constructed of glass or non-corroding metal. The bottom of the cell forms an airtight connection (for example, via an adapter) with the manometer (Figure 2.9.14.-2). A ledge 0.5-1 mm in width is located 50 ± 15 mm from the top of the cell. It is an integral part of the cell or firmly fixed so as to be airtight. It supports a perforated metal disk (*B*), constructed of non-corroding metal. The disk has a thickness

of 0.9 ± 0.1 mm and is perforated with 30 to 40 holes 1 mm in diameter evenly distributed over this area.

The plunger (*C*) is made of non-corroding metal and fits into the cell with a clearance of not more than 0.1 mm.

The bottom of the plunger has sharp square edges at right angles to the principal axis. There is an air vent 3 mm long and 0.3 mm deep on one side of the plunger. The top of the plunger has a collar such that when the plunger is placed in the cell and the collar is brought into contact with the top of the cell, the distance between the bottom of the plunger and the top of the perforated disk (*B*) is 15 ± 1 mm.

The filter paper disks (*D*) have smooth edges and the same diameter as the inside of the cell.

(b) a *U-tube manometer* (*E*) (Figure 2.9.14.-2) is made of nominal 9 mm outer diameter and 7 mm inner diameter glass tubing with standard walls. The top of one arm of the manometer forms an airtight connection with the permeability cell (*F*). The manometer arm connected to the permeability cell has a line etched around the tube at 125-145 mm below the top of the side outlet and three other lines at distances of 15 mm, 70 mm and 110 mm above that line (*G*). The side outlet, 250-305 mm above the bottom of the manometer, is used to evacuate the manometer arm connected to the permeability cell. A tap is provided on the side outlet not more than 50 mm from the manometer arm.

The manometer is mounted firmly in such a manner that the arms are vertical. It is filled to the lowest mark with the liquid recommended by the manufacturer of the equipment or water.

METHOD

If prescribed, dry the powder to be examined and sift through a suitable sieve (for example no. 125) to disperse agglomerates. Calculate the mass (*M*) of the powder to be used from the following expression:

$$V \times \rho \times (1 - \varepsilon) \qquad (1)$$

V = bulk volume of the compacted bed of powder,
ρ = density of the substance to be examined in grams per millilitre,
ε = porosity of the compacted bed of powder.

Assume first a porosity of 0.5 and introduce this value in expression (1) to calculate the mass (*M*) of the powder to be examined.

Place a filter paper disk on top of the perforated metal disk (*B*). Weigh the calculated mass (*M*) of the powder to be examined to the nearest 1 mg. Carefully transfer the powder into the cleaned, tared permeability cell and carefully tap the cell so that the surface of the powder bed is level and cover it with a second filter paper disk. Slowly compact the powder by means of the plunger, avoiding rotary movement. Maintain the pressure until the plunger is completely inserted into the permeability cell. If this is not possible, decrease the quantity of the powder used. If, on the contrary, there is not enough resistance, increase the quantity of the powder. In this case calculate the porosity again. After at least 10 s, remove the plunger.

Attach the permeability cell to the tube of the manometer by means of an airtight connection. Evacuate the air from the manometer by means of a rubber bulb until the level of the coloured liquid is at the highest mark. Close the tap and check that the apparatus is airtight by closing the upper end of the cell, for example with a rubber stopper. Remove the stopper and, using a timer, measure the time taken for the liquid to fall from the second to the third mark.

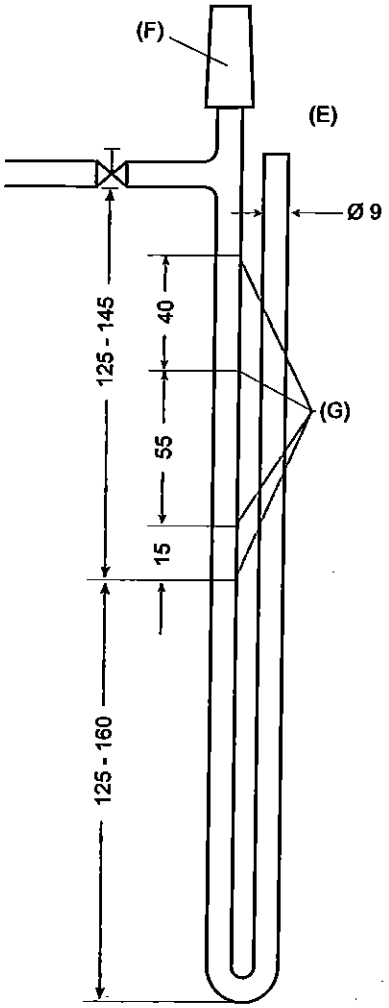


Figure 2.9.14.-2. – *Manometer*
Dimensions in millimetres

Using the measured flow time, calculate the specific surface area (*S*), expressed in square metres per gram, from the following expression:

$$\frac{K \times \sqrt{\varepsilon^3} \times \sqrt{t}}{\rho \times (1 - \varepsilon) \times \sqrt{\eta}} \qquad (2)$$

t = flow time in seconds,
η = dynamic viscosity of air in millipascal seconds (see Table 2.9.14.-1),
K = apparatus constant determined according to expression (4),
ρ = density of the substance to be examined in grams per millilitre,
ε = porosity of the compacted bed of powder.

CALIBRATION OF THE APPARATUS

Bulk volume of the compacted powder bed

It is determined by the mercury displacement method as follows:

Place two filter paper disks in the permeability cell, pressing down the edges with a rod slightly smaller than the cell diameter until the filter disks lie flat on the perforated metal disk; fill the cell with mercury, removing any air bubbles adhering to the wall of the cell and wipe away the excess to create a plane surface of mercury at the top of the cell. If the cell is made of material that will amalgamate, grease the cell and the metal disk first with a thin layer of liquid paraffin. Pour out the mercury into a tared beaker and determine the mass (M_A) and the temperature of the mercury.

Make a compacted bed using the reference powder and again fill the cell with mercury with a planar surface at the top of the cell. Pour out the mercury in a tared beaker and again determine the mass of the mercury (M_B). Calculate the bulk volume (V) of the compacted bed of powder from the following expression:

$$\frac{M_A - M_B}{\rho_{Hg}} \qquad (3)$$

$M_A - M_B$ = difference between the determined masses of mercury in grams,

ρ_{Hg} = density of mercury at the determined temperature in grams per millilitre.

Repeat the procedure twice, changing the powder each time; the range of values for the calculated volume (V) is not greater than 0.01 mL. Use the mean value of the three determined volumes for the calculations.

Apparatus constant (K)

It is determined using a reference powder with known specific surface area and density as follows:

Calculate the required quantity of the reference powder to be used (expression (1)) using the stated density and the determined volume of the compacted powder bed (expression (3)).

Homogenise and loosen up the powder by shaking it for 2 min in a 100 mL bottle. Prepare a compacted powder bed and measure the flow time of air as previously described. Calculate the apparatus constant (K) from the following expression:

$$\frac{S_{sp} \times \rho \times (1 - \epsilon) \times \sqrt{\eta}}{\sqrt{\epsilon^3} \times \sqrt{t}} \qquad (4)$$

S_{sp} = stated specific surface area of the reference powder,
ρ = density of the substance to be examined in grams per millilitre,
ϵ = porosity of the compacted bed of powder,
t = flow time in seconds,
η = dynamic viscosity of air in millipascal seconds (see Table 2.9.14.-1).

The density of mercury and the viscosity of air over a range of temperatures are shown in Table 2.9.14.-1.

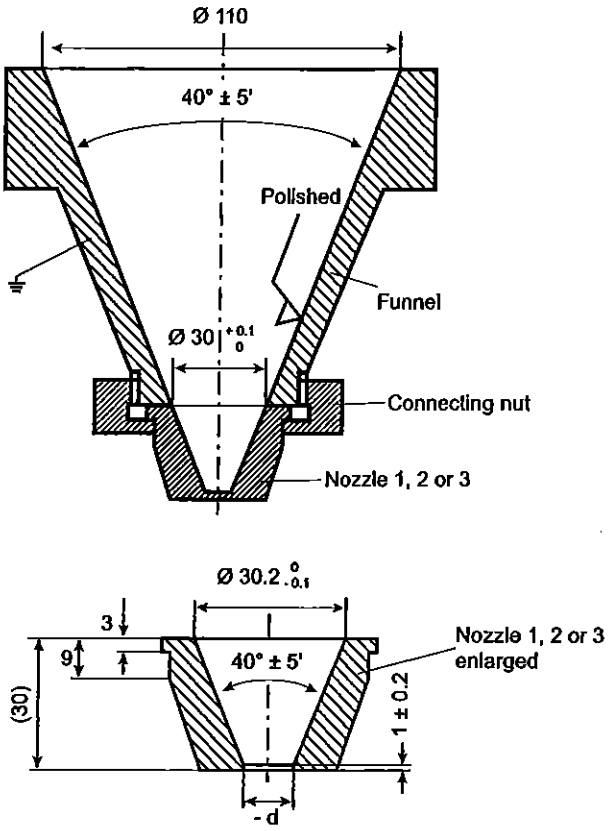
Table 2.9.14.-1.

Temperature (°C)	Density of mercury (g/mL)	Viscosity of air (η) (mPa·s)	$\sqrt{\eta}$
16	13.56	0.01800	0.1342
17	13.56	0.01805	0.1344
18	13.55	0.01810	0.1345
19	13.55	0.01815	0.1347
20	13.55	0.01819	0.1349
21	13.54	0.01824	0.1351
22	13.54	0.01829	0.1353
23	13.54	0.01834	0.1354
24	13.54	0.01839	0.1356

E. Flowability

(Ph. Eur. Method 2.9.16)

The test for flowability is intended to determine the ability of divided solids (for example, powders and granules) to flow vertically under defined conditions.



Nozzle	Diameter (d) of the outflow opening (millimetres)
1	10 ± 0.01
2	15 ± 0.01
3	25 ± 0.01

Figure 2.9.16.-1. – *Flow funnel and nozzle. Nozzle is made of stainless, acid-resistant steel (V4A,CrNi)*

Dimensions in millimetres

APPARATUS

According to the flow properties of the material to be tested, funnels with or without stem, with different angles and orifice diameters are used. Typical apparatuses are shown in Figures 2.9.16.-1 and 2.9.16.-2. The funnel is maintained upright by a suitable device. The assembly must be protected from vibrations.

METHOD

Into a dry funnel, whose bottom opening has been blocked by suitable means, introduce without compacting a test sample weighed with 0.5 per cent accuracy. The amount of the sample depends on the apparent volume and the apparatus used. Unblock the bottom opening of the funnel and measure the time needed for the entire sample to flow out of the funnel. Carry out three determinations.

EXPRESSION OF RESULTS

The flowability is expressed in seconds and tenths of seconds, related to 100 g of sample.

The results depend on the storage conditions of the material to be tested.

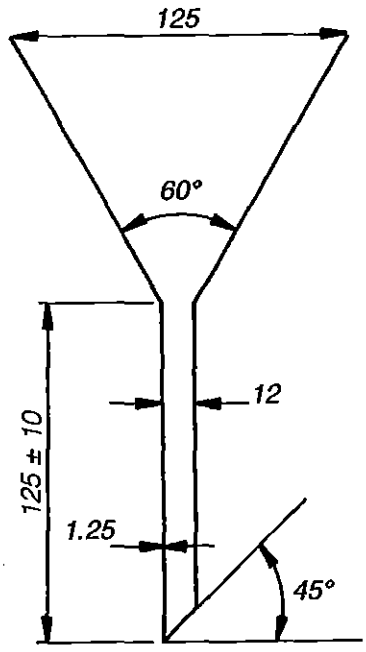


Figure 2.9.16.-2
Dimensions in millimetres

The results can be expressed as the following:

a) the mean of the determinations, if none of the individual values deviates from the mean value by more than 10 per cent;

b) as a range, if the individual values deviate from the mean value by more than 10 per cent;

c) as a plot of the mass against the flow time;

d) as an infinite time, if the entire sample fails to flow through.

F. Measurement of Consistency and Texture Analysis

1. Measurement of Consistency by Penetrometry

(Ph. Eur. method 2.9.9)

This test is intended to measure, under determined and validated conditions, the penetration of an object into the product to be examined in a container with a specified shape and size.

APPARATUS

The apparatus consists of a penetrometer made up of a stand and a penetrating object. A suitable apparatus is shown in Figure 2.9.9.-1.

A. Scale showing the depth of penetration, graduated in tenths of millimetres.

B. Vertical shaft to maintain and guide the penetrating object.

C. Device to retain and to release the penetrating object automatically and for a constant time.

D. Device to ensure that the penetrating object is vertical and that the base is horizontal.

E. Penetrating object (see Figures 2.9.9.-2 and 3).

F. Container.

G. Horizontal base.

H. Control for the horizontal base.

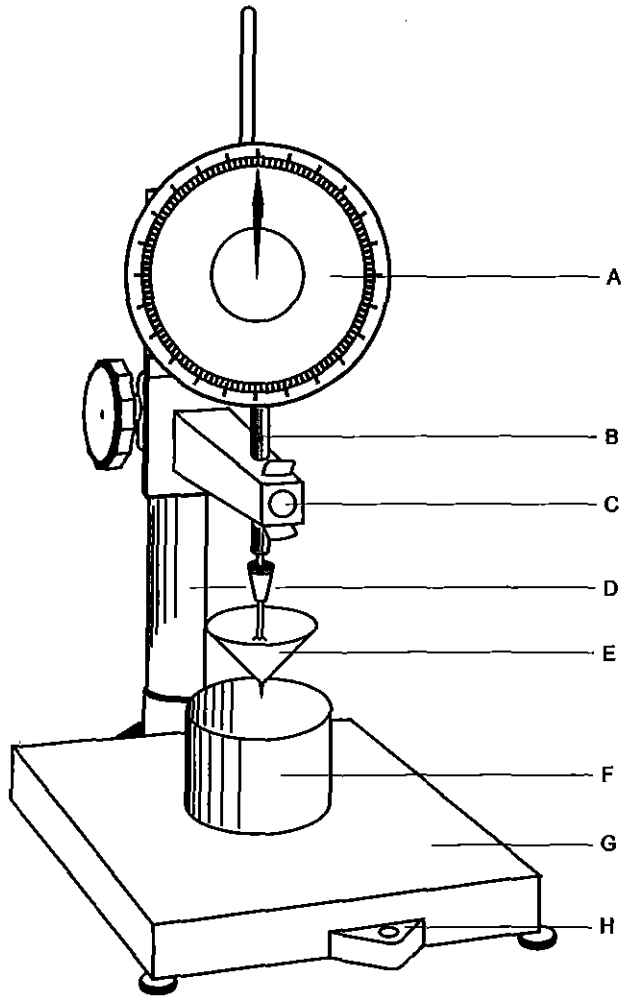


Figure 2.9.9.-1. – *Penetrometer*

The stand is made up of:
- a vertical shaft to maintain and guide the penetrating object;
- a horizontal base;
- a device to ensure that the penetrating object is vertical;
- a device to check that the base is horizontal;
- a device to retain and release the penetrating object;
- a scale showing the depth of penetration, graduated in tenths of a millimetre.

The penetrating object, made of a suitable material, has a smooth surface, and is characterised by its shape, size and mass (*m*).

Suitable penetrating objects are shown in Figures 2.9.9.-2 and 2.9.9.-3.

PROCEDURE

Prepare the test samples according to one of the following procedures.

A. Carefully and completely fill 3 containers, without forming air bubbles. Level if necessary to obtain a flat surface. Store the samples at 25 ± 0.5 °C for 24 h, unless otherwise prescribed.

B. Store 3 samples at 25 ± 0.5 °C for 24 h, unless otherwise prescribed. Apply a suitable shear to the samples for 5 min. Carefully and completely fill 3 containers, without forming air bubbles, and level if necessary to obtain a flat surface.

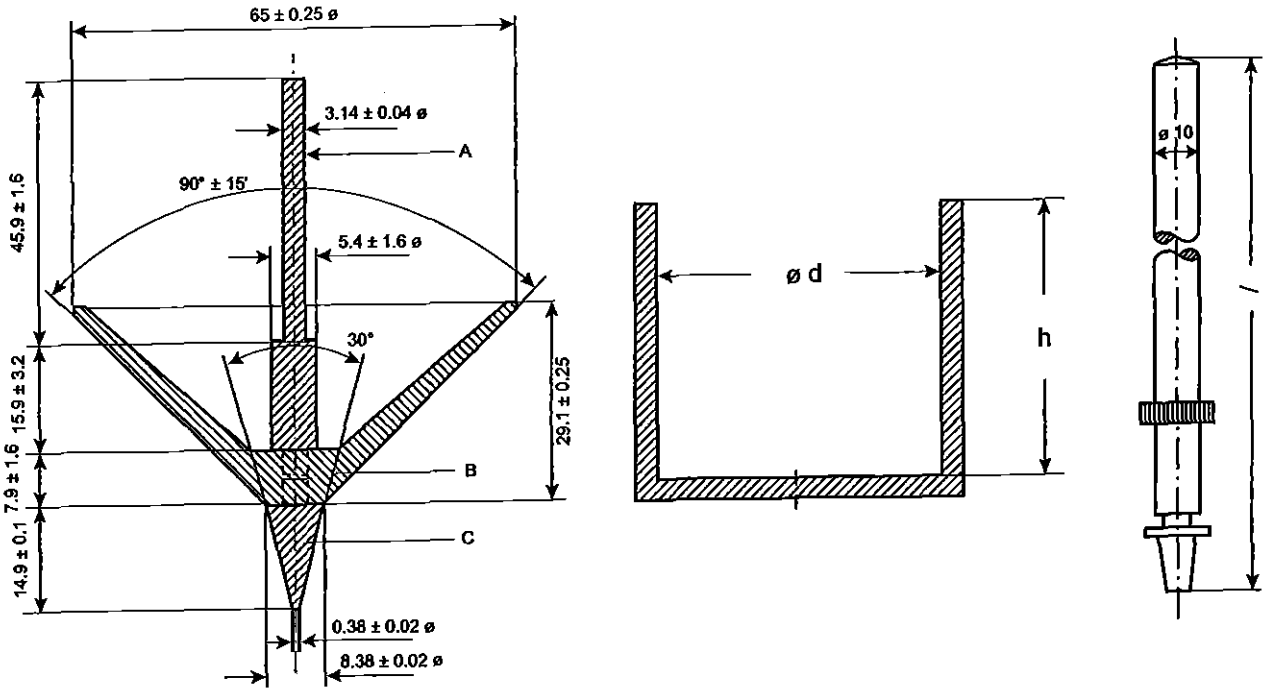


Figure 2.9.9.-2. – *Cone (m = 102.5 ± 0.05 g), suitable container (d = 102 mm or 75 mm; h ≥ 62 mm) and shaft (l = 162 mm; m = 47.5 ± 0.05 g).*
Dimensions in millimetres

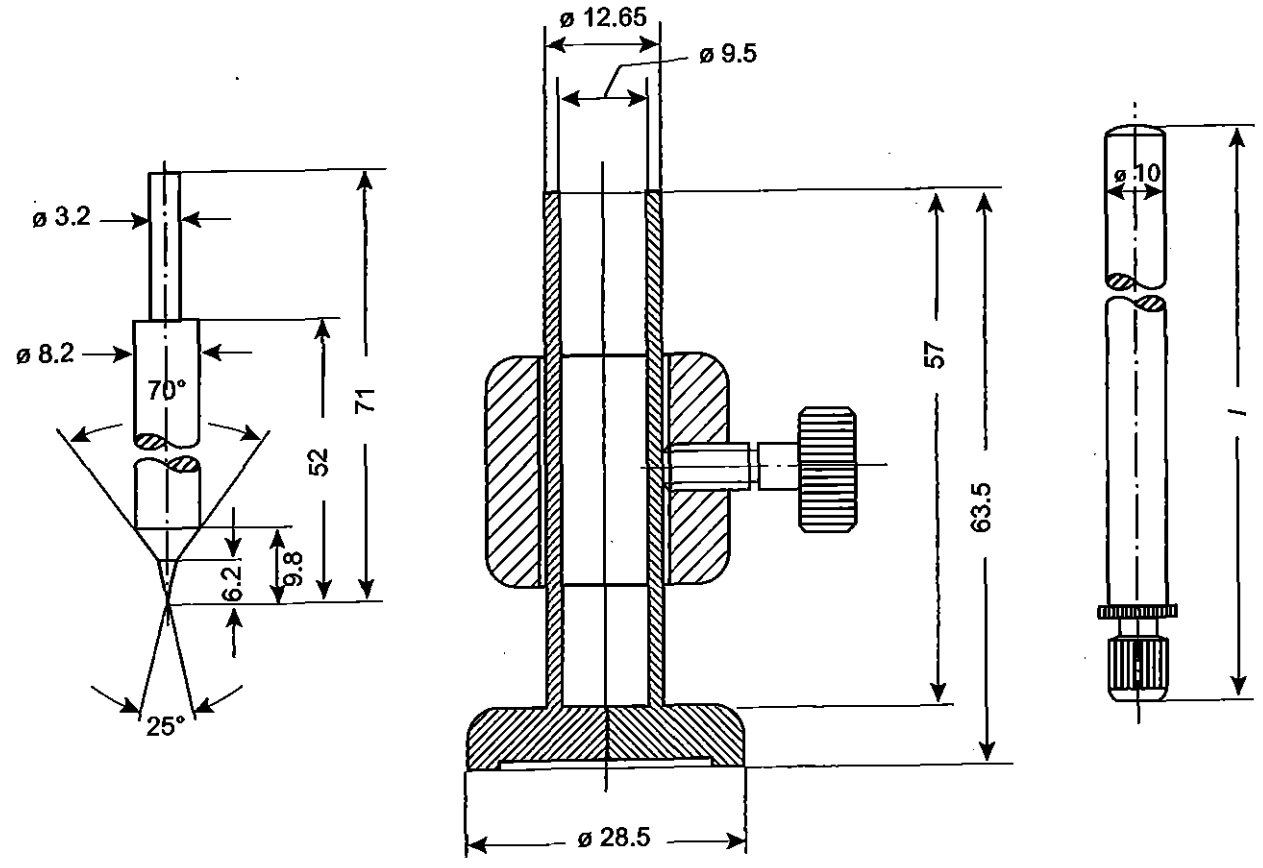


Figure 2.9.9.-3 – *Micro-cone (m = 7.0 g), suitable container and shaft (l = 116 mm; m = 16.8 g)*
Dimensions in millimetres

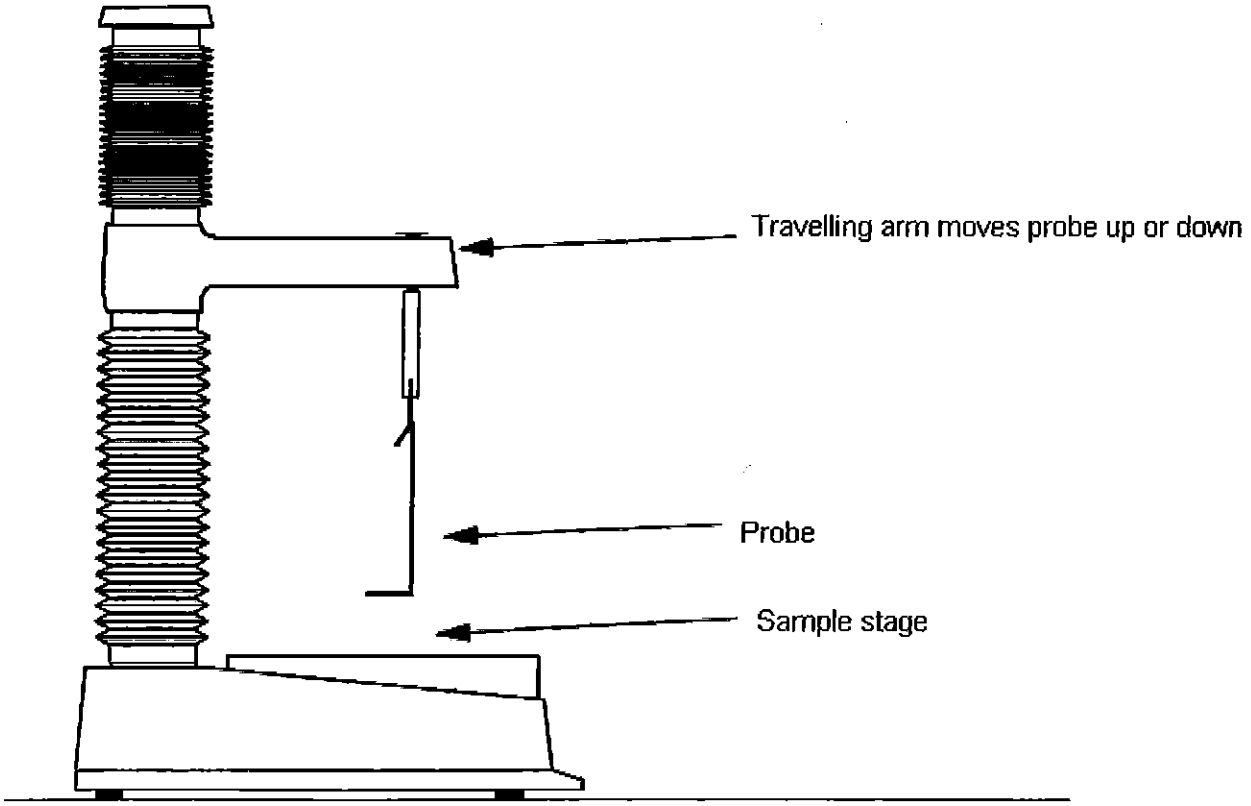


Fig. 17F-1 *Texture Analyser*

C. Melt 3 samples and carefully and completely fill 3 containers, without forming air bubbles. Store the samples at 25 ± 0.5 °C for 24 h, unless otherwise prescribed.

Determination of penetration

Place the test sample on the base of the penetrometer. Verify that its surface is perpendicular to the vertical axis of the penetrating object. Bring the temperature of the penetrating object to 25 ± 0.5 °C and then adjust its position such that its tip just touches the surface of the sample. Release the penetrating object and hold it free for 5 s. Clamp the penetrating object and measure the depth of penetration. Repeat the test with the 2 remaining containers.

EXPRESSION OF THE RESULTS

The penetration is expressed in tenths of a millimetre as the arithmetic mean of the 3 measurements. If any of the individual results differ from the mean by more than 3 per cent, repeat the test and express the results of the 6 measurements as the mean and the relative standard deviation.

2. Texture Analysis of Semi-solids or Gels

(No Ph. Eur. method)

This test determines, under defined conditions, the force required to penetrate a semi-solid or gel sample.

This test applies to samples consisting of a semi solid or gel-like mass which retains its form. It is not applicable to suspensions consisting of fine solid particles in a liquid.

Apparatus A suitable texture analyser, (see Fig. 17F-1) consisting of:

- a suitable platform or device to hold the sample being examined,
- a mobile arm that can be moved in a vertical direction towards or away from the sample,
- a probe attachment, which may be of various shapes such as a platen, a cylinder, a cone, a needle, a sphere or a wire,
- a load cell capable of measuring the load or tensile forces experienced by the probe as the mobile arm moves up or down.

Calibration The apparatus is calibrated using a suitable certified weight.

Method Check that the apparatus is vertical.

Place the sample being examined in a suitable holder as specified in the monograph. Programme the apparatus in accordance with the manufacturer's specifications to move the probe up or down at a defined speed or force as specified in the monograph. Measure the forces experienced by the probe through the sample.

The maximum peak force (in g) required to penetrate the sample is measured by the load cell.

Carry out each measurement six times.

G. Friability

1. Uncoated Tablets[1]

(Ph. Eur. method 2.9.7)

This chapter provides guidelines for the friability determination of compressed, uncoated tablets. The test procedure presented in this chapter is generally applicable to most compressed tablets. Measurement of tablet friability

[1] *This chapter has undergone pharmacopoeial harmonisation. See chapter 5.8 Pharmacopoeial harmonisation.*

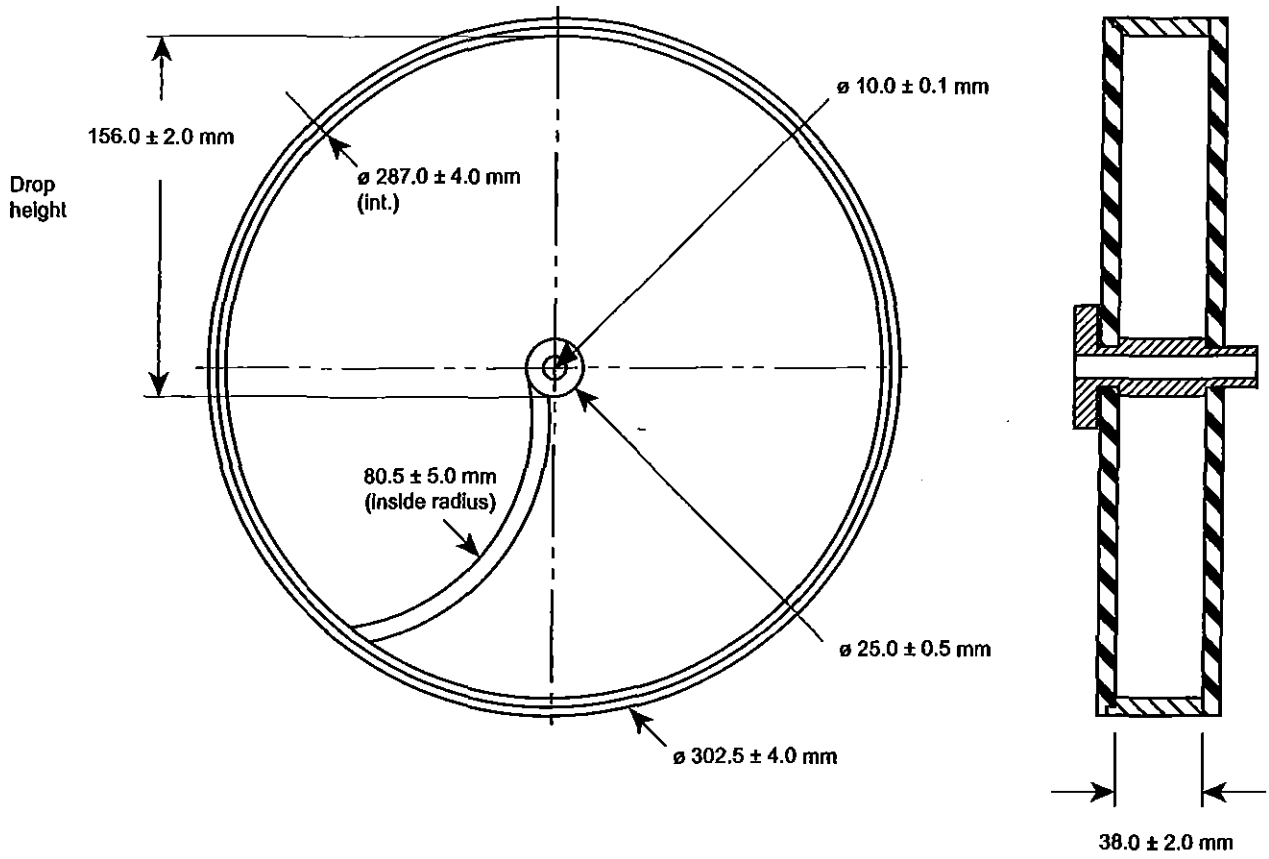


Figure 2.9.7.-1. – *Tablet friability apparatus*

supplements other physical strength measurements, such as tablet breaking force.

Use a drum, with an internal diameter between 283-291 mm and a depth between 36-40 mm, of transparent synthetic polymer with polished internal surfaces, and subject to minimum static build-up (see Figure 2.9.7.-1.). One side of the drum is removable. The tablets are tumbled at each turn of the drum by a curved projection with an inside radius between 75.5-85.5 mm that extends from the middle of the drum to the outer wall. The outer diameter of the central ring is between 24.5-25.5 mm. The drum is attached to the horizontal axis of a device that rotates at 25 ± 1 r/min. Thus, at each turn the tablets roll or slide and fall onto the drum wall or onto each other.

For tablets with a unit mass equal to or less than 650 mg, take a sample of whole tablets corresponding as near as possible to 6.5 g. For tablets with a unit mass of more than 650 mg, take a sample of 10 whole tablets. The tablets are carefully dedusted prior to testing. Accurately weigh the tablet sample, and place the tablets in the drum. Rotate the drum 100 times, and remove the tablets. Remove any loose dust from the tablets as before, and accurately weigh.

Generally, the test is run once. If obviously cracked, cleaved, or broken tablets are present in the tablet sample after tumbling, the sample fails the test. If the results are difficult to interpret or if the weight loss is greater than the targeted value, the test is repeated twice and the mean of the 3 tests determined. A maximum loss of mass (obtained from a single test or from the mean of 3 tests) not greater than 1.0 per cent is considered acceptable for most products.

If tablet size or shape causes irregular tumbling, adjust the drum base so that the base forms an angle of about 10° with the horizontal and the tablets no longer bind together when lying next to each other, which prevents them from falling freely.

Effervescent tablets and chewable tablets may have different specifications as far as friability is concerned. In the case of hygroscopic tablets, a humidity-controlled environment is required for testing.

A drum with dual scooping projections, or apparatus with more than one drum, for the running of multiple samples at one time, are also permitted.

2. Granules and Spheroids

(Ph. Eur. method 2.9.41)

This chapter describes 2 methods for determination of the friability of granules and spheroids, which may be used during development studies. It is recognised, however, that many methods with equal suitability may be used.

This test is intended to determine, under defined conditions, the friability of granules and spheroids. Friability is defined as a reduction in the mass of the granules or spheroids or in the formation of fragments of granules or spheroids, occurring when the granules or spheroids are subjected to mechanical strain during handling (tumbling, vibration, fluidisation, etc.). Examples of changes are abrasion, breakage or deformation of granules or spheroids.

METHOD A

Apparatus (fluidised-bed apparatus)

The apparatus (see Figure 2.9.41.-1) consists of a glass cylinder (A) with a conical lower part. The cylinder is provided with a sieve lid (B) having an aperture size of 500 µm or any other suitable sieve. The conical end is connected to a U-shaped glass tube (C) that can be disconnected from the cylinder for removal of the granules or spheroids. The U-tube is attached to a T-coupling (D).

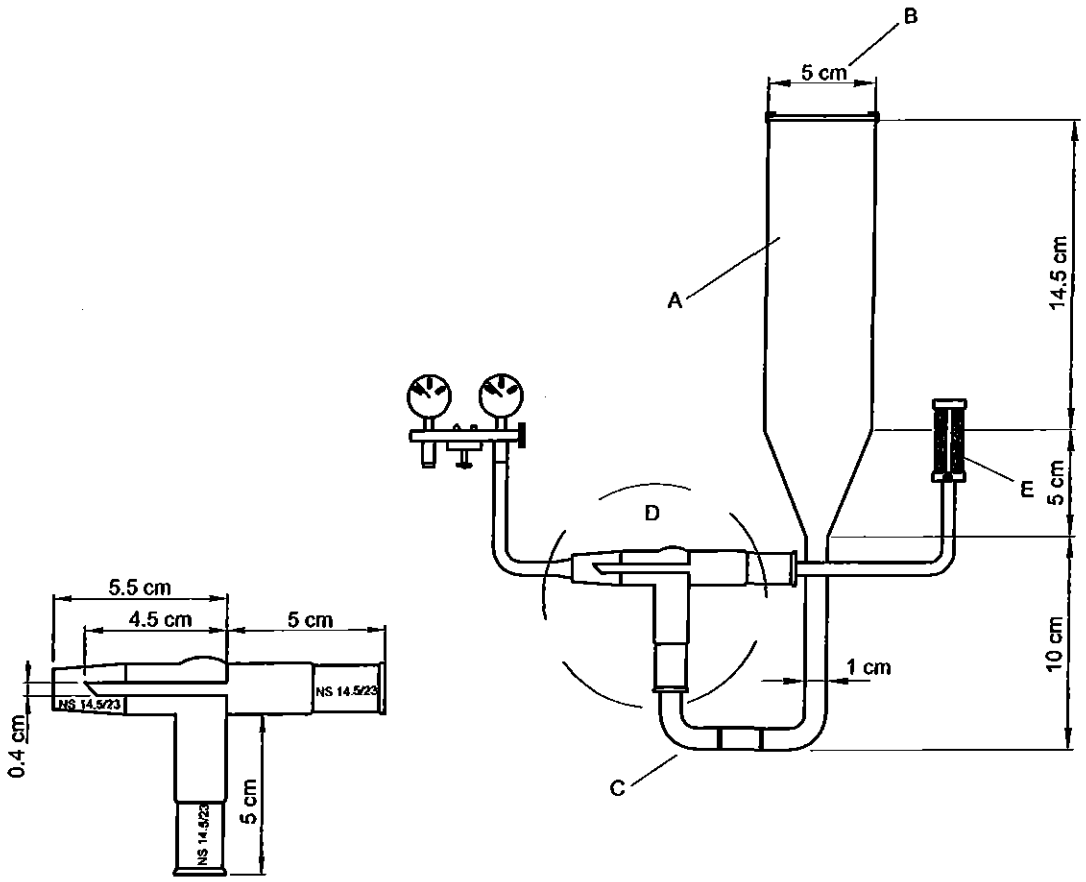


Figure 2.9.41.-1. – *Fluidised-bed apparatus*

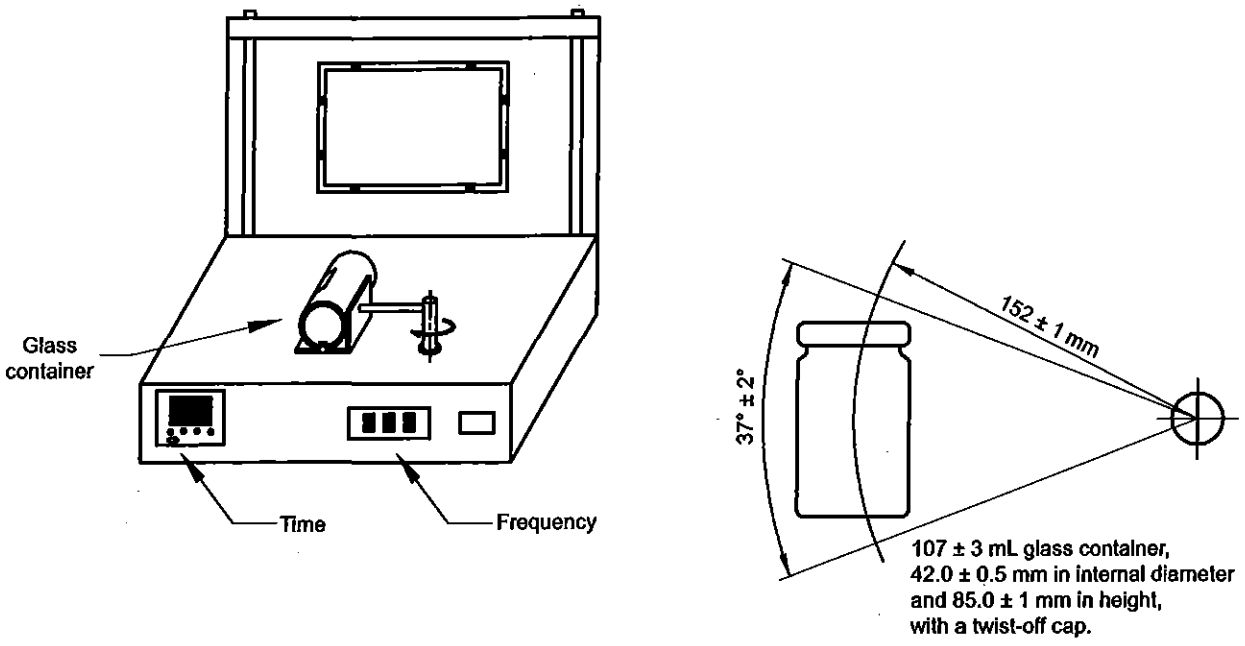


Figure 2.9.41.-2. – *Oscillating apparatus*

One inlet of the T-coupling is joined by a silicone tube to a manometer for regulating the compressed-air flow (use compressed air complying with the test for water in the monograph *Medicinal air (1238)*), the other one is connected via a silicone tube to a by-pass flowmeter (E) (0.10-1.00 $m^3 \cdot h^{-1}$).

Procedure

The following procedure is usually suitable Remove the fine particles by sieving (sieve having an aperture size of 710 µm or any other suitable sieve). Introduce about 8.0 g (m_1) of granules or spheroids into the cylinder (A). Close the apparatus with the sieve lid (B). Adjust the flow rate of the compressed air to 0.45 $m^3 \cdot h^{-1}$. After 15 min, remove the granules or spheroids from the apparatus by

disconnecting the U-tube and weigh again (m_2). Test 3 samples and calculate the mean value. It is recommended to spray the inside of the apparatus with an antistatic agent every 3 determinations in order to prevent electrostatic charging.

Loss on drying Dry in an oven at 105 °C, unless otherwise prescribed. Alternatively, other drying conditions as described in general chapter *2.2.32* may be used.

Calculation

$$F = \frac{m_1(100 - T_1) - m_2(100 - T_2)}{m_1} \times 100$$

F = friability;
T_1 = percentage loss on drying before the test (mean of 2 determinations);
T_2 = percentage loss on drying after the test (mean of 2 determinations);
m_1 = mass of the granules or spheroids before the test, in grams;
m_2 = mass of the granules or spheroids after the test, in grams.

METHOD B

Apparatus (oscillating apparatus)

The apparatus (see Figure 2.9.41.-2) consists of a glass container, containing the granules or spheroids to be examined, which is subjected to horizontal oscillations. The frequency and duration of the oscillations can be varied continuously. The frequency can be adjusted, using a scale, to a value in the range 0-400 oscillations/min. The duration can be set to a value in the range 0-9999 s.

Procedure

The following procedure is usually suitable Remove the fine particles by sieving (sieve having an aperture size of 355 µm or any other suitable sieve). In the glass container, weigh about 10.00 g (m_1) of the granules or spheroids. Install the container in the apparatus. Shake for 240 s at the highest frequency for hard granules or spheroids, or for 120 s at a lower frequency (e.g. 140 oscillations/min) for soft granules or spheroids. Sieve (355 µm, or the same sieve as used previously) and weigh the granules or spheroids again (m_2). Test 3 samples and calculate the mean value.

Loss on drying Dry in an oven at 105 °C, unless otherwise prescribed. Alternatively, other drying conditions as described in general chapter *2.2.32* may be used.

Calculation

$$F = \frac{m_1(100 - T_1) - m_2(100 - T_2)}{m_1} \times 100$$

F = friability;
T_1 = percentage loss on drying before the test (mean of 2 determinations);
T_2 = percentage loss on drying after the test (mean of 2 determinations);
m_1 = mass of the granules or spheroids before the test, in grams;
m_2 = mass of the granules or spheroids after the test, in grams.

H. Resistance to Crushing of Tablets

(Ph. Eur. method 2.9.8)

This test is intended to determine, under defined conditions, the resistance to crushing of tablets, measured by the force needed to disrupt them by crushing.

APPARATUS

The apparatus consists of 2 jaws facing each other, one of which moves towards the other. The flat surfaces of the jaws are perpendicular to the direction of movement. The crushing surfaces of the jaws are flat and larger than the zone of contact with the tablet. The apparatus is calibrated using a system with a precision of 1 newton.

OPERATING PROCEDURE

Place the tablet between the jaws, taking into account, where applicable, the shape, the break-mark and the inscription; for each measurement orient the tablet in the same way with respect to the direction of application of the force. Carry out the measurement on 10 tablets, taking care that all fragments of tablets have been removed before each determination.

This procedure does not apply when fully automated equipment is used.

EXPRESSION OF RESULTS

Express the results as the mean, minimum and maximum values of the forces measured, all expressed in newtons.

Indicate the type of apparatus and, where applicable, the orientation of the tablets.

J. Softening Time Determination of Lipophilic Suppositories

(Ph. Eur. method 2.9.22)

The test is intended to determine, under defined conditions, the time which elapses until a suppository maintained in water softens to the extent that it no longer offers resistance when a defined weight is applied.

APPARATUS A

The apparatus (see Figure 2.9.22.-1) consists of a glass tube 15.5 mm in internal diameter with a flat bottom and a length of about 140 mm. The tube is closed by a removable plastic cover having an opening 5.2 mm in diameter. The apparatus comprises a rod 5.0 mm in diameter which becomes wider towards the lower end, reaching a diameter of 12 mm. A metal needle 2 mm in length and 1 mm in diameter is fixed on the flat underside.

The rod consists of 2 parts, a lower part made of plastic material and an upper part made of plastic material or metal with a weight disk. The upper and lower parts are either fitted together (manual version) or separate (automated version). The weight of the entire rod is 30 ± 0.4 g. The upper part of the rod carries a sliding mark ring. When the rod is introduced into the glass tube so that it touches the bottom, the mark ring is adjusted to coincide with the upper level of the plastic cover.

Method

Place the glass tube containing 10 mL of water in a water-bath and equilibrate at 36.5 ± 0.5 °C. Fix the glass tube vertically and immerse to a depth of at least 7 cm below the surface but without touching the bottom of the water-bath. Introduce a suppository, tip first, into the tube followed by the rod with the free gliding plastic cover into the glass tube until the metal needle touches the flat end of the suppository. Put the cover on the tube (beginning of time measurement). Note the time which elapses until the rod sinks down to the bottom of the glass tube and the mark ring reaches the upper level of the plastic cover.

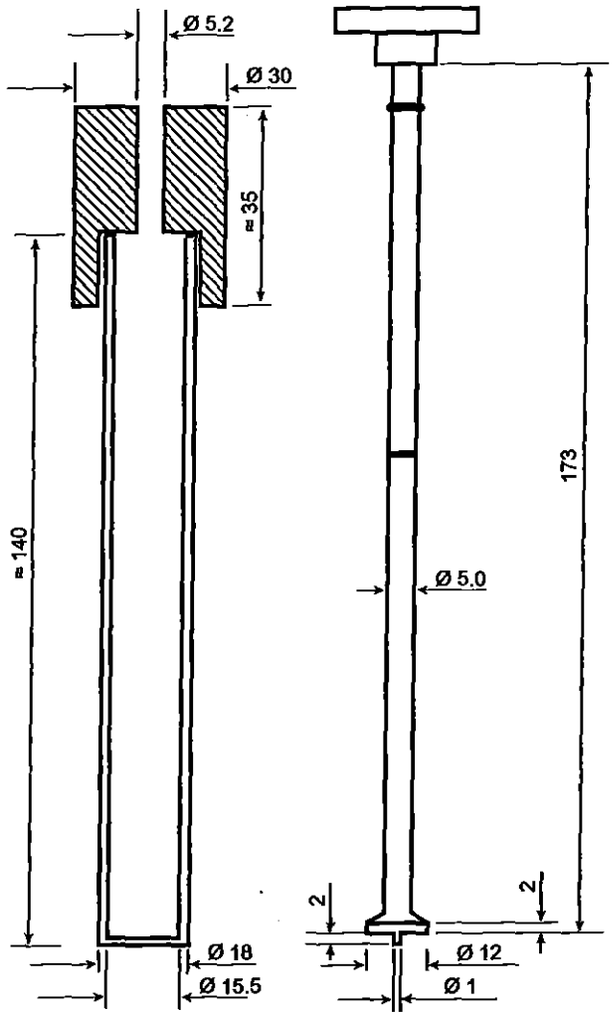


Figure 2.9.22.-1. – *Apparatus A for measuring the softening time of lipophilic suppositories*

Dimensions in millimetres

APPARATUS B

The apparatus (see Figure 2.9.22.-2) consists of a water-bath (B) into which an inner tube (A) is inserted and fixed with a stopper. The inner tube is closed by a stopper at the bottom. The apparatus is fitted with a thermometer. 2 insets are available:

— a glass rod (C1) in the form of a tube sealed at both ends, carrying a rim at its lower end weighed with lead shot, which has a weight of 30 ± 0.4 g,

— a penetration inset (C2) consisting of a rod (7.5 ± 0.1 g) in a tube which has an enlargement for the suppository, both made of stainless steel.

Method

Pour 5 mL of water at 36.5 ± 0.5 °C into the inner tube (A), introduce a suppository with the tip downwards and onto that, place the inset (C1 or C2). Note the time which elapses between this moment and the moment when the lower, rimmed end of the glass rod (C1) or the steel rod (C2) reaches the narrowed part of the inner glass tube. Melting or dissolution is then considered as complete.

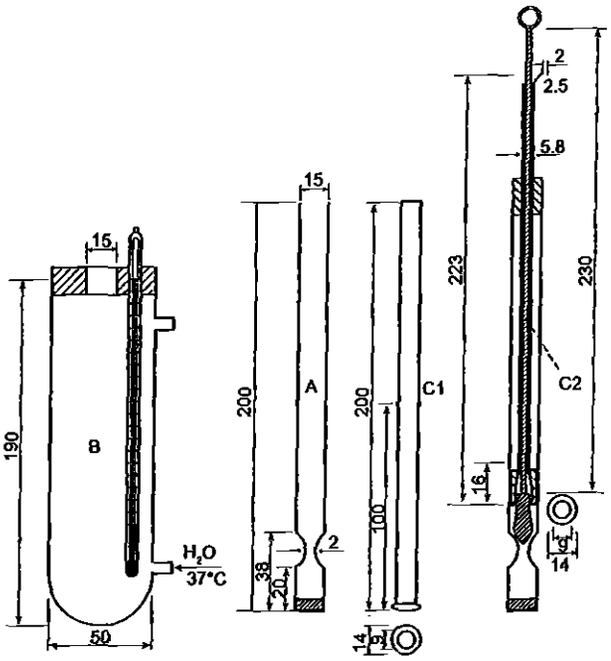


Figure 2.9.22.-2. – *Apparatus B for measuring the softening time of lipophilic suppositories*

Dimensions in millimetres

K. Pycnometric Density of Solids[1]

(Ph. Eur. method 2.9.23)

Gas pycnometric density is determined by measuring the volume occupied by a known mass of powder, which is equivalent to the volume of gas displaced by the powder using a gas displacement pycnometer. In gas pycnometric density measurements, the volume determined excludes the volume occupied by open pores; however, it includes the volume occupied by sealed pores or pores inaccessible to the gas.

Usually, helium is used as a test gas due to its high diffusivity into small open pores. If gases other than helium are used, different values would be obtained, since the penetration of the gas is dependent on the size of the pore as well as the cross-sectional area of the gas molecules.

The measured density is a volume-weighted average of the densities of individual powder particles. It is called the particle density, distinct from the true density of a solid or the bulk density of a powder. The density of solids is expressed in grams per cubic centimetre (g/cm^3), although the International Unit is the kilogram per cubic metre (1 g/cm^3 = 1000 kg/m^3).

APPARATUS

The apparatus (see Figure 2.9.23.-1) consists of the following:

— a sealed test cell, with empty cell volume V_c, connected through a valve to an expansion cell, with volume V_r;

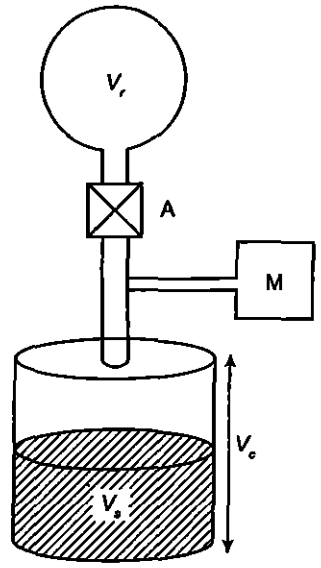
— a system capable of pressurising the test cell with the measurement gas until a defined pressure (P) indicated by a manometer;

[1] *This chapter has undergone pharmacopoeial harmonisation. See chapter 5.8. Pharmacopoeial harmonisation.*

— the system is connected to a source of measurement gas, preferably helium, unless another gas is specified.

The gas pycnometric density measurement is performed at a temperature between 15 °C and 30 °C that does not vary by more than 2 °C during the course of measurement.

The apparatus is calibrated, which means that the volumes V_c and V_r are determined using a suitable calibration standard whose volume is known to the nearest 0.001 cm^3. The procedure described below is followed in 2 runs, firstly with an empty test cell, and secondly with the calibration standard placed in the test cell. The volumes V_c and V_r are calculated using the equation for the sample volume (V_s), taking into account that V_s is zero in the first run.



A = valve;
V_r = expansion volume, in cubic centimetres;
V_c = cell volume, in cubic centimetres;
V_s = sample volume, in cubic centimetres;
M = manometer.

Figure 2.9.23.-1. – *Schematic diagram of a gas pycnometer*

METHOD

Volatile contaminants in the powder are removed by degassing the powder under a constant purge of helium prior to the measurement. Occasionally, powders may have to be degassed under vacuum. Because volatiles may be evolved during the measurement, weighing of the sample is carried out after the pycnometric measurement of volume.

Weigh the test cell of the pycnometer and record the mass. Fill the test cell with a given mass of powder of the substance to be examined. Seal the test cell in the pycnometer. Record the system reference pressure (P_r) as indicated by the manometer while the valve that connects the expansion cell with the test cell is open. Close the valve to separate the expansion cell from the test cell. Pressurise the test cell with the gas to an initial pressure (P_i) and record the value obtained. Open the valve to connect the expansion cell with the test cell. Record the final pressure (P_f). Repeat the measurement sequence for the same powder sample until consecutive measurements of the sample volume (V_s) agree to within 0.2 per cent. Unload the test cell and measure the final powder mass (m), expressed in grams. If the pycnometer differs in operation or construction from the one shown in Figure 2.9.23.-1, follow the instructions of the manufacturer of the pycnometer.

EXPRESSION OF THE RESULTS

The sample volume (V_s) is given by the equation:

$$V_s = V_c - \frac{V_r}{\frac{P_i - P_r}{P_f - P_r} - 1}$$

The density (ρ) is given by the equation:

$$\rho = \frac{m}{V_s}$$

The sample conditioning is indicated with the results. For example, indicate whether the sample was tested as is or dried under specific conditions such as those described for loss on drying.

M. Specific Surface Area by Gas Adsorption[1]

(Ph. Eur. method 2.9.26)

INTRODUCTION

The specific surface area of a powder is determined by physical adsorption of a gas on the surface of the solid and by calculating the amount of adsorbate gas corresponding to a monomolecular layer on the surface. Physical adsorption results from relatively weak forces (van der Waals forces) between the adsorbate gas molecules and the adsorbent surface of the test powder. The determination is usually carried out at the temperature of liquid nitrogen.

The amount of gas adsorbed can be measured by a volumetric or continuous flow procedure.

BRUNAUER, EMMETT AND TELLER (BET) THEORY AND SPECIFIC SURFACE AREA DETERMINATION

MULTI-POINT MEASUREMENT

The data are treated according to the Brunauer, Emmett and Teller (BET) adsorption isotherm equation:

$$\frac{1}{\left[V_a\left(\frac{P_o}{P} - 1\right)\right]} = \frac{C-1}{V_m C} \times \frac{P}{P_o} + \frac{1}{V_m C} \qquad (1)$$

P = partial vapour pressure of adsorbate gas in equilibrium with the surface at 77.4 K (b.p. of liquid nitrogen), in pascals,
P_o = saturated pressure of adsorbate gas, in pascals,
V_a = volume of gas adsorbed at standard temperature and pressure (STP) [273.15 K and atmospheric pressure (1.013×10^5 Pa)], in millilitres,
V_m = volume of gas adsorbed at STP to produce an apparent monolayer on the sample surface, in millilitres,
C = dimensionless constant that is related to the enthalpy of adsorption of the adsorbate gas on the powder sample.

A value of V_a is measured at each of not less than 3 values of P/P_o.

[1] *This chapter has undergone pharmacopoeial harmonisation. See chapter 5.8 Pharmacopoeial harmonisation.*

Then the BET value

$$\frac{1}{\left[V_a\left(\frac{P_o}{P}-1\right)\right]}$$

is plotted against P/P_o according to equation (1). This plot should yield a straight line usually in the approximate relative pressure range 0.05 to 0.3. The data are considered acceptable if the correlation coefficient, r, of the linear regression is not less than 0.9975; that is, r^2 is not less than 0.995. From the resulting linear plot, the slope, which is equal to $(C-1)/V_mC$, and the intercept, which is equal to $1/V_mC$, are evaluated by linear regression analysis. From these values, V_m is calculated as $1/(slope + intercept)$, while C is calculated as $(slope/intercept) + 1$. From the value of V_m so determined, the specific surface area, S, in $m^2 \cdot g^{-1}$, is calculated by the equation:

$$S = \frac{V_m N a}{m \times 22400} \quad (2)$$

N	=	Avogadro constant (6.022×10^{23} mol^{-1}),
a	=	effective cross-sectional area of one adsorbate molecule, in square metres (0.162 nm^2 for nitrogen and 0.195 nm^2 for krypton),
m	=	mass of test powder, in grams,
22400	=	volume occupied by 1 mole of the adsorbate gas at STP allowing for minor departures from the ideal, in millilitres.

A minimum of 3 data points is required. Additional measurements may be carried out, especially when non-linearity is obtained at a P/P_o value close to 0.3. Because non-linearity is often obtained at a P/P_o value below 0.05, values in this region are not recommended. The test for linearity, the treatment of the data, and the calculation of the specific surface area of the sample are described above.

SINGLE-POINT MEASUREMENT

Normally, at least 3 measurements of V_a each at different values of P/P_o are required for the determination of specific surface area by the dynamic flow gas adsorption technique (*Method I*) or by volumetric gas adsorption (*Method II*). However, under certain circumstances described below, it may be acceptable to determine the specific surface area of a powder from a single value of V_a measured at a single value of P/P_o such as 0.300 (corresponding to 0.300 mole of nitrogen or 0.001038 mole fraction of krypton), using the following equation for calculating V_m:

$$V_m = V_a\left(1-\frac{P}{P_o}\right) \quad (3)$$

The specific surface area is then calculated from the value of V_m by equation (2) given above.

The single-point method may be employed directly for a series of powder samples of a given material for which the material constant C is much greater than unity. These circumstances may be verified by comparing values of specific surface area determined by the single-point method with that determined by the multiple-point method for the series of powder samples. Close similarity between the single-point values and multiple-point values suggests that $1/C$ approaches zero.

The single-point method may be employed indirectly for a series of very similar powder samples of a given material for which the material constant C is not infinite but may be assumed to be invariant. Under these circumstances, the error associated with the single-point method can be reduced or eliminated by using the multiple-point method to evaluate C for one of the samples of the series from the BET plot, from which C is calculated as $(1 + slope/intercept)$. Then V_m is calculated from the single value of V_a measured at a single value of P/P_o by the equation:

$$V_m = V_a\left(\frac{P_o}{P}-1\right)\left[\frac{1}{C}+\frac{C-1}{C}\times\left(\frac{P}{P_o}\right)\right] \quad (4)$$

The specific surface area is calculated from V_m by equation (2) given above.

EXPERIMENTAL TECHNIQUES

This section describes the methods to be used for the sample preparation, the dynamic flow gas adsorption technique (*Method I*) and the volumetric gas adsorption technique (*Method II*).

SAMPLE PREPARATION

Outgassing

Before the specific surface area of the sample can be determined, it is necessary to remove gases and vapours that may have become physically adsorbed onto the surface after manufacture and during treatment, handling and storage. If outgassing is not achieved, the specific surface area may be reduced or may be variable because an intermediate area of the surface is covered with molecules of the previously adsorbed gases or vapours. The outgassing conditions are critical for obtaining the required precision and accuracy of specific surface area measurements on pharmaceuticals because of the sensitivity of the surface of the materials.

Conditions The outgassing conditions must be demonstrated to yield reproducible BET plots, a constant weight of test powder, and no detectable physical or chemical changes in the test powder.

The outgassing conditions defined by the temperature, pressure and time should be chosen so that the original surface of the solid is reproduced as closely as possible. Outgassing of many substances is often achieved by applying a vacuum, by purging the sample in a flowing stream of a non-reactive, dry gas, or by applying a desorption-adsorption cycling method. In either case, elevated temperatures are sometimes applied to increase the rate at which the contaminants leave the surface. Caution should be exercised when outgassing powder samples using elevated temperatures to avoid affecting the nature of the surface and the integrity of the sample.

If heating is employed, the recommended temperature and time of outgassing are as low as possible to achieve reproducible measurement of specific surface area in an acceptable time. For outgassing sensitive samples, other outgassing methods such as the desorption-adsorption cycling method may be employed.

Adsorbate

The standard technique is the adsorption of nitrogen of analytical quality at liquid nitrogen temperature.

For powders of low specific surface area (< 0.2 m$^2 \cdot$g^{-1}) the proportion adsorbed is low. In such cases the use of krypton at liquid nitrogen temperature is preferred because the low vapour pressure exerted by this gas greatly reduces error. The use of larger sample quantities where feasible (equivalent to 1 m^2 or greater total surface area using nitrogen) may compensate for the errors in determining low surface areas.

All gases used must be free from moisture.

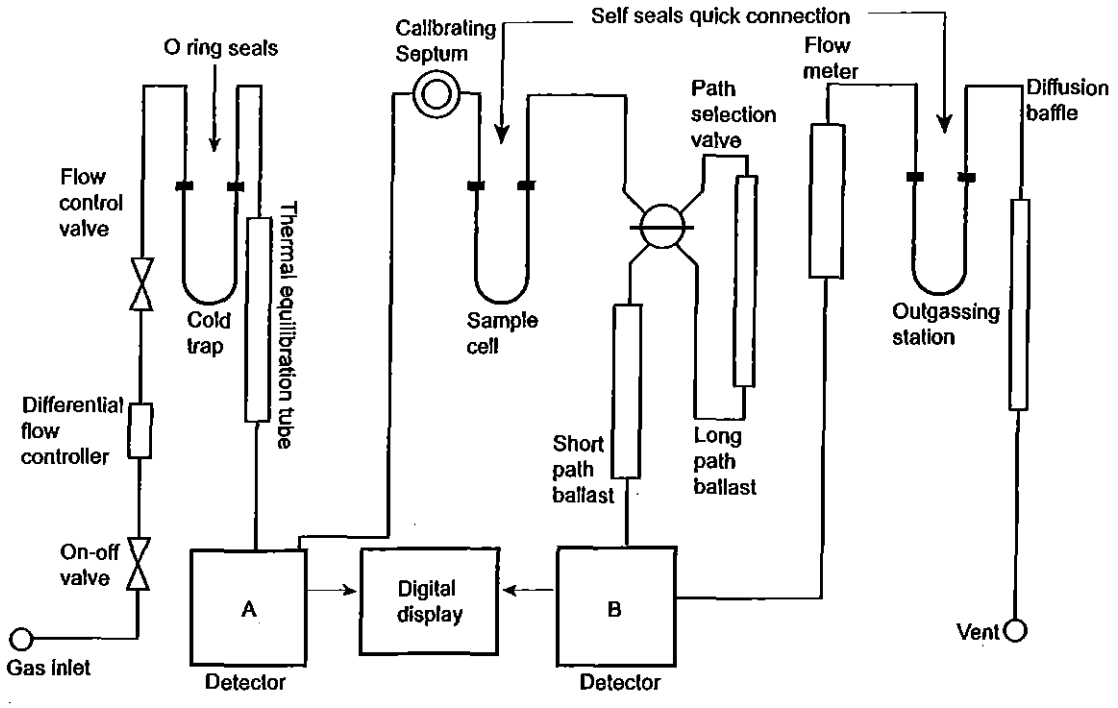


Figure 2.9.26.-1. – *Schematic diagram of the dynamic flow method apparatus*

Quantity of sample

Accurately weigh a quantity of the test powder such that the total surface of the sample is at least 1 m^2 when the adsorbate is nitrogen and 0.5 m^2 when the adsorbate is krypton.

Lower quantities of sample may be used after appropriate validation.

MEASUREMENTS

Because the amount of gas adsorbed under a given pressure tends to increase on decreasing the temperature, adsorption measurements are usually made at a low temperature. Measurement is performed at 77.4 K, the boiling point of liquid nitrogen.

Method I: the dynamic flow method

Principle

In the dynamic flow method (see Figure 2.9.26.-1), the recommended adsorbate gas is dry nitrogen or krypton, while helium is employed as a diluent gas, which is not adsorbed under the recommended conditions.

A minimum of 3 mixtures of the appropriate adsorbate gas with helium are required within the P/P_o range 0.05 to 0.30.

The gas detector-integrator should provide a signal that is approximately proportional to the volume of the gas passing through it under defined conditions of temperature and pressure. For this purpose, a thermal conductivity detector with an electronic integrator is one among various suitable types. A minimum of 3 data points within the recommended range of 0.05 to 0.30 for P/P_o is to be determined.

Procedure

A known mixture of the gases, usually nitrogen and helium, is passed through a thermal conductivity cell, through the sample, again through the thermal conductivity cell and then to a recording potentiometer.

Immerse the sample cell in liquid nitrogen, then the sample adsorbs nitrogen from the mobile phase. This unbalances the thermal conductivity cell, and a pulse is generated on a recorder chart.

Remove from the coolant; this gives a desorption peak equal in area and in the opposite direction to the adsorption peak. Since this is better defined than the adsorption peak, it is the one used for the determination.

To effect the calibration, inject a known quantity of adsorbate into the system, sufficient to give a peak of similar magnitude to the desorption peak and obtain the proportion of gas volume per unit peak area.

Use a nitrogen/helium mixture for a single-point determination and several such mixtures or premixing 2 streams of gas for a multiple-point determination.

Calculation is essentially the same as for the volumetric method.

Method II: the volumetric method

Principle

In the volumetric method (see Figure 2.9.26.-2), the recommended adsorbate gas is nitrogen which is admitted into the evacuated space above the previously outgassed powder sample to give a defined equilibrium pressure, P, of the gas. The use of a diluent gas, such as helium, is therefore unnecessary, although helium may be employed for other purposes, such as to measure the dead volume.

Since only pure adsorbate gas, instead of a gas mixture, is employed, interfering effects of thermal diffusion are avoided in this method.

Procedure

Admit a small amount of dry nitrogen into the sample tube to prevent contamination of the clean surface, remove the sample tube, insert the stopper, and weigh it. Calculate the weight of the sample. Attach the sample tube to the volumetric apparatus. Cautiously evacuate the sample down to the specified pressure (e.g. between 2 Pa and 10 Pa). Alternatively, some instruments operate by evacuating to a defined rate of pressure change (e.g. less than 13 Pa/30 s) and holding for a defined period of time before commencing the next step.

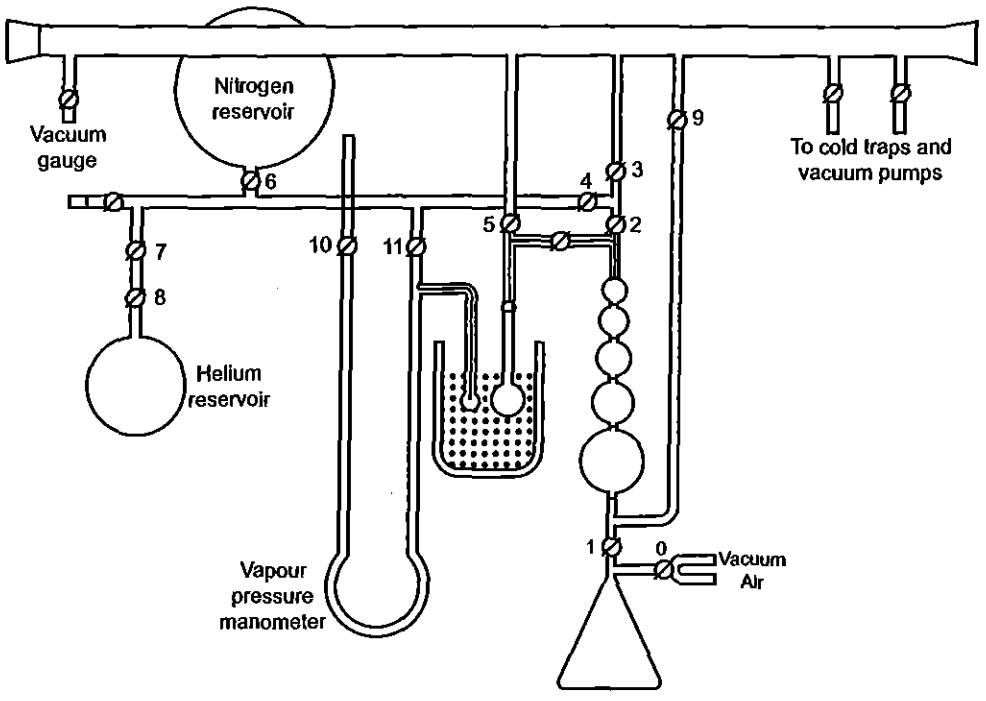


Figure 2.9.26.-2. – *Schematic diagram of the volumetric method apparatus*

If the principle of operation of the instrument requires the determination of the dead volume in the sample tube, for example, by the admission of a non-adsorbed gas, such as helium, this procedure is carried out at this point, followed by evacuation of the sample. The determination of dead volume may be avoided using difference measurements, that is, by means of reference and sample tubes connected by a differential transducer. The adsorption of nitrogen gas is then measured as described below.

Raise a Dewar vessel containing liquid nitrogen at 77.4 K up to a defined point on the sample cell. Admit a sufficient volume of adsorbate gas to give the lowest desired relative pressure. Measure the volume adsorbed, V_a. For multipoint measurements, repeat the measurement of V_a at successively higher P/P_o values. When nitrogen is used as the adsorbate gas, P/P_o values of 0.10, 0.20, and 0.30 are often suitable.

REFERENCE MATERIALS

Periodically verify the functioning of the apparatus using appropriate reference materials of known surface area, such as α-alumina, which should have a specific surface area similar to that of the sample to be examined.

N1. Powder Flow[1]

(Ph. Eur. method 2.9.36)

The widespread use of powders in the pharmaceutical industry has generated a variety of methods for characterising powder flow. Not surprisingly, scores of references appear in the pharmaceutical literature, attempting to correlate the various measures of powder flow to manufacturing properties. The development of such a variety of test methods was inevitable; powder behavior is multifaceted and thus complicates the effort to characterise powder flow.

The purpose of this chapter is to review the methods for characterising powder flow that have appeared most frequently in the pharmaceutical literature. In addition, while it is clear that no single and simple test method can adequately characterise the flow properties of pharmaceutical powders, this chapter proposes the standardisation of test methods that may be valuable during pharmaceutical development.

4 commonly reported methods for testing powder flow are:
— angle of repose,
— compressibility index or Hausner ratio,
— flow rate through an orifice,
— shear cell.

In addition, numerous variations of each of these basic methods are available. Given the number of test methods and variations, standardising the test methodology, where possible, would be advantageous.

With this goal in mind, the most frequently used methods are discussed below. Important experimental considerations are identified and recommendations are made regarding standardisation of the methods. In general, any method of measuring powder flow must be practical, useful, reproducible and sensitive, and must yield meaningful results. It bears repeating that no simple powder flow method will adequately or completely characterise the wide range of flow properties experienced in the pharmaceutical industry. An appropriate strategy may well be the use of multiple standardised test methods to characterise the various aspects of powder flow as needed by the pharmaceutical scientist.

ANGLE OF REPOSE

The angle of repose has been used in several branches of science to characterise the flow properties of solids. Angle of repose is a characteristic related to interparticulate friction, or resistance to movement between particles. Angle of repose test results are reported to be very dependent upon the method used. Experimental difficulties arise due to segregation of material and consolidation or aeration of the powder as the cone is formed. Despite its difficulties, the

[1] *This chapter has undergone pharmacopoeial harmonisation. See chapter 5.8 Pharmacopoeial harmonisation.*

method continues to be used in the pharmaceutical industry, and a number of examples demonstrating its value in predicting manufacturing problems appear in the literature.

The angle of repose is the constant, three-dimensional angle (relative to the horizontal base) assumed by a cone-like pile of material formed by any of several different methods, described briefly below.

Basic methods for angle of repose

A variety of angle of repose test methods are described in the literature. The most common methods for determining the static angle of repose can be classified based on 2 important experimental variables:

— the height of the 'funnel' through which the powder passes may be fixed relative to the base, or the height may be varied as the pile forms;
— the base upon which the pile forms may be of fixed diameter or the diameter of the powder cone may be allowed to vary as the pile forms.

Variations in angle of repose methods

Variations of the above methods have also been used to some extent in the pharmaceutical literature:

— *drained angle of repose*: this is determined by allowing an excess quantity of material positioned above a fixed diameter base to 'drain' from the container. Formation of a cone of powder on the fixed diameter base allows determination of the drained angle of repose;
— *dynamic angle of repose*: this is determined by filling a cylinder (with a clear, flat cover on one end) and rotating it at a specified speed. The dynamic angle of repose is the angle (relative to the horizontal) formed by the flowing powder. The internal angle of kinetic friction is defined by the plane separating those particles sliding down the top layer of the powder and those particles that are rotating with the drum (with roughened surface).

General scale of flowability for angle of repose

While there is some variation in the qualitative description of powder flow using the angle of repose, much of the pharmaceutical literature appears to be consistent with the classification by Carr[2], which is shown in Table 2.9.36.-1. There are examples in the literature of formulations with an angle of repose in the range of 40-50 degrees that manufactured satisfactorily. When the angle of repose exceeds 50 degrees, the flow is rarely acceptable for manufacturing purposes.

Table 2.9.36.-1. – *Flow properties and corresponding angles of repose*[2]

Flow property	Angle of repose (degrees)
Excellent	25-30
Good	31-35
Fair (aid not needed)	36-40
Passable (may hang up)	41-45
Poor (must agitate, vibrate)	46-55
Very poor	56-65
Very, very poor	> 66

Experimental considerations for angle of repose

Angle of repose is not an intrinsic property of the powder, that is to say, it is very much dependent upon the method used to form the cone of powder. On this subject, the existing literature raises these important considerations:

— the peak of the cone of powder can be distorted by the impact of powder from above. By carefully building the powder cone, the distortion caused by impact can be minimised;
— the nature of the base upon which the powder cone is formed influences the angle of repose. It is recommended that the powder cone be formed on a 'common base', which can be achieved by forming the cone of powder on a layer of powder. This can be done by using a base of fixed diameter with a protruding outer edge to retain a layer of powder upon which the cone is formed.

Recommended procedure for angle of repose

Form the angle of repose on a fixed base with a retaining lip to retain a layer of powder on the base. The base must be free of vibration. Vary the height of the funnel to carefully build up a symmetrical cone of powder. Care must be taken to prevent vibration as the funnel is moved. The funnel height is maintained at approximately 2-4 cm from the top of the powder pile as it is being formed in order to minimise the impact of falling powder on the tip of the cone. If a symmetrical cone of powder cannot be successfully or reproducibly prepared, this method is not appropriate. Determine the angle of repose by measuring the height of the cone of powder and calculating the angle of repose, α, from the following equation:

$$\tan(\alpha) = \frac{\text{height}}{0.5 \times \text{base}}$$

COMPRESSIBILITY INDEX AND HAUSNER RATIO

In recent years the compressibility index and the closely related Hausner ratio have become the simple, fast, and popular methods of predicting powder flow characteristics. The compressibility index has been proposed as an indirect measure of bulk density, size and shape, surface area, moisture content, and cohesiveness of materials, because all of these can influence the observed compressibility index. The compressibility index and the Hausner ratio are determined by measuring both the bulk volume and tapped volume of a powder.

Basic methods for compressibility index and Hausner ratio

While there are some variations in the method of determining the compressibility index and Hausner ratio, the basic procedure is to measure the unsettled apparent volume, (V_0), and the final tapped volume, (V_f), of the powder after tapping the material until no further volume changes occur. The compressibility index and the Hausner ratio are calculated as follows:

$$\textit{Compressibility Index} = 100 \times \frac{V_0 - V_f}{V_0}$$

$$\textit{Hausner Ratio} = \frac{V_0}{V_f}$$

Alternatively, the compressibility index and Hausner ratio may be calculated using measured values of bulk density (ρ_{bulk}) and tapped density (ρ_{tapped}) as follows:

$$\textit{Compressibility Index} = 100 \times \frac{\rho_{tapped} - \rho_{bulk}}{\rho_{tapped}}$$

$$\textit{Hausner Ratio} = \frac{\rho_{tapped}}{\rho_{bulk}}$$

[2] *Carr RL. Evaluating flow properties of solids. Chem. Eng 1965; 72:163-168.*

In a variation of these methods, the rate of consolidation is sometimes measured rather than, or in addition to, the change in volume that occurs on tapping. For the compressibility index and the Hausner ratio, the generally accepted scale of flowability is given in Table 2.9.36.-2.

Table 2.9.36.-2. – *Scale of flowability*[3]

Compressibility index (per cent)	Flow character	Hausner ratio
1-10	Excellent	1.00-1.11
11-15	Good	1.12-1.18
16-20	Fair	1.19-1.25
21-25	Passable	1.26-1.34
26-31	Poor	1.35-1.45
32-37	Very poor	1.46-1.59
> 38	Very, very poor	> 1.60

Experimental considerations for the compressibility index and Hausner ratio

Compressibility index and Hausner ratio are not intrinsic properties of the powder, that is to say, they are dependent upon the methodology used. The existing literature points out several important considerations affecting the determination of the unsettled apparent volume, V_0, of the final tapped volume, V_f, of the bulk density, ρ_{bulk}, and of the tapped density, ρ_{tapped}:

- the diameter of the cylinder used,
- the number of times the powder is tapped to achieve the tapped density,
- the mass of material used in the test,
- rotation of the sample during tapping.

Recommended procedure for compressibility index and Hausner ratio

Use a 250 mL volumetric cylinder with a test sample mass of 100 g. Smaller amounts and volumes may be used, but variations in the method must be described with the results. An average of 3 determinations is recommended.

FLOW THROUGH AN ORIFICE

The flow rate of a material depends upon many factors, some of which are particle-related and some related to the process. Monitoring the rate of flow of material through an orifice has been proposed as a better measure of powder flowability. Of particular significance is the utility of monitoring flow continuously, since pulsating flow patterns have been observed even for free-flowing materials. Changes in flow rate as the container empties can also be observed. Empirical equations relating flow rate to the diameter of the opening, particle size, and particle density have been determined. However, determining the flow rate through an orifice is useful only with free-flowing materials.

The flow rate through an orifice is generally measured as the mass per time flowing from any of a number of types of containers (cylinders, funnels, hoppers). Measurement of the flow rate can be in discrete increments or continuous.

Basic methods for flow through an orifice

There are a variety of methods described in the literature. The most common for determining the flow rate through an orifice can be classified based on 3 important experimental variables:

- the type of container used to contain the powder. Common containers are cylinders, funnels, and hoppers from production equipment;
- the size and shape of the orifice used. The orifice diameter and shape are critical factors in determining powder flow rate;
- the method of measuring powder flow rate. Flow rate can be measured continuously using an electronic balance with some sort of recording device (strip chart recorder, computer). It can also be measured in discrete samples (for example, the time it takes for 100 g of powder to pass through the orifice to the nearest tenth of a second or the amount of powder passing through the orifice in 10 s to the nearest tenth of a gram).

Variations in methods for flow through an orifice

Either mass flow rate or volume flow rate can be determined. Mass flow rate is the easier of the methods, but it biases the results in favour of high-density materials. Since die fill is volumetric, determining volume flow rate may be preferable. A vibrator is occasionally attached to facilitate flow from the container, however, this appears to complicate interpretation of results. A moving orifice device has been proposed to more closely simulate rotary press conditions. The minimum diameter orifice through which powder flows can also be identified.

General scale of flowability for flow through an orifice

No general scale is available because flow rate is critically dependent on the method used to measure it. Comparison between published results is difficult.

Experimental considerations for flow through an orifice

Flow rate through an orifice is not an intrinsic property of the powder. It is very much dependent upon the methodology used. The existing literature points out several important considerations affecting these methods:

- the diameter and shape of the orifice,
- the type of container material (metal, glass, plastic),
- the diameter and height of the powder bed.

Recommended procedure for flow through an orifice

Flow rate through an orifice can be used only for materials that have some capacity to flow. It is not useful for cohesive materials. Provided that the height of the powder bed (the 'head' of powder) is much greater than the diameter of the orifice, the flow rate is virtually independent of the powder head. It is advisable to use a cylinder as the container, because the walls of the container must have little effect on flow. This configuration results in flow rate being determined by the movement of powder over powder, rather than powder along the wall of the container. Powder flow rate often increases when the height of the powder column is less than twice the diameter of the column. The orifice must be circular and the cylinder must be free of vibration. General guidelines for dimensions of the cylinder are as follows:

- diameter of the opening greater than 6 times the diameter of the particles,
- diameter of the cylinder greater than twice the diameter of the opening.

Use of a hopper as the container may be appropriate and representative of flow in a production situation. It is not advisable to use a funnel, particularly one with a stem, because flow rate will be determined by the size and length of the stem as well as the friction between the stem and the powder. A truncated cone may be appropriate, but flow will be influenced by the powder-wall friction coefficient, thus, selection of an appropriate construction material is important.

For the opening in the cylinder, use a flat-faced bottom plate with the option to vary orifice diameter to provide maximum

[3] *Carr RL. Evaluating flow properties of solids. Chem. Eng 1965; 72:163-168.*

flexibility and better ensure a powder-over-powder flow pattern. Rate measurement can be either discrete or continuous. Continuous measurement using an electronic balance can more effectively detect momentary flow rate variations.

SHEAR CELL METHODS

In an effort to put powder flow studies and hopper design on a more fundamental basis, a variety of powder shear testers and methods that permit more thorough and precisely defined assessment of powder flow properties have been developed. Shear cell methodology has been used extensively in the study of pharmaceutical materials. From these methods, a wide variety of parameters can be obtained, including the yield loci representing the shear stress-shear strain relationship, the angle of internal friction, the unconfined yield strength, the tensile strength, and a variety of derived parameters such as the flow factor and other flowability indices. Because of the ability to control experimental parameters more precisely, flow properties can also be determined as a function of consolidation load, time, and other environmental conditions. These methods have been successfully used to determine critical hopper and bin parameters.

Basic methods for shear cell

One type of shear cell is the cylindrical shear cell which is split horizontally, forming a shear plane between the lower stationary base and the upper moveable portion of the shear cell ring. After powder bed consolidation in the shear cell (using a well-defined procedure), the force necessary to shear the powder bed by moving the upper ring is determined. Annular shear cell designs offer some advantages over the cylindrical shear cell design, including the need for less material. A disadvantage, however, is that because of its design, the powder bed is not sheared as uniformly because material on the outside of the annulus is sheared more than material in the inner region. A third type of shear cell (plate-type) consists of a thin sandwich of powder between a lower stationary rough surface and an upper rough surface that is moveable.

All of the shear cell methods have their advantages and disadvantages, but a detailed review is beyond the scope of this chapter. As with the other methods for characterising powder flow, many variations are described in the literature. A significant advantage of shear cell methodology in general is a greater degree of experimental control. The methodology generally is rather time-consuming and requires significant amounts of material and a well-trained operator.

Recommendations for shear cell

The many existing shear cell configurations and test methods provide a wealth of data and can be used very effectively to characterise powder flow. They are also helpful in the design of equipment such as hoppers and bins. Because of the diversity of available equipment and experimental procedures, no specific recommendations regarding methodology are presented in this chapter. It is recommended that the results of powder flow characterisation using shear cell methodology include a complete description of equipment and methodology used.

N2. Powder Flow Properties by Shear Cell Methods

(Ph. Eur. method 2.9.49)

The methodology in this general chapter is based on standard test methods ASTM D6773-08 and ASTM D6128-14.

Powder flow properties play an important role in the design of formulations, processes and pharmaceutical production equipment, and an approach commonly used to characterise these properties is to submit the powder to shear tests, which are experiments designed to determine the flow properties of the powder by applying different states of stress and strain to it. Using these tests, a wide variety of parameters describing the flow properties of a powder can be studied, including the yield locus, the angle of internal friction, the compressive strength, the tensile strength, and a variety of derived parameters such as the flowability ratio. Because of the ability to control experimental parameters precisely, flow properties can also be determined as a function of consolidation load, time, and environmental conditions such as temperature and humidity.

Many suitable shear cell configurations and test methods are available; the most widely used are those based on the Jenike-type shear cell or on ring shear cells such as the Schulze-type ring shear tester.

PRINCIPLE

UNIAXIAL COMPRESSION TEST

The uniaxial compression test illustrates the concept of flowability. As shown in Figure 2.9.49.-1, a hollow cylinder (cross-sectional area A, internal wall assumed as frictionless) is filled with a powder. The powder is loaded by the consolidation stress (σ_1) in the vertical direction. The more the volume of the powder can be reduced, the more compressible the powder is. In addition to the increase in bulk density (ρ_b) from the consolidation stress, an increase in the strength of the powder is also observed. Hence, the powder is both consolidated and compressed through the effect of the consolidation stress.

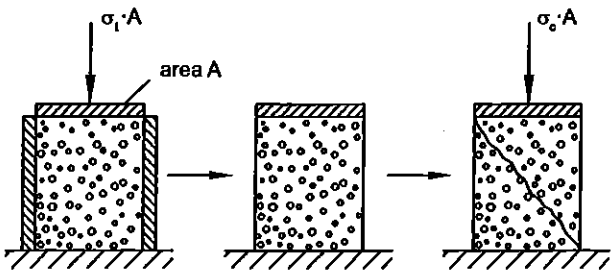


Figure 2.9.49.-1. – *Uniaxial compression test*

After consolidation, the powder is relieved of the consolidation stress (σ_1) and the hollow cylinder is removed. The consolidated cylindrical powder sample is subsequently loaded with an increasing vertical compressive stress and, at a certain stress, the sample fails along the shear plane and the powder starts to flow. The compressive strength (σ_c), or unconfined yield strength, is defined as the stress causing failure. Since the powder fails only at a sufficiently large vertical stress, there is a specific yield limit for the powder.

The yield limit of a powder is dependent on its stress history, i.e. its previous consolidation. The greater the consolidation stress (σ_1) the greater the bulk density (ρ_b) and compressive strength (σ_c). Thus, uniaxial compression tests conducted at different consolidation stresses (σ_1) yield different pairs of values (σ_1, σ_c) and (σ_1, ρ_b). Plotting these pairs of values as points in a (σ_1, σ_c) diagram and a (σ_1, ρ_b) diagram, respectively, and drawing in each diagram a curve through these points, usually results in curves similar to those shown for product A in Figure 2.9.49.-2, where bulk density (ρ_b) and compressive strength (σ_c) typically increase with consolidation stress (σ_1). Very rarely a progressive slope similar to that of the left part of curve B is observed. The graph of σ_c versus σ_1 is called the flow function.

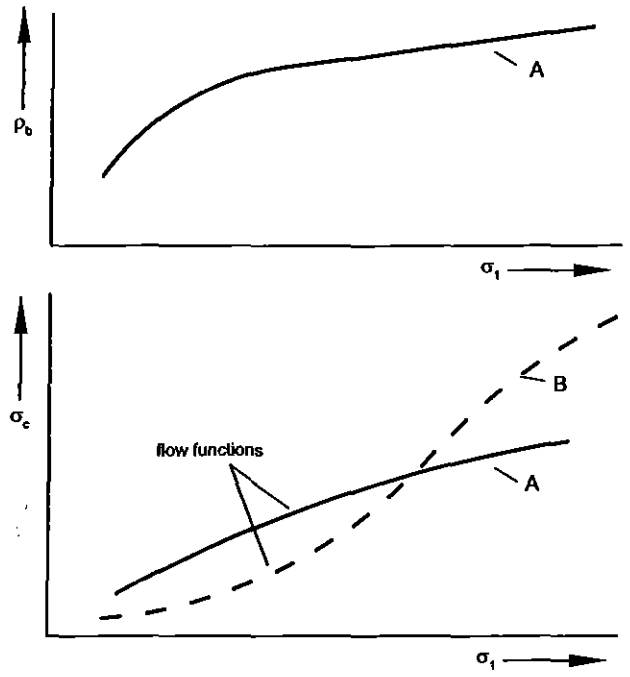


Figure 2.9.49.-2. – *Bulk density (ρ_b) and compressive strength (σ_c) as a function of consolidation stress (σ_1)*

NUMERICAL CHARACTERISATION OF FLOWABILITY

The flow function can be used to characterise the flow behaviour of a powder in terms of the flowability ratio (ff_c), which is given by:

$$ff_c = \sigma_1 / \sigma_c$$

The larger the ff_c, the better a powder flows. The ff_c value can be used to classify the flow behaviour as follows:

$ff_c < 1$	not flowing
$1 < ff_c < 2$	very cohesive
$2 < ff_c < 4$	cohesive
$4 < ff_c < 10$	easy-flowing
$10 < ff_c$	free-flowing

Figure 2.9.49.-3 shows the flow function A taken from the (σ_1, σ_c) diagram in Figure 2.9.49.-2. Additionally, the boundaries of the ranges of the classifications listed above are shown as straight lines, each representing a constant value of the flowability ratio (ff_c). This diagram clearly shows that the flowability ratio (ff_c) of a specific powder is dependent on the consolidation stress (σ_1) applied.

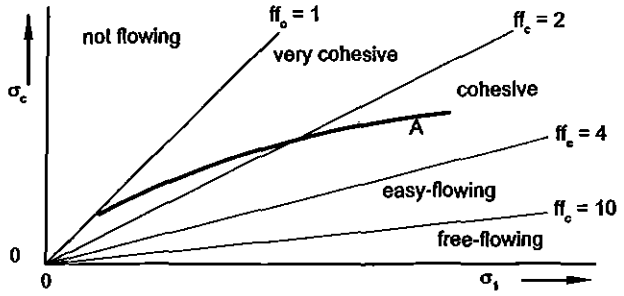


Figure 2.9.49.-3. – *Flow function and lines of constant flowability ratio*

Furthermore, the flow function may change depending on the consolidation time, for example, at the same σ_1 level, higher σ_c values may be obtained with longer consolidation times; this is known as the time consolidation effect. Possible mechanisms are solid or liquid bridges, solid crystallisation, viscoelastic or viscoplastic deformation, or chemical reactions at the particle contacts.

YIELD LIMIT AND MOHR STRESS CIRCLE

Assuming that the force of gravity and wall friction effects can be disregarded, the uniaxial compression test can be represented as shown in Figure 2.9.49.-4, in a (σ, τ) diagram where σ is the normal stress and τ is the shear stress. In such a diagram, all pairs of values (σ, τ) form a circle representing the stresses in the powder; this is called a 'Mohr stress circle'. The Mohr stress circle is centred on the sigma axis. The two intersect points with the sigma axis are called the minor and major principal stresses. Figure 2.9.49.-4 shows the Mohr stress circles corresponding to the uniaxial compression test and a possible yield limit of the sample (the real course of the yield limit cannot be determined with only the uniaxial compression test).

Mohr stress circle A describes the stresses in the powder sample at consolidation. Since no shear stress is applied, σ_1 corresponds to the normal stress or vertical stress (σ_v) and σ_2 corresponds to the horizontal stress (σ_h).

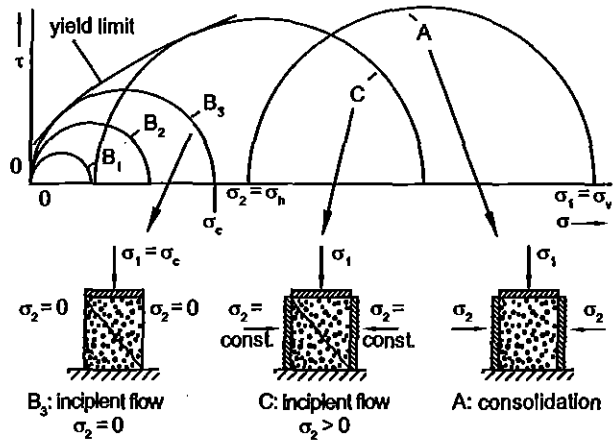


Figure 2.9.49.-4. – *Measurement of compressive strength and corresponding (σ, τ) diagram*

In the second part of the test shown in Figure 2.9.49.-1, the sample is loaded with increasing vertical stress after it has been relieved of the consolidation stress and the hollow cylinder has been removed. As the vertical load increases, the stress states at different load steps are represented by stress circles with increasing diameter (stress circles B_1, B_2, B_3 in Figure 2.9.49.-4). The minor principal stress, i.e. the horizontal stress, is equal to zero for all stress circles since the lateral surface of the sample is uncovered and not loaded.

Mohr stress circle B_3 represents the stresses in the powder sample at failure of the sample. Since the load corresponding to this Mohr stress circle causes incipient flow of the sample, Mohr stress circle B_3 must be tangential to the yield limit in the (σ, τ) diagram.

If, during the second part of the experiment shown in Figure 2.9.49.-1 (i.e. the measurement of compressive strength), a constant horizontal stress $\sigma_h > 0$ were also applied to the sample in addition to the vertical stress (σ_v), stress circles that indicate failure of the sample and are tangential to the yield limit (e.g. stress circle C in Figure 2.9.49.-4) would likewise be found. Thus the yield limit is

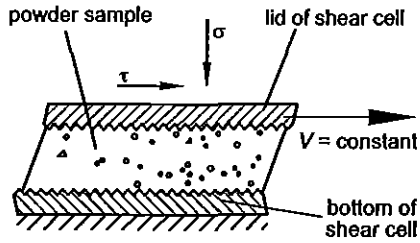


Figure 2.9.49.-5. – *Principle of shear deformation in a shear cell*

the envelope of all stress circles that indicate failure of a powder sample.

MEASUREMENT WITH A SHEAR TESTER

PRINCIPLE

The test sample must be representative of the powder with respect to particle-size distribution, moisture, temperature and other properties that may have an influence on the flow behaviour. The test sample is filled into a shear cell of circular or annular cross-section, depending on the type of tester used. In order to achieve a homogenous and representative powder bed, filling should be carried out uniformly in small horizontal layers using a spoon or spatula, without applying force to the surface of the material, until the cell is slightly overfilled. Excess powder is then removed by scraping off with a blade until the powder is flush with the top of the shear cell. The shear cell is then completed with a lid placed on top of the sample. The latter step may vary with the type of apparatus and is therefore carried out according to the manufacturer's instructions to achieve a homogenous and representative powder bed.

In general, a shear test measures the yield limit of a consolidated powder bed. This yield limit is also called the yield locus. It depends to a certain extent on the apparatus and experimental conditions. Typically the yield locus is measured immediately after consolidation of the powder bed. If however the yield locus is measured after a certain consolidation time, then it is called the time yield locus. When running a shear test, a normal stress (σ) is applied vertically to the powder in the shear cell. A shear deformation of the sample is then induced by moving the lid of the shear cell relative to the bottom of the shear cell in a horizontal direction with constant velocity (V) (see Figure 2.9.49.-5). This results in a horizontal shear stress (τ), which develops due to the friction between the particles in the shearing plane. The lid of the shear cell is allowed to move vertically in order to adjust to changes in the sample's bulk density.

When a point of a yield locus is measured, as in the uniaxial compression test, 2 steps are necessary: first the powder sample is consolidated in a preshear step, and then a point of the yield locus is measured in a shear or shear-to-failure step.

PRESHEAR STEP

For preshear, the powder sample is loaded in the vertical direction under a well-defined normal stress ($\sigma = \sigma_{pre}$), and is then sheared. Preshear is stopped when steady-state flow, characterised by constant shear stress (τ_{pre}), is achieved.

The pair of values of normal stress and shear stress at steady-state flow (σ_{pre}, τ_{pre}) is plotted in a normal stress - shear stress diagram ((σ, τ) diagram, Figure 2.9.49.-6, right). Point (σ_{pre}, τ_{pre}) is called the preshear point.

SHEAR-TO-FAILURE STEP

After the powder has been consolidated by the preshear procedure, the shear stress (τ) is reduced to zero by reversing the relative motion of the lid with respect to the bottom of the shear cell.

For the next step of the test procedure, the so-called shear or shear-to-failure step, the normal stress acting on the sample is decreased to a value σ_{sh}, which is less than the normal stress at preshear (σ_{pre}).

If the consolidated sample is sheared under the normal stress $\sigma_{sh} < \sigma_{pre}$, it will start to flow when a sufficiently large shear stress (τ_{sh}) is attained. At that point particles start to move against each other. The material will start to dilate, i.e. decrease in bulk density and shear resistance, and thus shear stress will decrease. The maximum shear stress (τ_{sh}) characterises incipient flow. The corresponding pair of values (σ_{sh}, τ_{sh}) is a point of the yield locus in the (σ, τ) diagram (Figure 2.9.49.-6, right). Such a point is called a shear point or a point of incipient flow.

In order to measure the course of the yield locus, several of the tests described above must be performed, where the samples must first be consolidated at the same normal stress (σ_{pre}, preshear). Then the samples are sheared to failure under different normal stresses $\sigma_{sh} < \sigma_{pre}$. The yield locus follows a curve plotted through all measured shear points (Figure 2.9.49.-6, right). In general, at least 3 shear points should be measured.

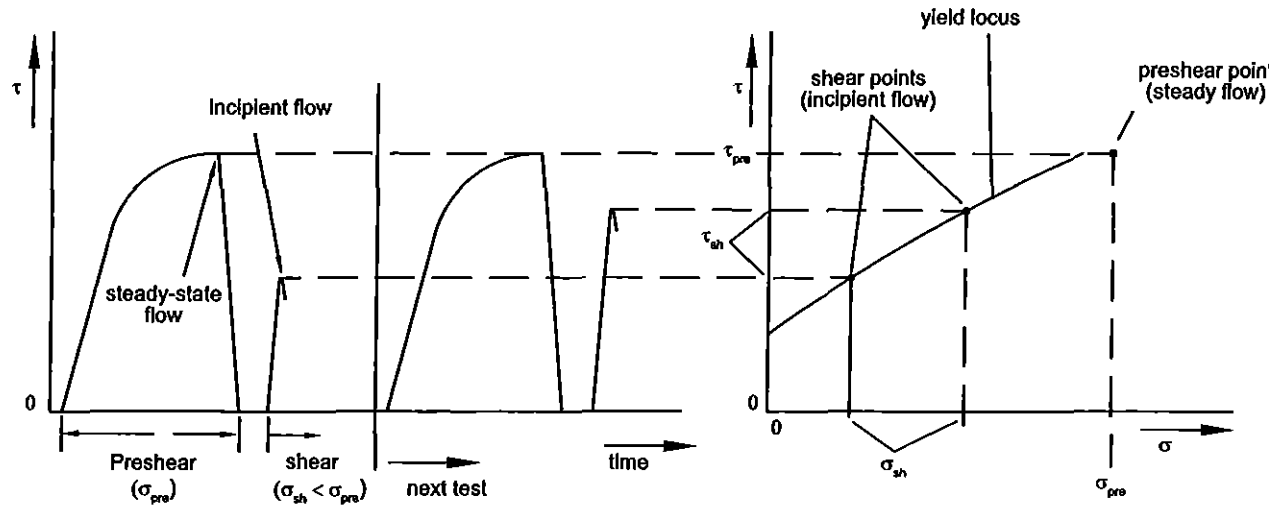


Figure 2.9.49.-6. – *Plot of shear stress versus time (left) and corresponding yield locus (right)*

With the Jenike-type shear cell a new sample has to be prepared for each shear point (indicated as 'next test' in Figure 2.9.49.-6). However, with ring shear testers a complete yield locus is usually measured with 1 sample. In addition, with the Schulze-type ring shear tester the bulk density is measured during the test, depending on the consolidation stress applied.

DATA EVALUATION

Parameters that describe the flow properties can be determined from the yield locus as shown in Figure 2.9.49.-7.

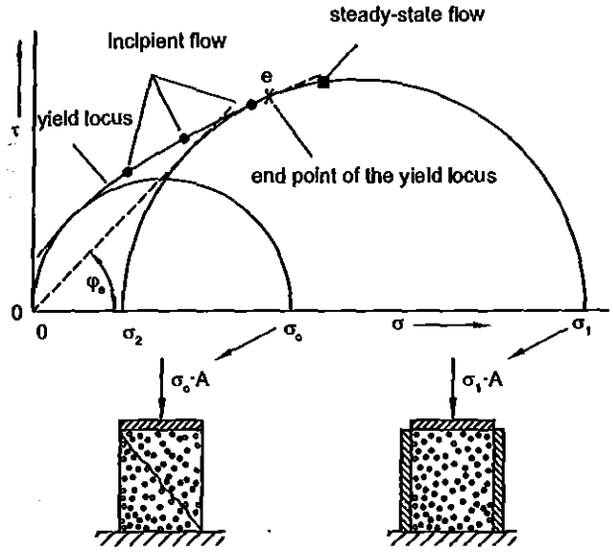


Figure 2.9.49.-7. – *Yield locus, Mohr stress circles and analogy with uniaxial compression test*

The consolidation stress (σ_1) is equal to the major principal stress of the Mohr stress circle, which is tangential to the yield locus, and intersects at the point of steady-state flow (σ_{pre}, τ_{pre}). This stress circle represents the stress distribution in the sample at the end of the consolidation procedure (stresses at steady-state flow). It corresponds to the stress circle at the end of consolidation in the uniaxial compression test.

The compressive strength (σ_c) is determined from the stress circle that is tangential to the yield locus and runs through the origin (minor principal stress $\sigma_2 = 0$). This stress circle represents a similar stress state to the one that prevails in the second step of the uniaxial compression test.

A straight line through the origin of the (σ, τ) diagram, tangent to the greater Mohr circle, is the effective yield locus, shown as a broken line in Figure 2.9.49.-7. It encloses the σ-axis with the effective angle of internal friction (φ_e). Because the largest Mohr stress circle indicates a state of steady-state flow, the angle φ_e can be regarded as a measure of the internal friction at steady-state flow.

Further flow properties can be determined from the yield locus as illustrated in Figure 2.9.49.-8.

For many applications the yield locus is linearised as the tangent to both Mohr circles. The linearised yield locus is characterised by its slope angle φ_{lin}.

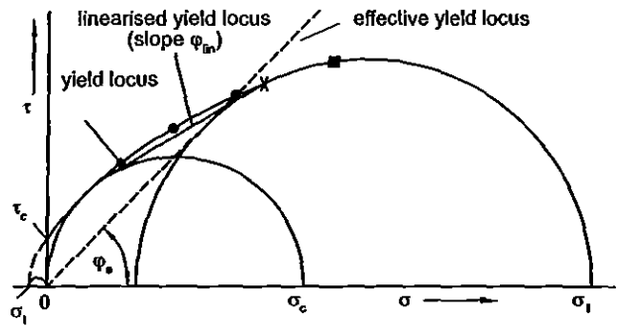


Figure 2.9.49.-8. – *Yield locus and characteristic values for flow properties*

The cohesion (τ_c) is the value of the shear stress where the yield locus intersects with the τ-axis, i.e. at normal stress $\sigma = 0$. The uniaxial tensile strength (σ_t) is the normal stress at the left end of the yield locus at shear stress $\tau = 0$. As it is difficult to measure the yield locus at small and negative stress levels, cohesion may be obtained by extrapolating the yield locus to the intersection with the τ-axis. Due to the pronounced non-linearity of the yield locus at low stresses, the cohesion obtained in this way is less accurate than the compressive strength, and it is hardly possible to determine tensile strength by extrapolation.

If several yield loci are measured at different stress levels, i.e. with different normal stresses at preshear (σ_{pre}), each yield locus represents another state of consolidation and another bulk density. The above-mentioned flow properties, namely compressive strength, effective angle of internal friction or slope angle of the linearised yield locus, can be indicated as functions of the consolidation stress (σ_1), similar to Figure 2.9.49.-2 where bulk density and compressive strength are plotted versus consolidation stress.

01. Optical Microscopy[1]

(Ph. Eur. method 2.9.37)

Optical microscopy for particle characterisation can generally be applied to particles of 1 µm and greater. The lower limit is imposed by the resolving power of the microscope. The upper limit is less definite and is determined by the increased difficulty associated with the characterisation of larger particles. Various alternative techniques are available for particle characterisation outside the applicable range of optical microscopy. Optical microscopy is particularly useful for characterising particles that are not spherical. This method may also serve as a base for the calibration of faster and more routine methods that may be developed.

Apparatus

Use a microscope that is stable and protected from vibration. The microscope magnification (product of the objective magnification, ocular magnification, and additional magnifying components) must be sufficient to allow adequate characterisation of the smallest particles to be classified in the test sample. The greatest numerical aperture of the objective is sought for each magnification range. Polarising filters may be used in conjunction with suitable analysers and retardation plates. Colour filters of relatively narrow spectral transmission are used with achromatic objectives, and are

[1] *This chapter has undergone pharmacopoeial harmonisation. See chapter 5.8 Pharmacopoeial harmonisation.*

preferable with apochromats; they are required for appropriate colour rendition in photomicrography. Condensers, corrected at least for spherical aberration are used in the microscope substage and with the lamp. The numerical aperture of the substage condenser matches that of the objective under the conditions of use; this is affected by the actual aperture of the condenser diaphragm and the presence of immersion oils.

Adjustment
The precise alignment of all elements of the optical system and proper focusing are essential. The focusing of the elements is done in accordance with the recommendations of the microscope manufacturer. Critical axial alignment is recommended.

Illumination
A requirement for good illumination is a uniform and adjustable intensity of light over the entire field of view; Köhler illumination is preferred. With coloured particles, choose the colour of the filters so as to control the contrast and detail of the image.

Visual characterisation
The magnification and numerical aperture must be sufficiently high to allow adequate resolution of the images of the particles to be characterised. Determine the actual magnification using a calibrated stage micrometer to calibrate an ocular micrometer. Errors can be minimised if the magnification is sufficient that the image of the particle is at least 10 ocular divisions. Each objective must be calibrated separately. To calibrate the ocular scale, the stage micrometer scale and the ocular scale must be aligned. In this way, a precise determination of the distance between ocular stage divisions can be made. Several different magnifications may be necessary to characterise materials having a wide particle size distribution.

Photographic characterisation
If particle size is to be determined by photographic methods, take care to ensure that the object is sharply focused at the plane of the photographic emulsion. Determine the actual magnification by photographing a calibrated stage micrometer, using photographic film of sufficient speed, resolving power, and contrast. Exposure and processing must be identical for photographs of both the test sample and the determination of magnification. The apparent size of a photographic image is influenced by the exposure, development, and printing processes as well as by the resolving power of the microscope.

Preparation of the mount
The mounting medium will vary according to the physical properties of the test sample. Sufficient, but not excessive, contrast between the sample and the mounting medium is required to ensure adequate detail of the sample edge. The particles must rest in one plane and be adequately dispersed to distinguish individual particles of interest. Furthermore, the particles must be representative of the distribution of sizes in the material and must not be altered during preparation of the mount. Care must be taken to ensure that this important requirement is met. Selection of the mounting medium must include a consideration of the analyte solubility.

Crystallinity characterisation
The crystallinity of a material may be characterised to determine compliance with the crystallinity requirement where stated in the individual monograph of a drug substance. Unless otherwise specified in the individual monograph, mount a few particles of the sample in mineral oil on a clean glass slide. Examine the mixture using a polarising microscope: the particles show birefringence (interference colors) and extinction positions when the microscope stage is revolved.

Limit test of particle size by microscopy
Weigh a suitable quantity of the powder to be examined (for example, 10-100 mg), and suspend it in 10 mL of a suitable medium in which the powder does not dissolve, adding, if necessary, a wetting agent. A homogeneous suspension of particles can be maintained by suspending the particles in a medium of similar or matching density and by providing adequate agitation. Introduce a portion of the homogeneous suspension into a suitable counting cell, and scan under a microscope an area corresponding to not less than 10 µg of the powder to be examined. Count all the particles having a maximum dimension greater than the prescribed size limit. The size limit and the permitted number of particles exceeding the limit are defined for each substance.

Particle size characterisation
The measurement of particle size varies in complexity depending on the shape of the particle, and the number of particles characterised must be sufficient to ensure an acceptable level of uncertainty in the measured parameters. Additional information on particle size measurement, sample size, and data analysis is available, for example, in ISO 9276. For spherical particles, size is defined by the diameter. For irregular particles, a variety of definitions of particle size exist. In general, for irregularly shaped particles, characterisation of particle size must also include information on the type of diameter measured as well as information on particle shape. Several commonly used measurements of particle size are defined in Figure 2.9.37.-1.

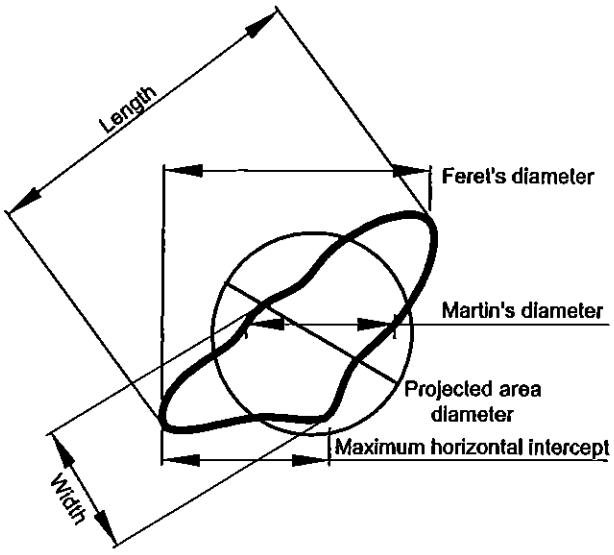


Figure 2.9.37.-1. – *Commonly used measurements of particle size*

- *Feret's diameter*: the distance between imaginary parallel lines tangent to a randomly oriented particle and perpendicular to the ocular scale,
- *Martin's diameter*: the diameter of the particle at the point that divides a randomly oriented particle into 2 equal projected areas,
- *projected area diameter*: the diameter of a circle that has the same projected area as the particle,
- *length*: the longest dimension from edge to edge of a particle oriented parallel to the ocular scale,
- *width*: the longest dimension of the particle measured at right angles to the length.

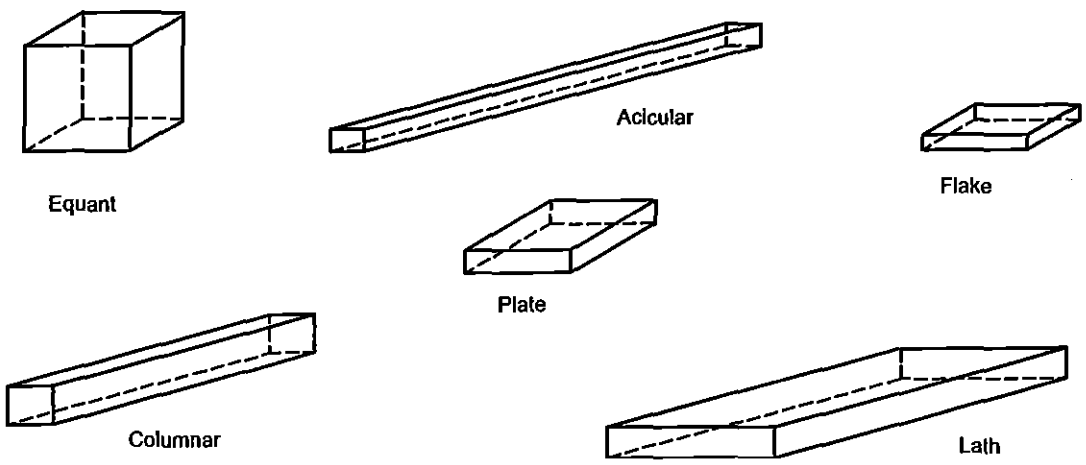


Figure 2.9.37.-2. – *Commonly used descriptions of particle shape*

Particle shape characterisation

For irregularly shaped particles, characterisation of particle size must also include information on particle shape. The homogeneity of the powder must be checked using appropriate magnification. The following defines some commonly used descriptors of particle shape (see Figure 2.9.37.-2).

— *acicular*: slender, needle-like particle of similar width and thickness,
— *columnar*: long, thin particle with a width and thickness that are greater than those of an acicular particle,
— *flake*: thin, flat particle of similar length and width,
— *plate*: flat particle of similar length and width but with greater thickness than a flake particle,
— *lath*: long, thin, blade-like particle,
— *equant*: particle of similar length, width, and thickness; both cubical and spherical particles are included.

General observations

A particle is generally considered to be the smallest discrete unit. A particle may be a liquid or semi-solid droplet; a single crystal or polycrystalline; amorphous or an agglomerate. Particles may be associated. This degree of association may be described by the following terms:

— *lamellar*: stacked plates,
— *aggregate*: mass of adhered particles,
— *agglomerate*: fused or cemented particles,
— *conglomerate*: mixture of 2 or more types of particles,
— *spherulite*: radial cluster,
— *drusy*: particle covered with tiny particles.

Particle condition may be described by the following terms:

— *edges*: angular, rounded, smooth, sharp, fractured,
— *optical*: color (using proper color balancing filters), transparent, translucent, opaque,
— *defects*: occlusions, inclusions.

Surface characteristics may be described as:

— *cracked*: partial split, break, or fissure,
— *smooth*: free of irregularities, roughness, or projections,
— *porous*: having openings or passageways,
— *rough*: bumpy, uneven, not smooth,
— *pitted*: small indentations.

O2. Scanning Electron Microscopy

(Ph. Eur. method 2.9.52)

This general chapter focuses on scanning electron microscopy applied to pharmaceutical materials, from research to quality control.

Scanning electron microscopy (SEM) is a powerful imaging technique that is superior to and complements light microscopy in terms of resolution, magnification and depth of field.

In addition, by exploiting the different interactions between the electrons and the specimen, it provides topographical and compositional information and can thus also be used as an analytical tool. When it is combined with elemental X-ray microanalysis, in particular, chemical analysis can be performed (see general chapter *2.2.37. X-ray fluorescence spectrometry*).

SEM is a well-established technique and, increasingly, it is being applied to examine and characterise a wide range of pharmaceutical materials in solid form but also, with the use of specialised specimen preparation methods, in semi-solid and liquid form.

PRINCIPLE

To produce magnified images, a scanning electron microscope uses a finely-focused beam of accelerated electrons instead of light; the specimen is scanned (rastered) in a rectangular pattern with the electron beam. This technique exploits the interactions between the electron beam and the specimen using different types of detectors. Due to the nature of these interactions, a variety of signals are produced and these can be used to provide characteristic information about the specimen at and from just below its surface.

INTERACTION WITH THE SPECIMEN

As the surface of the specimen is scanned by the electron beam, these electrons (also known as primary electrons) interact with the specimen and can penetrate to a depth of up to a few tens of micrometres. Electrons are scattered and absorbed within a teardrop-shaped volume just below the surface of the specimen, known as the interaction volume (Figure 2.9.52-1). The depth to which the electron beam penetrates is directly proportional to the beam voltage (a high-energy beam will penetrate deeper than a low-energy beam) and inversely proportional to the average atomic number of the constituent elements of the specimen (the beam will penetrate much deeper into a specimen rich in light elements than into one rich in heavy elements).

Types of emitted signals

Figure 2.9.52-1 illustrates the sources of the principal emitted signals, which are described as follows:

— *secondary electrons* are low-energy electrons that are ejected from just below the specimen surface as a result of the inelastic scattering of the incident electrons;
— *backscattered electrons* are high-energy electrons that result from the elastic scattering of incident electrons deeper in

the interaction volume; as a consequence, backscattered electron images typically have a lower spatial resolution than secondary electron images and show less surface detail;
— characteristic *X-rays* are produced when the incident electron beam interacts with the elements in the specimen;
— *cathodoluminescence* is the emission of photons in the visible spectrum when atoms and molecules deep within the interaction volume return to their ground state after being excited by the electron beam.

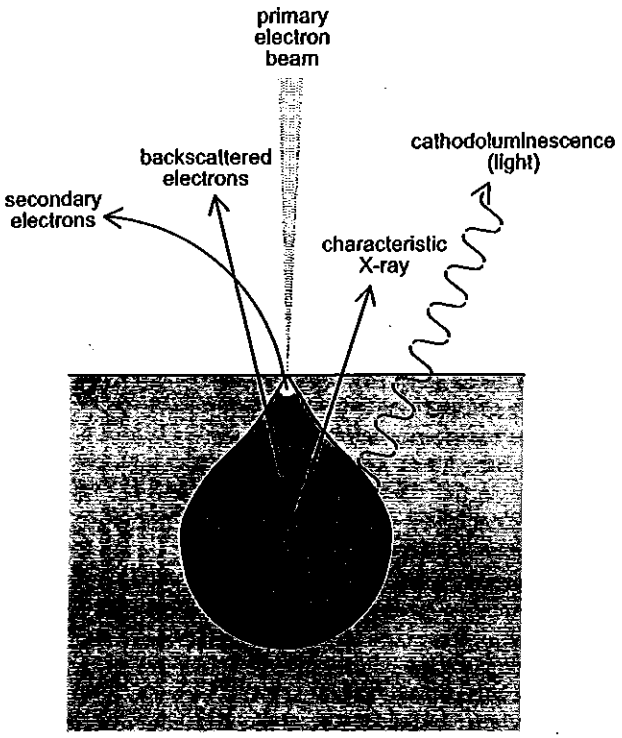


Figure 2.9.52-1. – *Schematic diagram showing the signals generated from a teardrop-shaped interaction volume*

APPLICATIONS

SEM is used to support many pharmaceutical processes during the formulation and analytical development, manufacturing and quality control of medicinal products. Its applications include:
— physical and chemical analysis of samples;
— comparison of raw materials from different suppliers;
— support for the development of particle sizing methods;
— particle size analysis (using image analysis);
— characterisation of the surface roughness of tablet film coatings;
— investigation of complaints;
— analysis and identification of particulate matter contamination;
— trouble-shooting of manufacturing problems;
— examination of falsified products.

SEM is used to examine a wide range of materials, such as active pharmaceutical ingredients (APIs), excipients, powder blends, solid dosage forms, medical devices, primary and secondary packaging materials and contaminants.
The examination of SEM images permits the qualitative and quantitative assessment of the homogeneity and consistency of powders as raw materials or after processing (e.g. compressed into tablets), with respect to the shapes, sizes and size distributions of particles in powders and of the texture, porosity and shapes of the crystals. This information can be correlated with dissolution behaviour, bioavailability and the crystallinity of the components in solid dosage forms. SEM has particular value in supporting the development and optimisation of manufacturing processes for most solid dosage forms, such as tablets, powder mixtures for oral suspensions, granules, powders for inhalation, spray- or freeze-dried powders, and powders for injections.

SPECIFICITIES OF THE TECHNIQUE

SEM offers many distinct advantages over conventional optical light microscopy because specimens can be examined at higher magnifications (250 000 × compared to 1000 ×, for example) with greater lateral resolution (3 nm or better compared to about 200 nm) and superior depths of field. However, the interaction between the electrons and the specimen could induce a strong charging effect due to the accumulation of electrical charge on its surface. Specific preparation procedures may thus be required for non-conducting specimens so that the accumulated charges can be dissipated.

Therefore, the ultimate performance of an electron microscope depends upon a number of factors, with the nature of the specimen having a major influence on the quality of the final image.

EQUIPMENT

Figure 2.9.52-2 is a schematic diagram of an electron microscope. It typically consists of:

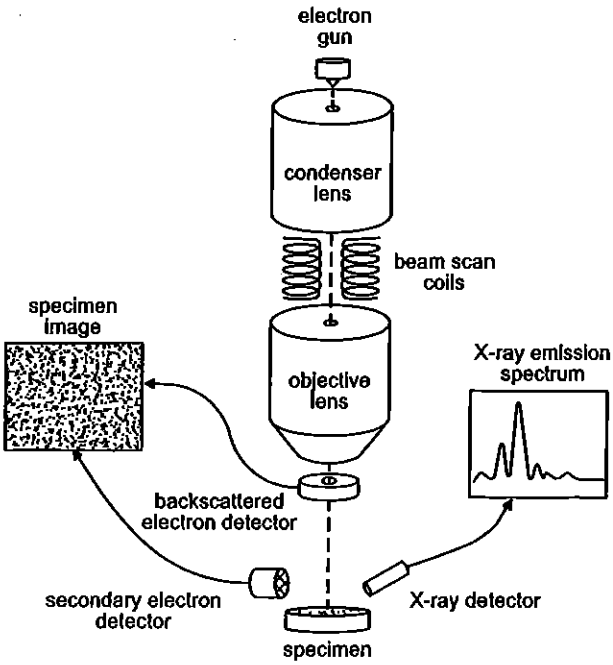


Figure 2.9.52-2. – *Schematic diagram of a scanning electron microscope*

— an electron gun that is housed at the top of an electron-optical column (electron column) and emits a beam of electrons; the electrons are accelerated down the column under the influence of a high voltage that is typically variable from 100 V to 30 kV;
— an electron column, which contains various components used to control and focus the electron beam:
  — a condenser lens system that controls the diameter and energy of the electron beam;
  — scan coils that are used to move the beam in a raster-fashion across a rectangular area on the specimen;

— an objective lens that focuses the beam to a fine point and directs it towards the specimen;
— a specimen chamber positioned at the bottom of the electron column; it contains the specimen and an array of detectors that collect the emitted signals;
— a system of pumps that maintains the electron gun chamber and electron column under high vacuum to preserve the reliability and stability of the electron source.

VACUUM IN THE SPECIMEN CHAMBER

Conventional SEM is performed with the electron column and specimen chamber maintained at a high vacuum so that conductive specimens can be examined. However, recent developments have enabled the examination of non-conductive specimens and hydrated materials either in a humid or wet atmosphere using environmental SEM (ESEM), or in a dry partial vacuum using variable pressure SEM (VPSEM) at pressures ranging from 10 Pa to 10^3 Pa. A differential vacuum pumping system keeps the gun chamber at a high vacuum relative to the low vacuum (sometimes close to atmospheric pressure) in the specimen chamber. An advantage of using ESEM or VPSEM is that the microscope can also be operated in high vacuum mode.

ELECTRON EMITTERS

There are 2 main types of electron sources:
— *thermionic emission*. A widely-used thermionic source consists of a filament, usually a tungsten wire, heated by passing an electrical current directly through it.
An alternative is a lanthanum hexaboride (LaB_6) emitter, which consists of an indirectly heated single crystal; it has a much greater electron yield and is much brighter than a tungsten emitter.
— *field emission (FE)*. Electrons are emitted from the ultra-sharp tip of a fine-pointed tungsten wire immersed within an intense electrostatic field. Used in high-resolution electron microscopes, FE emitters give an intensely bright, small-diameter and low-current electron beam. They are very stable and have an operating life of many thousands of hours. FE-SEM offers significant advantages, because it produces high-resolution images at low accelerating voltages (down to a few hundreds of volts), with increased signal-to-noise ratios (giving low-noise, high-quality images) and with a superior depth of field.

DETECTORS

A cluster of different detectors (see Figure 2.9.52-2) are positioned at optimised distances close to the specimen and collect the variety of signals emitted during interaction with the electron beam.

Secondary electron detectors

Most high-vacuum electron microscopes are fitted with an Everhart-Thornley (E-T) detector positioned to one side of the specimen. E-T detectors are sensitive to the low-energy secondary electrons that originate from a shallow depth (typically less than 50 nm) below the surface of a specimen.

The E-T detector has a positively biased wire grid in front of it to attract electrons towards a scintillator which converts the secondary electrons into flashes of light that are directed into a photomultiplier. Secondary electrons can follow curved paths from areas on specimens that may not be in the direct line of sight of the detector. As a consequence, secondary electron images tend to have high contrast and show considerable surface details that emphasise topographical features and variations in surface roughness across specimens.

In addition to secondary electrons, E-T detectors are also sensitive to backscattered electrons emitted from areas on the specimen that happen to be in the direct line of sight of the detector. As a consequence, secondary electron images include contributions from both secondary electrons and backscattered electrons and it is this combination that produces the three-dimensional effect that is a distinctive characteristic of secondary electron images.

An E-T detector cannot be used in ESEM or VPSEM because secondary electrons emitted from a specimen are scattered and absorbed by the gas molecules in the chamber. To observe secondary electron images in ESEM or VPSEM, a detector that is sensitive to the small amount of light that is emitted from the ionised gas molecules surrounding the specimen (a process called gas luminescence) in the specimen chamber is used. It is this cloud of positive ions around the specimen that also neutralises any negative charge accumulation.

Backscattered electron detectors

Backscattered electrons have higher energies than secondary electrons and are emitted over a broad angular range. Consequently, the detector is positioned close to and above the specimen. Most high-vacuum and low-vacuum electron microscopes are fitted with a dedicated backscattered electron detector that uses either a scintillator or solid-state diodes.

Scintillator detectors work in the same way as E-T secondary electron detectors by detecting electrons as flashes of light, but have no biased grid to attract electrons towards them. Solid-state backscattered electron detectors consist of an annulus having 4 or 5 separate photodiodes arranged as an array of segments. The diodes can be turned on or off in different combinations to allow topographic imaging, compositional imaging or both together.

The emission of backscattered electrons increases with the average atomic number of the various components (pure or composite) of the specimen. This behaviour can be exploited to examine the spatial distribution and homogeneity of components containing light and heavy atoms in mixtures such as powder blends, compressed tablets and specimens contaminated with foreign matter. Backscattered electron compositional images do not reveal which elements are present in a specimen but they do show where high atomic number materials are relative to low atomic number materials. Materials consisting mainly of heavy atoms (e.g. iron, bromine) appear brighter than those having lighter atoms (e.g. carbon, nitrogen, oxygen, aluminium).
To determine which elements are actually present, elemental X-ray microanalysis is used.

Unlike secondary electron detectors, backscattered electron detectors are not greatly affected by electrical charging at the specimen surface. Therefore, non-conducting specimens can be imaged in high-vacuum mode and in low-vacuum mode using a backscattered electron detector.

Both scintillator and solid-state backscattered electron detectors are also very sensitive to visible light and can therefore also function as cathodoluminescence detectors.

Cathodoluminescence detectors

The weak-intensity light emitted from cathodoluminescent materials is collected by a retractable paraboloidal mirror and directed into a light-sensitive scintillator detector (not shown in Figure 2.9.52-2). The mirror is inserted just above the specimen and is retracted when not in use. The electron beam passes through a small hole in the mirror so that it strikes the specimen to induce the emission of visible light.

In its simplest form, the detector does not discriminate between different wavelengths of light and greyscale images are produced with cathodoluminescent areas on a specimen

being shown as bright. More complex detectors have the capability to select different wavelengths of light being emitted to produce monochromatic images tuned to a single wavelength that represents a specific material.
Cathodoluminescence spectral analysis is achieved by passing polychromatic light emitted from the specimen into a spectrometer where it is dispersed to produce a visible light spectrum.

Spectral analysis of the emitted light is not widely used but has applications in the examination and characterisation of some organic and inorganic materials. It can be used to acquire diagnostic information about the chemistry and structure of single materials and spectral imaging can be used for analysing mixtures of materials.

Elemental X-ray microanalysis detectors
An X-ray analyser fitted to the specimen chamber of an electron microscope enables the rapid and non-destructive elemental analysis of materials being examined. 2 main detector types are available and these detect either the dispersed energies (energy-dispersive X-ray microanalysis, EDX) or the dispersed wavelengths (wavelength-dispersive X-ray microanalysis, WDX) of the characteristic X-rays emitted from specimens (for more details, see general chapter *2.2.37*).

PROCEDURE

SPECIMEN PREPARATION / PRESENTATION

Prior to preparing a specimen for examination by SEM, the purpose of the examination must be considered because this can affect the way the sample is treated.

In addition, the electrical conductivity of the specimen must be considered to minimise charge accumulation as the electron beam scans across the specimen. Numerous methods have been developed to prepare specimens to maintain integrity whilst minimising artefacts.
The preparation of most pharmaceutical samples is simple and quick and does not require specialised equipment or complex processes.

Specimen holders, called stubs, are used to support powders, single particles, tablets, freeze-dried cakes, capsules and beads. Rapid-curing, vacuum compatible glue or electrically conductive silver or carbon paints are ideal for holding large objects that are up to a few millimetres in diameter. Fine powders and small objects can be attached to stubs on double-sided adhesive tape or on sticky tabs. Large samples may have to be reduced in size to fit onto a stub or into the specimen chamber and tablets can be broken open to expose their cores before being attached securely to a stub.

Powders can be simply sprinkled or poured onto a stub that has had a thin layer of adhesive applied to it and excess loose powder is then tapped off or blown off with a gentle stream of inert compressed gas. A rapid and simple method for powders is to attach double-sided adhesive tape onto a stub and dip it gently into the powder so that a thin layer sticks to the tape, and excess powder is then blown off (taking care not to inhale the airborne powder).

When preparing powder samples, cross-contamination must be avoided because the presence in the specimen of just a single particle from another material can lead to an incorrect interpretation of an image or chemical analysis. For this reason, it is not good practice to disperse powder particles onto a specimen stub using a brush, because the brush must be decontaminated or discarded after a single use.
For microanalysis, the use of inert/plastic tools (needles, spatulas, tweezers, etc.) is preferred for sample manipulation since contaminant particles could potentially be released from metal tools.

SPECIMEN COATING

Most pharmaceutical materials are readily examined without the need for prolonged and complicated preparation techniques. However, such materials tend to be electrically non-conductive and so specimens that are examined using high-vacuum SEM need to be coated with an ultra-thin layer of conductive material. Without a conductive coating, specimens will acquire a net negative charge as the electron beam scans across them; this causes the specimen to glow brightly and, when it discharges, disturbing flashes and bright streaks will be seen in the image. Suitable conductive materials for coating include metals, such as platinum and gold, and these can be applied to rough and smooth specimen surfaces using a plasma sputter coater. If carbon is to be used, it needs to be evaporated in a very high vacuum because it cannot be sputtered. In addition to reducing charging effects, a conductive coating will increase the emission of secondary electrons to give brighter images and will also transfer localised electron beam-induced heat away from the specimen. Some powders consisting of very small particles (such as colloidal silica) may need to be sputter coated 3 or 4 times to prevent charging because the coating material initially fails to form a continuous conductive layer.

Some beam-sensitive specimens, such as semi-solids or wet materials, may have to be cooled or frozen using a cooling stage to minimise degradation or evaporation. With respect to this, FE-SEM allows the surfaces of labile specimens to be examined with an electron beam that has a very low energy (e.g. 500 volts or less), and this can help to minimise or even eliminate beam-induced degradation.

Specimens to be analysed by elemental X-ray microanalysis are typically not coated with metal because the coat would add extra peaks to the X-ray spectrum and this could interfere with the analysis or even obscure minor amounts of some elements, thereby precluding the possibility of quantitative determinations of unknowns. Most elemental analysers are capable of detecting light elements, so even a thin carbon coat could hinder the interpretation of the spectrum. To minimise or exclude potential problems and artefacts associated with specimen coating, non-conductive materials can be examined uncoated using ESEM or VPSEM. Software correction for any coating may need to be applied.

OPERATION

The electron microscope is operated with a beam-accelerating voltage that is appropriate to produce images that reveal the information of interest about a specimen. For example, a low voltage (e.g. less than 5 kV) can be used to image surface details and a higher voltage (e.g. greater than 10 kV) increases the resolving power and can be used for the analysis of a wide range of elements. The most useful accelerating voltage range is about 2 kV to 20 kV because most elements of interest can be ionized by electrons with energies in this range.

Adjustment of the objective lens focuses the beam on the specimen over a wide range of working distances to accommodate large and small specimens. The depth of field can also be increased by selecting smaller beam apertures to enable the full depth of thick specimens to be in acceptable focus simultaneously.

When SEM is used to examine specimens in a high vacuum, very high magnifications in excess of 10 000 × can be achieved to resolve fine detail down to about 3 nm,

depending upon the specimen. This is possible because the electron beam can be focused without being scattered by gas molecules. For ESEM or VPSEM, a consequence of gas or water vapour being present in the specimen chamber is that the electron beam suffers some lateral scattering (called skirting). This scattering can inhibit high-resolution imaging because the beam cannot be focused as finely as it would be in a high vacuum. By selecting a shorter working distance and optimising the gas pressure, this adverse effect can be minimised.

Lateral spatial resolutions of 1 nm or better become possible (depending upon the specimen being examined) when using FE-SEM. To achieve this high resolving power, a beam-accelerating voltage in excess of about 15 kV may be required, but a high beam voltage could damage beam-sensitive materials. For most organic solids, the electron beam will penetrate many micrometres into the specimen and surface detail will be lost due to the scattering and absorption of the emitted electrons. In order to reveal the surface details of specimens, the electron microscope must be operated at a much lower voltage, and this is at the expense of resolution. Most pharmaceutical specimens studied by SEM are likely to be examined at a magnification less than 10 000 × and so a high resolving power is typically not needed.

COMBINATION OF SEM AND X-RAY MICROANALYSIS

This combination enables the identification of the elemental compositions of specimens and elemental mapping to visualise the distributions of elements, for example in tablets and blends.

Element maps are easily and quickly generated to visualise the spatial distribution of the elements present in a specimen at low and high magnifications. Cross-sections cut or fractured through tablet cores and powders that have been lightly pressed flat can be mapped to reveal the relationships between their components. This technique is useful to compare the differences between samples to indicate why some perform better than others. It is important to have surfaces that are not rough because emitted X-rays follow straight paths and so may not be detected in areas (effectively in shadows) that are not in direct line of sight of the X-ray detector. This artefact can be reduced by tilting the specimen towards the detector.

X-ray microanalysis is also used for the investigation of particulate matter. Black spots in tablets, for example, are often caused by particles of metal or rubber debris, caused by the wearing of tablet presses, that have subsequently been incorporated into the tablet as a visible defect. SEM-EDX is an ideal technique to identify them because it is non-destructive and can often be used to distinguish between different metals so that the source and root cause can be identified. Also, particles in vials and syringes of solutions for injection can be examined and analysed after they have been isolated by filtration or removed using a micropipette.

EQUIPMENT PERFORMANCE

SEM user requirements, in addition to manufacturer's recommendations, provide a basis for the system performance evaluation. For size measurements, accuracy of the displayed scale bar represents a critical parameter that should be checked at both low and high magnifications using certified calibration test specimens (available from SEM accessory suppliers). As accuracy of scale can be influenced by instrumental parameters, such as accelerating voltage and working distance, or by the specimen itself, it should be checked under different operating conditions to allow for instrument variations using, for example, a bracketing concept. Accuracy of scale should be checked in orthogonal directions and the typical accuracy of the displayed scale should be 10 per cent or better. If justified, other acceptance criteria, such as image resolution, might be applied.

For elemental analysis by EDX, the accuracy and reproducibility of the energy scale over a specific energy range (e.g. 0-5 keV or 0-20 keV) represents a parameter that should be checked and calibrated according to the manufacturer's instrument operating instructions using suitable reference materials (see general chapter *2.2.37*). These single element materials are used because they have multiple X-ray emission peaks at well-defined energies and intensities across the spectral range being calibrated.
The intensities of the emitted X-rays can be influenced by other instrumental parameters, such as accelerating voltage or the working distance, and should be checked for relevant instrumental parameters using, for example, a bracketing concept. If justified, other acceptance criteria (such as detector resolution) might be used.

P. Particle Size Analysis by Laser Light Diffraction[1]

(Ph. Eur. method 2.9.31)

The method is based on the ISO standards 13320-1(1999) and 9276-1(1998).

INTRODUCTION

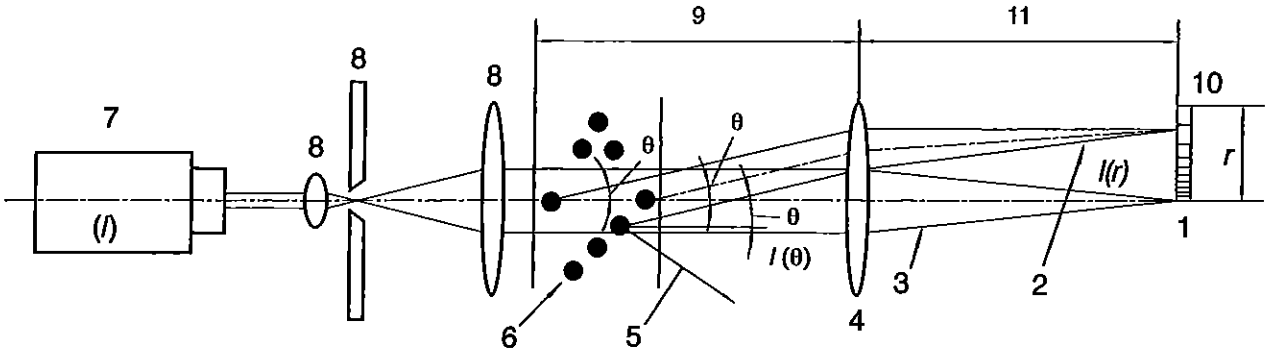
The laser light diffraction technique used for the determination of particle-size distribution is based on the analysis of the diffraction pattern produced when particles are exposed to a beam of monochromatic light. Historically, the early laser diffraction instruments only used scattering at small angles. However, the technique has since been broadened to include laser light scattering in a wider angular range and application of the Mie theory, in addition to the Fraunhofer approximation and anomalous diffraction.

The technique cannot distinguish between scattering by single particles and scattering by clusters of primary particles, i.e. by agglomerates or aggregates. As most particulate samples contain agglomerates or aggregates and as the focus of interest is generally on the size distribution of primary particles, the clusters are usually dispersed into primary particles before measurement.

For non-spherical particles, an equivalent sphere-size distribution is obtained because the technique assumes spherical particles in its optical model. The resulting particle-size distribution may differ from those obtained by methods based on other physical principles (e.g. sedimentation, sieving).

This chapter provides guidance for the measurement of size distributions of particles in different dispersed systems, for example, powders, sprays, aerosols, suspensions, emulsions, and gas bubbles in liquids, through analysis of their angular light-scattering patterns. It does not address specific requirements of particle size measurement of specific products.

[1] *This chapter has undergone pharmacopoeial harmonisation. See chapter 5.8. Pharmacopoeial harmonisation.*



1. Obscuration detector
2. Scattered beam
3. Direct beam
4. Fourier lens
5. Scattered light not collected by lens (4)
6. Particle ensemble
7. Light source laser
8. Beam processing unit
9. Working distance of lens (4)
10. Multi-element detector
11. Focal distance of lens (4)

Figure 2.9.31.-1. – *Example of a set-up of a laser light diffraction instrument*

PRINCIPLE

A representative sample, dispersed at an adequate concentration in a suitable liquid or gas, is passed through a beam of monochromatic light, usually a laser. The light scattered by the particles at various angles is measured by a multi-element detector. Numerical values representing the scattering pattern are then recorded for subsequent analysis. These scattering pattern values are then transformed, using an appropriate optical model and mathematical procedure, to yield the proportion of total volume to a discrete number of size classes, forming a volumetric particle-size distribution.

INSTRUMENT

The instrument is located in an environment where it is not affected by electrical noise, mechanical vibrations, temperature fluctuations, humidity or direct bright light.

An example of a set-up of a laser light diffraction instrument is given in Figure 2.9.31.-1. Other equipment may be used.

The instrument comprises a laser light source, beam processing optics, a sample measurement region (or cell), a Fourier lens, and a multi-element detector for measuring the scattered light pattern. A data system is also required for deconvolution of the scattering data into a volumetric size distribution and associated data analysis and reporting.

The particles can enter the laser beam in 2 positions. In the conventional case the particles enter the parallel beam before the collecting lens and within its working distance.
In so-called reversed Fourier optics the particles enter behind the collecting lens and thus, in a converging beam.
The advantage of the conventional set-up is that a reasonable path length for the sample is allowed within the working distance of the lens. The second set-up allows only small path lengths but enables measurement of scattered light at larger angles, which is useful when submicron particles are present.

The interaction of the incident light beam and the ensemble of dispersed particles results in a scattering pattern with different light intensities at various angles. The total angular intensity distribution, consisting of both direct and scattered light, is then focused onto a multi-element detector by a lens or a series of lenses. These lenses create a scattering pattern that, within limits, does not depend on the location of the particles in the light beam. Hence, the continuous angular intensity distribution is converted into a discrete spatial intensity distribution on a set of detector elements.

It is assumed that the measured scattering pattern of the particle ensemble is identical to the sum of the patterns from all individual single scattering particles presented in random relative positions. Note that only a limited angular range of scattered light is collected by the lens(es) and, therefore, by the detector.

DEVELOPMENT OF THE METHOD

The measurement of particle size by laser diffraction can give reproducible data, even in the sub-micron region, provided the instrument used and the sample tested are carefully controlled to limit variability of the test conditions (e.g. dispersion medium, method of preparation of the sample dispersion).

Traditionally, the measurement of particle size using laser diffraction has been limited to particles in the range of approximately 0.1 µm to 3 mm. Because of recent advances in lens and equipment design, newer instruments are capable of exceeding this range routinely. With the validation report the user demonstrates the applicability of the method for its intended use.

Sampling

The sampling technique must be adequate to obtain a representative sample of a suitable volume for the particle-size measurement. Sample splitting techniques such as rotating riffler or the cone and quartering method may be applied.

Evaluation of the dispersion procedure

Inspect the sample to be analysed, visually or with the aid of a microscope, to estimate its size range and particle shape. The dispersion procedure must be adjusted to the purpose of the measurement. The purpose may be such that it is preferable to deagglomerate clusters into primary particles as far as possible, or it may be desirable to retain clusters as intact as possible. In this sense, the particles of interest may be either primary particles or clusters.

For the development of a method it is highly advisable to check that comminution of the particles does not occur, and conversely, that dispersion of particles or clusters is satisfactory. This can usually be done by changing the dispersing energy and monitoring the change of the particle-size distribution. The measured size distribution must not change significantly when the sample is well dispersed and the particles are neither fragile nor soluble. Moreover, if the manufacturing process (e.g. crystallisation, milling) of the

material has changed, the applicability of the method must be verified (e.g. by microscopic comparison).

Sprays, aerosols and gas bubbles in a liquid should be measured directly, provided that their concentration is adequate, because sampling or dilution generally alters the particle-size distribution.

In other cases (such as emulsions, pastes and powders), representative samples may be dispersed in suitable liquids. Dispersing aids (wetting agents, stabilisers) and/or mechanical forces (e.g. agitation, sonication) are often applied for deagglomeration or deaggregation of clusters and stabilisation of the dispersion. For these liquid dispersions, a recirculating system is most commonly used, consisting of an optical measuring cell, a dispersion bath usually equipped with stirrer and ultrasonic elements, a pump, and tubing. Non-recirculating, stirred cells are useful when only small amounts of a sample are available or when special dispersion liquids are used.

Dry powders can also be converted into aerosols through the use of suitable dry powder dispersers, which apply mechanical force for deagglomeration or deaggregation. Generally, the dispersers use the energy of compressed gas or the differential pressure of a vacuum to disperse the particles to an aerosol, which is blown through the measuring zone, usually into the inlet of a vacuum unit that collects the particles. However, for free flowing, coarser particles or granules the effect of gravity may be sufficient to disperse the particles adequately.

If the maximum particle size of the sample exceeds the measuring range of the instrument, the material that is too coarse can be removed by sieving and the mass and percentage of removed material are reported. However, after pre-sieving, note that the sample is no longer representative, unless otherwise proven.

Optimisation of the liquid dispersion

Liquids, surfactants, and dispersing aids used to disperse powders must:

- be transparent at the laser wavelength and practically free from air bubbles or particles;
- have a refractive index that differs from that of the test material;
- be non-solvent of the test material (pure liquid or pre-filtered, saturated solution);
- not alter the size of the test materials (e.g. by solubility, solubility enhancement, or recrystallisation effects);
- favour easy formation and stability of the dispersion;
- be compatible with the materials used in the instrument (such as O-rings, gaskets, tubing, etc.);
- possess a suitable viscosity to facilitate recirculation, stirring and filtration.

Surfactants and/or dispersing aids are often used to wet the particles and to stabilise the dispersion. For weak acids and weak bases, buffering of the dispersing medium at low or high pH respectively can assist in identifying a suitable dispersant.

A preliminary check of the dispersion quality can be performed by visual or microscopic inspection. It is also possible to take fractional samples out of a well-mixed stock dispersion. Such stock dispersions are formed by adding a liquid to the sample while mixing it with, for example, a glass rod, a spatula or a vortex mixer. Care must be taken to ensure the transfer of a representative sample and that settling of larger particles does not occur. Therefore a sample paste is prepared or sampling is carried out quickly from a suspension maintained under agitation.

Optimisation of the gas dispersion

For sprays and dry powder dispersions, a compressed gas free from oil, water and particles may be used. To remove such materials from the compressed gas, a dryer with a filter can be used. Any vacuum unit should be located away from the measurement zone, so that its output does not disturb the measurement.

Determination of the concentration range

In order to produce an acceptable signal-to-noise ratio in the detector, the particle concentration in the dispersion must exceed a minimum level. Likewise, it must be below a maximum level in order to avoid multiple scattering. The concentration range is influenced by the width of the laser beam, the path length of the measurement zone, the optical properties of the particles, and the sensitivity of the detector elements.

In view of the above, measurements must be performed at different particle concentrations to determine the appropriate concentration range for any typical sample of material. (Note: in different instruments, particle concentrations are usually represented by differently scaled and differently named numbers, e.g. obscuration, optical concentration, proportional number of total mass).

Determination of the measuring time

The time of measurement, the reading time of the detector and the acquisition frequency are determined experimentally in accordance with the required precision. Generally, the time for measurement permits a large number of detector scans or sweeps at short time intervals.

Selection of an appropriate optical model

Most instruments use either the Fraunhofer or the Mie theory, though other approximation theories are sometimes applied for calculation of the scattering matrix. The choice of the theoretical model depends on the intended application and the different assumptions (size, absorbance, refractive index, roughness, crystal orientation, mixture, etc.) made for the test material. If the refractive index values (real and imaginary parts for the used wavelength) are not exactly known, then the Fraunhofer approximation or the Mie theory with a realistic estimate of the refractive index can be used. The former has the advantages that it is simple and it does not need refractive index values; the latter usually provides less-biased particle-size distributions for small particles. For instance, if the Fraunhofer model is used for samples containing an appreciable amount of small, transparent particles, a significantly larger amount of small particles may be calculated. In order to obtain traceable results, it is essential to document the refractive index values used, since small differences in the values assumed for the real and imaginary part of the complex refractive index may cause significant differences in the resulting particle-size distributions. Small values of the imaginary part of the refractive index (about 0.01-0.1 i) are often applied to allow the correction of the absorbance for the surface roughness of the particles. It should be noted, in general, that the optical properties of the substance to be tested, as well as the structure (e.g. shape, surface roughness and porosity), bear upon the final result.

Validation

Typically, the validity of a procedure may be assessed by the evaluation of its specificity, linearity, range, accuracy, precision and robustness. In particle-size analysis by laser light diffraction, specificity as defined by ICH is not applicable as it is not possible to discriminate between different components in a sample, nor is it possible to

discriminate agglomerates from dispersed particles unless properly complemented by microscopic techniques. Exploring a linear relationship between concentration and response, or a mathematical model for interpolation, is not applicable to this procedure. Rather than evaluating linearity, this method requires the definition of a concentration range within which the result of the measurements does not vary significantly. Concentrations below that range produce an error due to a poor signal-to-noise ratio, while concentrations above that range produce an error due to multiple scattering. The range depends mostly on the instrument hardware. Accuracy should be confirmed through an appropriate instrument qualification and comparison with microscopy, while precision may be assessed by means of a repeatability determination.

The attainable repeatability of the method mainly depends on the characteristics of the material (milled/not milled, robust/fragile, width of its size distribution, etc.), whereas the required repeatability depends on the purpose of the measurement. Mandatory limits cannot be specified in this chapter, as repeatabilities (different sample preparations) may vary appreciably from one substance to another. However, it is good practice to aim at acceptance criteria for repeatability such as $s_{rel} \leq 10$ per cent [$n = 6$] for any central value of the distribution (e.g. for x_{50}). Values at the sides of the distribution (e.g. x_{10} and x_{90}) are oriented towards less stringent acceptance criteria such as $s_{rel} \leq 15$ per cent [$n = 6$]. Below 10 µm, these values must be doubled. Robustness may be tested during the selection and optimisation of the dispersion media and forces. The change of the dispersing energy may be monitored by the change in the particle-size distribution.

MEASUREMENT

Precautions

The instructions given in the instrument manual are followed:

- never look into the direct path of the laser beam or its reflections;
- earth all instrument components to prevent ignition of solvents or dust explosions;
- check the instrument set-up (e.g. warm-up, required measuring range and lens, appropriate working distance, position of the detector, no direct bright daylight);
- in the case of wet dispersions, avoid air bubbles, evaporation of liquid, schlieren or other inhomogeneities in the dispersion; similarly, avoid improper mass-flow from the disperser or turbulent air-flow in the case of dry dispersions; such effects can cause erroneous particle-size distributions.

Measurement of the light scattering of dispersed sample(s)

After proper alignment of the optical part of the instrument, a blank measurement of the particle-free dispersion medium must be performed using the same method as that used for the measurement of the sample. The background signal must be below an appropriate threshold. The detector data are saved in order to substract them later from the data obtained with the sample. The sample dispersion is measured according to the developed method.

For each detector element, an average signal is calculated, sometimes together with its standard deviation.
The magnitude of the signal from each detector element depends upon the detection area, the light intensity and the quantum efficiency. The co-ordinates (size and position) of the detector elements together with the focal distance of the lens determine the range of scattering angles for each element. Most instruments also measure the intensity of the central (unscattered) laser beam. The ratio of the intensity of a dispersed sample to that in its absence (a blank measurement) indicates the proportion of scattered light and hence the particle concentration.

Conversion of scattering pattern into particle-size distribution

This deconvolution step is the inverse of the calculation of a scattering pattern for a given particle-size distribution. The assumption of spherical particle shape is particularly important as most algorithms use the mathematical solution for scattering from spherical particles. Furthermore, the measured data always contain some random and systematic errors, which may vitiate the size distributions. Several mathematical procedures have been developed for use in the available instruments. They contain some weighting of deviations between measured and calculated scattering patterns (e.g. least squares), some constraints (e.g. non-negativity for amounts of particles), and/or some smoothing of the size distribution curve.

The algorithms used are specific to each make and model of equipment, and are proprietary. The differences in the algorithms between different instruments may give rise to differences in the calculated particle-size distributions.

Replicates

The number of replicate measurements (with individual sample preparations) to be performed depends on the required measurement precision. It is recommended to set this number in a substance-specific method.

REPORTING OF RESULTS

The particle-size distribution data are usually reported as cumulative undersize distribution and/or as density distribution by volume. The symbol x is used to denote the particle size, which in turn is defined as the diameter of a volume-equivalent sphere. $Q3(x)$ denotes the volume fraction undersize at the particle size x. In a graphical representation, x is plotted on the abscissa and the dependent variable Q3 on the ordinate. Most common characteristic values are calculated from the particle-size distribution by interpolation. The particle sizes at the undersize values of 10 per cent, 50 per cent, and 90 per cent (denoted as x_{10}, x_{50}, and x_{90} respectively) are frequently used. x_{50} is also known as the median particle size. It is recognised that the symbol d is also widely used to designate the particle size, thus the symbol x may be replaced by d.

Moreover, sufficient information must be documented about the sample, the sample preparation, the dispersion conditions, and the cell type. As the results depend on the particular instrument, data analysis program, and optical model used, these details must also be documented.

CONTROL OF THE INSTRUMENT PERFORMANCE

Use the instrument according to the manufacturer's instructions and carry out the prescribed qualifications at an appropriate frequency, according to the use of the instrument and substances to be tested.

Calibration

Laser diffraction systems, although assuming idealised properties of the particles, are based on first principles of laser light scattering. Thus, calibration in the strict sense is not required. However, it is still necessary to confirm that the instrument is operating correctly. This can be undertaken using any certified reference material that is acceptable in industrial practice. The entire measurement procedure is

examined, including sample collection, sample dispersion, sample transport through the measuring zone, measurement, and the deconvolution procedure. It is essential that the total operational procedure is fully described.

The preferred certified reference materials consist of spherical particles of a known distribution. They must be certified as to the mass-percentage size distribution by an absolute technique, if available, and used in conjunction with an agreed, detailed operation procedure. It is essential that the real and imaginary parts of the complex refractive index of the material are indicated if the Mie theory is applied in data analysis. The representation of the particle-size distribution by volume will equal that of the distribution by mass, provided that the density of the particles is the same for all size fractions.

The response of a laser diffraction instrument is considered to meet the requirements if the mean value of x_{50} from at least 3 independent measurements does not deviate by more than 3 per cent from the certified range of values of the certified reference material. The mean values for x_{10} and x_{90} must not deviate by more than 5 per cent from the certified range of values. Below 10 µm, these values must be doubled.

Although the use of materials consisting of spherical particles is preferable, non-spherical particles may also be employed. Preferably, these particles have certified or typical values from laser diffraction analyses performed according to an agreed, detailed operating procedure. The use of reference values from methods other than laser diffraction may cause a significant bias. The reason for this bias is that the different principles inherent in the various methods may lead to different sphere-equivalent diameters for the same non-spherical particle.

Although the use of certified reference materials is preferred, other well-defined reference materials may also be employed. They consist of substances of typical composition and particle-size distribution for a specified class of substances. Their particle-size distribution has proven to be stable over time. The results must comply with previously determined data, with the same precision and bias as for the certified reference material.

Qualification of the system

In addition to the calibration, the performance of the instrument must be qualified at regular time intervals or as frequently as appropriate. This can be undertaken using any suitable reference material as mentioned in the previous paragraph.

The qualification of the system is based on the concept that the equipment, electronics, software and analytical operations constitute an integral system, which can be evaluated as an entity. Thus the entire measurement procedure is examined, including sample collection, sample dispersion, sample transport through the measuring zone, and the measurement and deconvolution procedure. It is essential that the total operational procedure is fully described.

In general, unless otherwise specified in the individual monograph, the response of a laser diffraction instrument is considered to meet the requirements if the x_{50} value does not deviate by more than 10 per cent from the range of values of the reference material. If optionally the values at the sides of the distribution are evaluated (e.g. x_{10} and x_{90}), then these values must not deviate by more than 15 per cent from the certified range of values. Below 10 µm, these values must be doubled.

NOTE: for calibration of the instrument, stricter requirements are laid down in the paragraph Calibration.

Q. Characterisation of Crystalline and Partially Crystalline Solids by X-ray Powder Diffraction (XRPD)[1]

(Ph. Eur. method 2.9.33)

Every crystalline phase of a given substance produces a characteristic X-ray diffraction pattern.

Diffraction patterns can be obtained from a randomly oriented crystalline powder composed of crystallites or crystal fragments of finite size. Essentially 3 types of information can be derived from a powder diffraction pattern: angular position of diffraction lines (depending on geometry and size of the unit cell); intensities of diffraction lines (depending mainly on atom type and arrangement, and particle orientation within the sample); and diffraction line profiles (depending on instrumental resolution, crystallite size, strain and specimen thickness).

Experiments giving angular positions and intensities of lines can be used for applications such as qualitative phase analysis (for example, identification of crystalline phases) and quantitative phase analysis of crystalline materials.
An estimate of the amorphous and crystalline fractions[2] can also be made.

The X-ray powder diffraction (XRPD) method provides an advantage over other means of analysis in that it is usually non-destructive in nature (specimen preparation is usually limited to grinding to ensure a randomly oriented sample). XRPD investigations can also be carried out under *in situ* conditions on specimens exposed to non-ambient conditions, such as low or high temperature and humidity.

PRINCIPLE

X-ray diffraction results from the interaction between X-rays and electron clouds of atoms. Depending on the atomic arrangement, interferences arise from the scattered X-rays. These interferences are constructive when the path difference between 2 diffracted X-ray waves differs by an integral number of wavelengths. This selective condition is described by the Bragg equation, also called Bragg's law (see Figure 2.9.33.-1):

$$2d_{hkl}\sin\theta_{hkl} = n\lambda$$

The wavelength λ of the X-rays is of the same order of magnitude as the distance between successive crystal lattice planes, or d_{hkl} (also called 'd-spacings'). θ_{hkl} is the angle between the incident ray and the family of lattice planes, and $\sin\theta_{hkl}$ is inversely proportional to the distance between successive crystal planes or d-spacings.

The direction and spacing of the planes with reference to the unit cell axes are defined by the Miller indices $\{hkl\}$. These indices are the reciprocals, reduced to the next-lower integer, of the intercepts that a plane makes with the unit cell axes. The unit cell dimensions are given by the spacings a, b and c and the angles between them, α, β, and γ.

The interplanar spacing for a specified set of parallel *hkl* planes is denoted by d_{hkl}. Each such family of planes may

[1] *This chapter has undergone pharmacopoeial harmonisation. See chapter 5.8. Pharmacopoeial harmonisation.*

[2] *There are many other applications of the X-ray powder diffraction technique that can be applied to crystalline pharmaceutical substances such as: determination of crystal structures, refinement of crystal structures, determination of crystallographic purity of crystalline phases, characterisation of crystallographic texture, etc. These applications are not described in this chapter.*

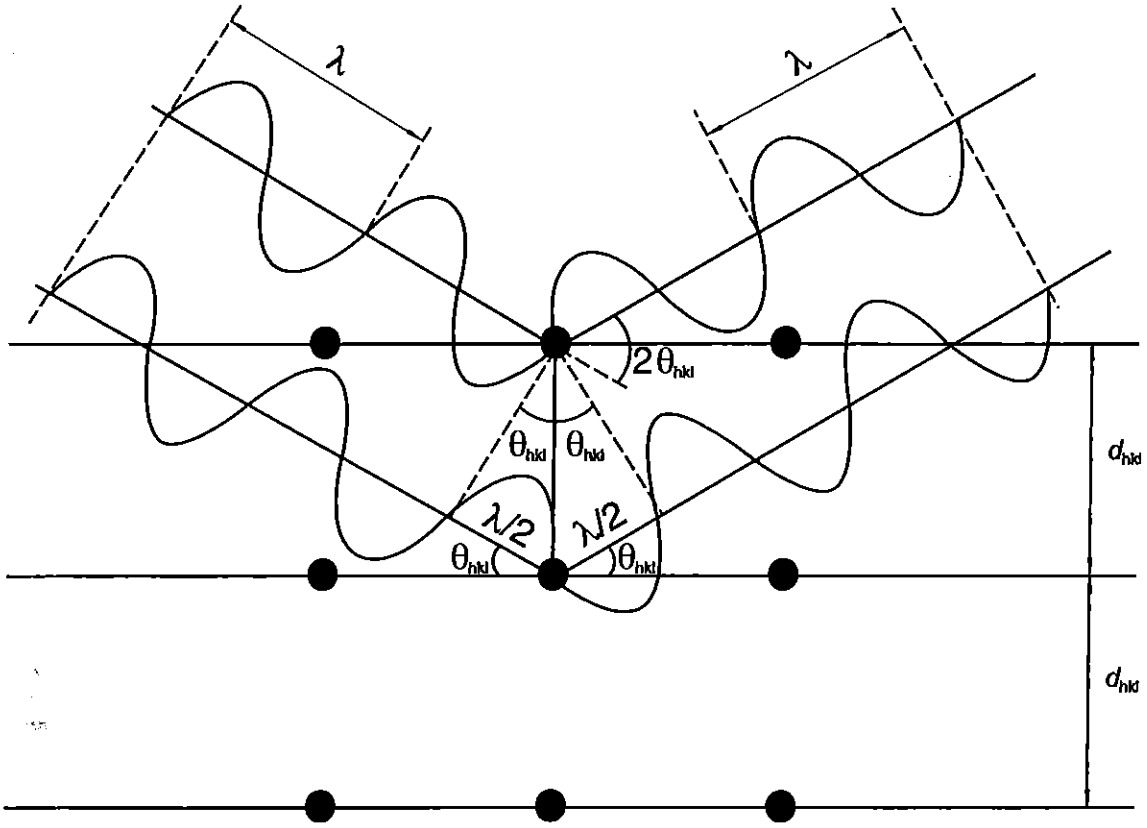


Figure 2.9.33.-1. – *Diffraction of X-rays by a crystal according to Bragg's law*

show higher orders of diffraction where the *d* values for the related families of planes n*h*, n*k*, n*l* are diminished by the factor 1/n (n being an integer: 2, 3, 4, etc.).

Every set of planes throughout a crystal has a corresponding Bragg diffraction angle, θ_{hkl}, associated with it (for a specific wavelength λ).

A powder specimen is assumed to be polycrystalline so that at any angle θ_{hkl} there are always crystallites in an orientation allowing diffraction according to Bragg's law[3]. For a given X-ray wavelength, the positions of the diffraction peaks (also referred to as 'lines', 'reflections' or 'Bragg reflections') are characteristic of the crystal lattice (d-spacings), their theoretical intensities depend on the crystallographic unit cell content (nature and positions of atoms), and the line profiles on the perfection and extent of the crystal lattice. Under these conditions the diffraction peak has a finite intensity arising from atomic arrangement, type of atoms, thermal motion and structural imperfections, as well as from instrument characteristics.

The intensity is dependent upon many factors such as structure factor, temperature factor, crystallinity, polarisation factor, multiplicity and Lorentz factor.

The main characteristics of diffraction line profiles are 2θ position, peak height, peak area and shape (characterised by, for example, peak width or asymmetry, analytical function, empirical representation). An example of the type of powder patterns obtained for 5 different solid phases of a substance are shown in Figure 2.9.33.-2.

[3] *An 'ideal' powder for diffraction experiments consists of a large number of small, randomly oriented spherical crystallites (coherently diffracting crystalline domains). If this number is sufficiently large, there are always enough crystallites in any diffracting orientation to give reproducible diffraction patterns.*

In addition to the diffraction peaks, an X-ray diffraction experiment also generates a more-or-less uniform background, upon which the peaks are superimposed. Besides specimen preparation, other factors contribute to the background, for instance the sample holder, diffuse scattering from air and equipment, other instrumental parameters such as detector noise, general radiation from the X-ray tube, etc. The peak-to-background ratio can be increased by minimising background and by choosing prolonged exposure times.

INSTRUMENT

Instrument set-up

X-ray diffraction experiments are usually performed using powder diffractometers or powder cameras.

A powder diffractometer generally comprises 5 main parts: an X-ray source; incident beam optics, which may perform monochromatisation, filtering, collimation and/or focusing of the beam; a goniometer; diffraction beam optics, which may perform monochromatisation, filtering, collimation and focusing or parallelising of the beam; and a detector. Data-collection and data-processing systems are also required and are generally included in current diffraction measurement equipment.

Depending on the type of analysis to be performed (phase identification, quantitative analysis, lattice parameters determination, etc.), different XRPD instrument configurations and performance levels are required.

The simplest instruments used to measure XRPD patterns are powder cameras. The replacement of photographic film as the detection method by photon detectors has led to the design of diffractometers in which the geometric arrangement of the optics is not truly focusing but parafocusing, such as in the Bragg-Brentano geometry. The Bragg-Brentano parafocusing configuration is currently the most widely used and is therefore briefly described here.

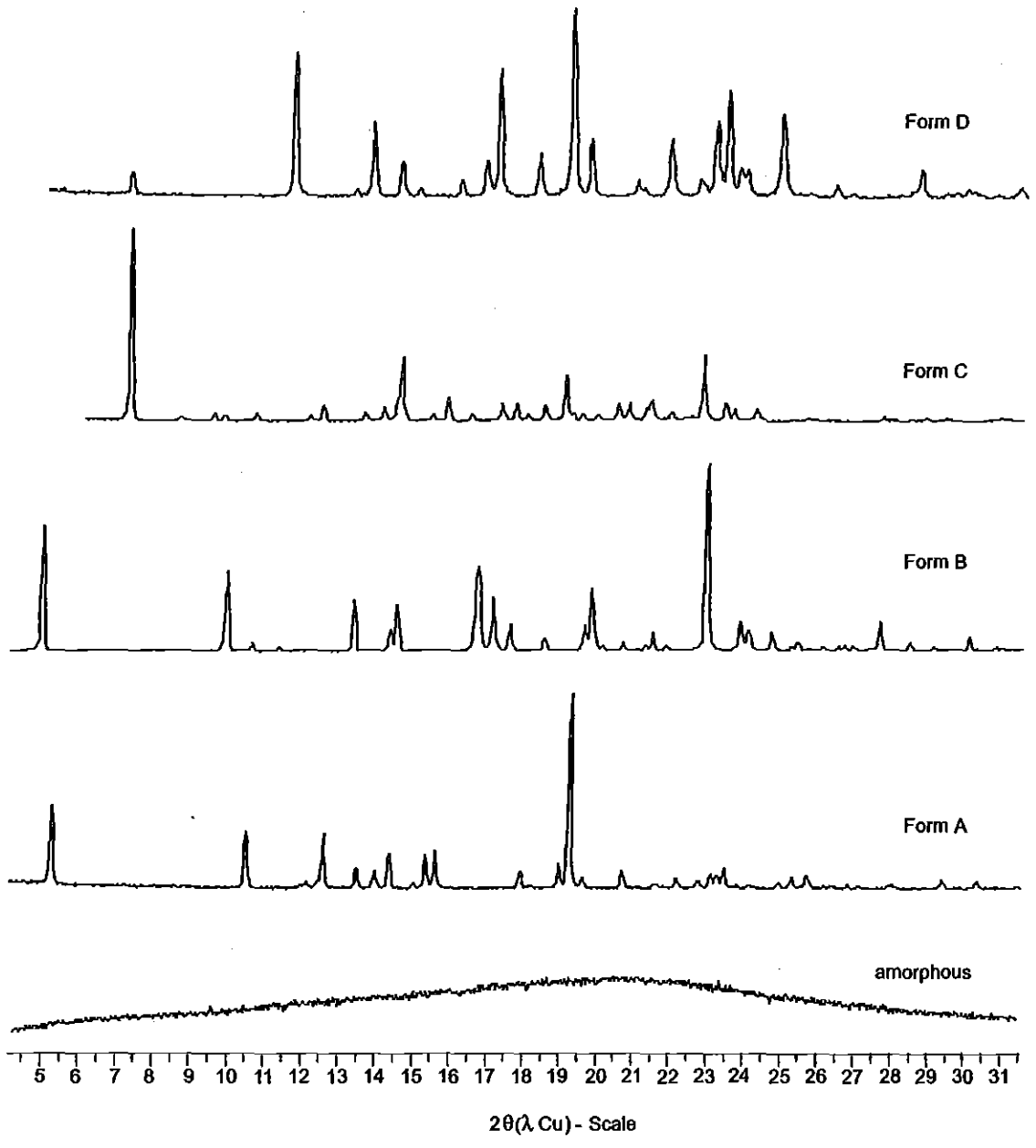


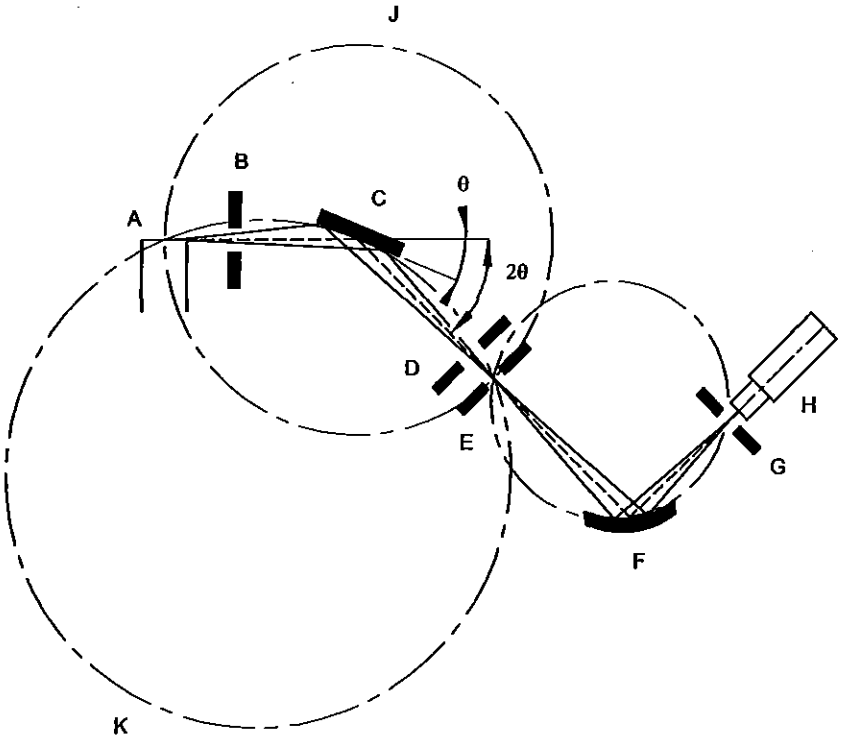
Figure 2.9.33.-2. – *X-ray powder diffraction patterns collected for 5 different solid phases of a substance (the intensities are normalised)*

A given instrument may provide a horizontal or vertical θ/2θ geometry or a vertical θ/θ geometry. For both geometries, the incident X-ray beam forms an angle θ with the specimen surface plane and the diffracted X-ray beam forms an angle 2θ with the direction of the incident X-ray beam (an angle θ with the specimen surface plane).
The basic geometric arrangement is represented in Figure 2.9.33.-3. The divergent beam of radiation from the X-ray tube (the so-called 'primary beam') passes through the parallel plate collimators and a divergence slit assembly and illuminates the flat surface of the specimen. All the rays diffracted by suitably oriented crystallites in the specimen at an angle 2θ converge to a line at the receiving slit. A second set of parallel plate collimators and a scatter slit may be placed either behind or before the receiving slit. The axes of the line focus and of the receiving slit are at equal distances from the axis of the goniometer. The X-ray quanta are counted by a radiation detector, usually a scintillation counter, a sealed-gas proportional counter, or a position-sensitive solid-state detector such as imaging plate or CCD detector. The receiving slit assembly and the detector are coupled together and move tangentially to the focusing circle. For θ/2θ scans the goniometer rotates the specimen about the same axis as that of the detector, but at half the rotational speed, in a θ/2θ motion. The surface of the specimen thus remains tangential to the focusing circle. The parallel plate collimator limits the axial divergence of the beam and hence partially controls the shape of the diffracted line profile.

A diffractometer may also be used in transmission mode. The advantage with this technology is to lessen the effects due to preferred orientation. A capillary of about 0.5-2 mm thickness can also be used for small sample amounts.

X-ray radiation

In the laboratory, X-rays are obtained by bombarding a metal anode with electrons emitted by the thermionic effect and accelerated in a strong electric field (using a high-voltage



A. X-ray tube
B. divergence slit
C. sample
D. anti-diffusion slit
E. receiving slit
F. monochromator
G. detector receiving slit
H. detector
J. diffractometer circle
K. focusing circle

Figure 2.9.33.-3. – *Geometric arrangement of the Bragg-Brentano parafocusing geometry*

generator). Most of the kinetic energy of the electrons is converted to heat, which limits the power of the tubes and requires efficient anode cooling. A 20- to 30-fold increase in brilliance can be obtained using rotating anodes and by using X-ray optics. Alternatively, X-ray photons may be produced in a large-scale facility (synchrotron).

The spectrum emitted by an X-ray tube operating at sufficient voltage consists of a continuous background of polychromatic radiation and additional characteristic radiation that depends on the type of anode. Only this characteristic radiation is used in X-ray diffraction experiments. The principal radiation sources utilised for X-ray diffraction are vacuum tubes utilising copper, molybdenum, iron, cobalt or chromium as anodes; copper, molybdenum or cobalt X-rays are employed most commonly for organic substances (the use of cobalt anodes can be especially preferred to separate distinct X-ray lines).

The choice of radiation to be used depends on the absorption characteristics of the specimen and possible fluorescence by atoms present in the specimen.

The wavelengths used in powder diffraction generally correspond to the K_α radiation from the anode. Consequently, it is advantageous to make the X-ray beam 'monochromatic' by eliminating all the other components of the emission spectrum. This can be partly obtained using K_β filters, i.e. metal filters selected as having an absorption edge between the K_α and K_β wavelengths emitted by the tube.

Such a filter is usually inserted between the X-ray tube and the specimen. Another, more-and-more-commonly used way to obtain a monochromatic X-ray beam is via a large monochromator crystal (usually referred to as a 'monochromator'). This crystal is placed before or behind the specimen and diffracts the different characteristic peaks of the X-ray beam (i.e. K_α and K_β) at different angles, so that only one of them may be selected to enter into the detector. It is even possible to separate $K_{\alpha 1}$ and $K_{\alpha 2}$ radiations by using a specialised monochromator. Unfortunately, the gain in getting a monochromatic beam by using a filter or a monochromator is counteracted by a loss in intensity. Another way of separating K_α and K_β wavelengths is by using curved X-rays mirrors that can simultaneously monochromate and focus or parallelise the X-ray beam.

RADIATION PROTECTION. Exposure of any part of the human body to X-rays can be injurious to health. It is therefore essential that whenever X-ray equipment is used, adequate precautions are taken to protect the operator and any other person in the vicinity. Recommended practice for radiation protection as well as limits for the levels of X-radiation exposure are those established by national legislation in each country. If there are no official regulations or recommendations in a country, the latest recommendations of the International Commission on Radiological Protection should be applied.

SPECIMEN PREPARATION AND MOUNTING

The preparation of the powdered material and mounting of the specimen in a suitable holder are critical steps in many analytical methods, and are particularly so for XRPD analysis, since they can greatly affect the quality of the data to be collected[4]. The main sources of error due to specimen preparation and mounting are briefly discussed here for instruments in Bragg-Brentano parafocusing geometry.

[4] *Similarly, changes in the specimen can occur during data collection in the case of a non-equilibrium specimen (temperature, humidity).*

SPECIMEN PREPARATION

In general, the morphology of many crystalline particles tends to give a specimen that exhibits some degree of preferred orientation in the specimen holder. This is particularly evident for needle-like or plate-like crystals when size reduction yields finer needles or platelets. Preferred orientation in the specimen influences the intensities of various reflections, so that some are more intense and others are less intense, compared to what would be expected from a completely random specimen. Several techniques can be employed to improve randomness in the orientation of crystallites (and therefore to minimise preferred orientation), but further reduction of particle size is often the best and simplest approach. The optimum number of crystallites depends on the diffractometer geometry, the required resolution and the specimen attenuation of the X-ray beam. In some cases, particle sizes as large as 50 µm will provide satisfactory results in phase identification. However, excessive milling (crystallite sizes less than approximately 0.5 µm) may cause line broadening and significant changes to the sample itself such as:

— specimen contamination by particles abraded from the milling instruments (mortar, pestle, balls, etc.);
— reduced degree of crystallinity;
— solid-state transition to another polymorph;
— chemical decomposition;
— introduction of internal stress;
— solid-state reactions.

Therefore, it is advisable to compare the diffraction pattern of the non-ground specimen with that corresponding to a specimen of smaller particle size (e.g. a milled specimen). If the XRPD pattern obtained is of adequate quality considering its intended use, then grinding may not be required.

It should be noted that if a sample contains more than one phase and if sieving is used to isolate particles to a specific size, the initial composition may be altered.

SPECIMEN MOUNTING

Effect of specimen displacement

A specimen surface that is offset by D with reference to the diffractometer rotation axis causes systematic errors that are very difficult to avoid entirely; for the reflection mode, this results in absolute $D \cdot \cos\theta$ shifts[5] in 2θ positions (typically of the order of 0.01° in 2θ at low angles ($\cos\theta \simeq 1$) for a displacement D = 15 µm) and asymmetric broadening of the profile towards low 2θ values. Use of an appropriate internal standard allows the detection and correction of this effect simultaneously with that arising from specimen transparency. This is by far the largest source of errors in data collected on well-aligned diffractometers.

Effect of specimen thickness and transparency

When the XRPD method in reflection mode is applied, it is often preferable to work with specimens of 'infinite thickness'. To minimise the transparency effect, it is advisable to use a non-diffracting substrate (zero background holder), for example a plate of single crystalline silicon cut parallel to the 510 lattice planes[6]. One advantage of the transmission mode is that problems with sample height and specimen transparency are less important. The use of an appropriate internal standard allows the detection and correction of this effect simultaneously with that arising from specimen displacement.

CONTROL OF THE INSTRUMENT PERFORMANCE

Goniometers and the corresponding incident and diffracted X-ray beam optics have many mechanical parts that need adjustment. The degree of alignment or misalignment directly influences the quality of the results of an XRPD investigation. Therefore, the different components of the diffractometer must be carefully adjusted (optical and mechanical systems, etc.) to minimise adequately systematic errors, while optimising the intensities received by the detector. The search for maximum intensity and maximum resolution is always antagonistic when aligning a diffractometer. Hence, the best compromise must be sought whilst performing the alignment procedure. There are many different configurations and each supplier's equipment requires specific alignment procedures.

The overall diffractometer performance must be tested and monitored periodically using suitable certified reference materials. Depending on the type of analysis, other well-defined reference materials may also be employed, although the use of certified reference materials is preferred.

QUALITATIVE PHASE ANALYSIS (IDENTIFICATION OF PHASES)

The identification of the phase composition of an unknown sample by XRPD is usually based on the visual or computer-assisted comparison of a portion of its XRPD pattern to the experimental or calculated pattern of a reference material. Ideally, these reference patterns are collected on well-characterised single-phase specimens. This approach makes it possible in most cases to identify a crystalline substance by its 2θ diffraction angles or d-spacings and by its relative intensities. The computer-aided comparison of the diffraction pattern of the unknown sample to the comparison data can be based either on a more-or-less extended 2θ-range of the whole diffraction pattern or on a set of reduced data derived from the pattern. For example, the list of d-spacings and normalised intensities (I_{norm}), a so-called (d, I_{norm})-list extracted from the pattern, is the crystallographic fingerprint of the material, and can be compared to (d, I_{norm})-lists of single-phase samples compiled in databases.

For most organic crystals, when using Cu K_α radiation, it is appropriate to record the diffraction pattern in a 2θ-range from as near 0° as possible to at least 40°. The agreement in the 2θ-diffraction angles between specimen and reference is within 0.2° for the same crystal form, while relative intensities between specimen and reference may vary considerably due to preferred orientation effects. By their very nature, variable hydrates and solvates are recognised to have varying unit cell dimensions and as such shifting occurs in peak positions of the measured XRPD patterns for these materials. In these unique materials, variance in 2θ-positions of greater than 0.2° is not unexpected. As such, peak position variances such as 0.2° are not applicable to these materials. For other types of samples (e.g. inorganic salts), it may be necessary to extend the 2θ-region scanned to well beyond 40°. It is generally sufficient to scan past the 10 strongest reflections identified in single phase XRPD database files.

It is sometimes difficult or even impossible to identify phases in the following cases:

— non-crystallised or amorphous substances;

[5] *Note that a goniometer zero alignment shift would result in constant shift on all observed 2θ-line positions, in other words, the whole diffraction pattern is in this case translated by an offset of Z° in 2θ.*

[6] *In the case of a thin specimen with low attenuation, accurate measurements of line positions can be made with focusing diffractometer configurations in either transmission or reflection geometry. Accurate measurements of line positions on specimens with low attenuation are preferably made using diffractometers with parallel beam optics. This helps to reduce the effects of specimen thickness.*

— the components to be identified are present in low mass fractions of the analyte amounts (generally less than 10 per cent *m/m*);
— pronounced preferred orientation effects;
— the phase has not been filed in the database used;
— formation of solid solutions;
— presence of disordered structures that alter the unit cell;
— the specimen comprises too many phases;
— presence of lattice deformations;
— structural similarity of different phases;

QUANTITATIVE PHASE ANALYSIS

If the sample under investigation is a mixture of 2 or more known phases, of which not more than 1 is amorphous, the percentage (by volume or by mass) of each crystalline phase and of the amorphous phase can, in many cases, be determined. Quantitative phase analysis can be based on the integrated intensities, on the peak heights of several individual diffraction lines[7], or on the full pattern. These integrated intensities, peak heights or full-pattern data points are compared to the corresponding values of reference materials. These reference materials shall be single-phase or a mixture of known phases. The difficulties encountered during quantitative analysis are due to specimen preparation (the accuracy and precision of the results require in particular homogeneity of all phases and a suitable particle size distribution in each phase) and to matrix effects.
In favourable cases, amounts of crystalline phases as small as 10 per cent may be determined in solid matrices.

POLYMORPHIC SAMPLES

For a sample composed of 2 polymorphic phases a and b, the following expression may be used to quantify the fraction F_a of phase a:

$$F_a = \frac{1}{1 + K(I_b/I_a)}$$

The fraction is derived by measuring the intensity ratio between the 2 phases, knowing the value of the constant K. K is the ratio of the absolute intensities of the 2 pure polymorphic phases I_{oa}/I_{ob}. Its value can be determined by measuring standard samples.

METHODS USING A STANDARD

The most commonly used methods for quantitative analysis are:
— the 'external standard method';
— the 'internal standard method';
— the 'spiking method' (often also called the 'standard addition method').

The 'external standard method' is the most general method and consists of comparing the X-ray diffraction pattern of the mixture, or the respective line intensities, with those measured in a reference mixture or with the theoretical intensities of a structural model, if it is fully known.

To limit errors due to matrix effects, an internal reference material with crystallite size and X-ray absorption coefficient comparable to those of the components of the sample, and with a diffraction pattern that does not overlap at all that of the sample to be analysed, can be used. A known quantity of this reference material is added to the sample to be analysed and to each of the reference mixtures. Under these conditions, a linear relationship between line intensity and concentration exists. This application, called the 'internal standard method', requires a precise measurement of diffraction intensities.

In the 'spiking method' (or 'standard addition method'), some of the pure phase a is added to the mixture containing the unknown concentration of a. Multiple additions are made to prepare an intensity-versus-concentration plot in which the negative x intercept is the concentration of the phase a in the original sample.

ESTIMATE OF THE AMORPHOUS AND CRYSTALLINE FRACTIONS

In a mixture of crystalline and amorphous phases, the crystalline and amorphous fractions can be estimated in several ways. The choice of the method used depends on the nature of the sample:
— if the sample consists of crystalline fractions and an amorphous fraction of different chemical compositions, the amounts of each of the individual crystalline phases may be estimated using appropriate standard substances as described above; the amorphous fraction is then deduced indirectly by subtraction;
— if the sample consists of one amorphous and one crystalline fraction, either as a 1-phase or a 2-phase mixture, with the same elemental composition, the amount of the crystalline phase ('the degree of crystallinity') can be estimated by measuring 3 areas of the diffractogram:

A = total area of the peaks arising from diffraction from the crystalline fraction of the sample;
B = total area below area A;
C = background area (due to air scattering, fluorescence, equipment, etc).

When these areas have been measured, the degree of crystallinity can be roughly estimated using the following formula:

$$\% \text{ crystallinity} = 100A/(A + B - C)$$

It is noteworthy that this method does not yield absolute degree-of-crystallinity values and hence is generally used for comparative purposes only.

More sophisticated methods are also available, such as the Ruland method.

SINGLE CRYSTAL STRUCTURE

In general, the determination of crystal structures is performed from X-ray diffraction data obtained using single crystals. However, crystal structure analysis of organic crystals is a challenging task, since the lattice parameters are comparatively large, the symmetry is low and the scattering properties are normally very low.

For any given crystalline form of a substance, knowledge of the crystal structure allows the calculation of the corresponding XRPD pattern, thereby providing a 'preferred-orientation-free' reference XRPD pattern, which may be used for phase identification.

[7] *If the crystal structures of all components are known, the Rietveld method can be used to quantify them with good accuracy. If the crystal structures of the components are not known, the Pawley or least squares methods can be used.*

R. Porosity and Pore-size Distribution of Solids by Mercury Porosimetry

(Ph. Eur. method 2.9.32)

INTRODUCTION

In general, different types of pores may be pictured as apertures, channels or cavities within a solid body, or as space (i.e. interstices or voids) between solid particles in a bed, compact or aggregate. Porosity is a term that is often used to indicate the porous nature of solid material, and is more precisely defined as the ratio of the volume of accessible pores and voids to the total volume occupied by a given amount of the solid. In addition to the accessible pores, a solid may contain closed pores, which are isolated from the external surface and into which fluids are not able to penetrate. The characterisation of closed pores, i.e. cavities with no access to an external surface, is not covered in this chapter.

Porous materials may take the form of fine or coarse powders, compacts, extrudates, sheets or monoliths. Their characterisation usually involves the determination of the total pore volume or porosity as well as the pore-size distribution.

It is well established that the performance of a porous solid (e.g. its strength, reactivity, permeability or adsorbent power) is dependent upon its pore structure. Many different methods have been developed for the characterisation of pore structure. In view of the complexity of most porous solids, it is not surprising to find that the results obtained are not always in agreement and that no single technique can be relied upon to provide a complete picture of the pore structure. The choice of the most appropriate method depends on the application of the porous solid, its chemical and physical nature and the range of pore-size.

This chapter provides guidance for measurement of porosity and pore-size distribution by mercury porosimetry. It is a comparative test, usually destructive, in which the volume of mercury penetrating a pore or void is determined as a function of an applied hydrostatic pressure, which can be related to a pore diameter. Other information such as pore shape and inter-connectivity, the internal and external surface area, powder granulometry, bulk and tapped density could also be inferred from volume-pressure curves; however, these aspects of the technique do not fall under the scope of this chapter.

Practical considerations presently limit the maximum applied absolute pressure reached by some equipment to about 400 MPa, corresponding to a minimum equivalent pore diameter of approximately 0.003 µm. The maximum diameter will be limited for samples having a significant depth due to the difference in hydrostatic head of mercury from the top to the bottom of the sample. For most purposes this limit may be regarded as 400 µm.

Inter-particle and intra-particle porosity can be determined, but the method does not distinguish between these porosities where they co-exist.

The method is suitable for the study of most porous materials. Samples that amalgamate with mercury, such as certain metals, may be unsuitable for this technique or may require a preliminary passivation. Other materials may deform or compact under the applied pressure. In some cases it may be possible to apply sample-compressibility corrections and useful comparative data may still be obtained.

Mercury porosimetry is considered to be comparative, as for most porous media a theory is not available to allow an absolute calculation of results of pore-size distribution. Therefore this technique is mainly recommended for development studies.

Mercury is toxic. Appropriate precautions must be observed to safeguard the health of the operator and others working in the area. Waste material must also be disposed of in a suitable manner, according to local regulations.

PRINCIPLE

The technique is based on the measurement of the mercury volume intruded into a porous solid as a function of the applied pressure. The measurement includes only those pores into which mercury can penetrate at the pressure applied.

A non-wetting liquid penetrates into a porous system only under pressure. The pressure to be applied is in inverse proportion to the inner diameter of the pore aperture. In the case of cylindrical pores, the correlation between pore diameter and pressure is given by the Washburn equation:

$$d_p = -\frac{4 \cdot \sigma}{p} \cos\theta$$

d_p = pore diameter, in metres;
σ = surface tension, in newtons per metre;
θ = contact angle of mercury on the sample, in degrees;
p = applied pressure, in pascals.

APPARATUS

The sample holder, referred to as penetrometer or dilatometer, has a calibrated capillary tube, through which the sample can be evacuated and through which mercury can enter. The capillary tube is attached to a wider tube in which the test sample is placed. The change in the volume of mercury intruded is usually measured by the change in capacitance between the mercury column in the capillary tube and a metal sleeve around the outside of the capillary tube. If precise measurements are required the expected total void and pore volume of the sample should be between 20 per cent and 90 per cent of the internal volume of the capillary tube. Since different materials exhibit a wide range of open porosities, a number of penetrometers with different capillary tube diameters and sample volumes may be required. A typical set-up for a mercury porosimeter instrument is given in Figure 2.9.32.-1. The porosimeter may have separate ports for high- and low-pressure operation, or the low-pressure measurement may be carried out on a separate unit.

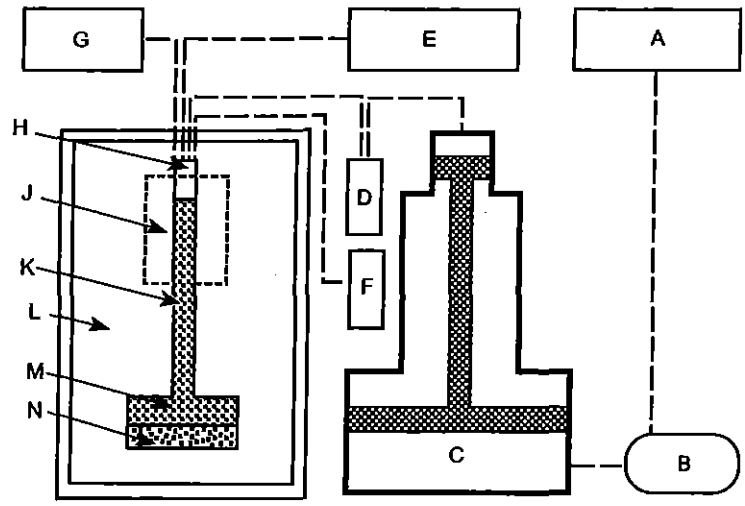
The pressure range is typically 4-300 kPa for low-pressure operation and above 300 kPa for high-pressure operation, depending on the design of the particular apparatus and on the intended use.

METHOD

Sample preparation

The sample is pre-treated to remove adsorbed material that can obscure its accessible porosity, for example by heating and/or evacuation, or by flowing inert gas. It may be possible to passivate the surface of wettable or amalgam-forming solids, for example by producing a thin layer of oxide, or by coating with stearate.

The sample of the pre-treated solid is weighed and transferred to the penetrometer. The pore system of the sample is then degassed in a vacuum to a maximum residual pressure of 7 Pa.



A. Low-pressure hydraulic fluid reservoir
B. Hydraulic pump
C. Pressure multiplier
D. Pressure transducer
E. High-pressure hydraulic fluid reservoir
F. Vacuum pump with gauge
G. Mercury reservoir
H. Oil
J. Penetration volume indicator
K. Capillary tube
L. High-pressure chamber
M. Mercury
N. Sample

Figure 2.9.32.-1. – *Example of the set-up of a mercury porosimeter instrument*

Filling the penetrometer with mercury

The mercury used is of analytical quality. Overlay the sample with mercury under vacuum. The vacuum is required to ensure the transfer of mercury from the reservoir to the penetrometer. In a filled penetrometer the filling pressure comprises the applied pressure plus the pressure contribution created by the head of mercury contacting the sample. A typical filling pressure would be about 4 kPa. The hydrostatic pressure of the mercury over the sample may be minimised by filling the penetrometer in the horizontal position.

Low-pressure measurement

Admit air or nitrogen in a controlled manner to increase the pressure either in stages corresponding to the particular pore sizes of interest, or continuously at a slow rate. The concomitant change in the length of the mercury column in the capillary tube is recorded. When the maximum required pressure has been reached, return to atmospheric pressure.

High-pressure measurement

After measurement at low pressure, the penetrometer filled with mercury is transferred to the high-pressure port or unit of the instrument and overlaid with hydraulic fluid. Mercury is intruded into the pore system via the hydraulic fluid. Increase the pressure in the system to the maximum pressure reached in the low-pressure measurement and record the intrusion volume at this pressure, since subsequent intrusion volumes are calculated from this initial volume. Increase the pressure either in stages corresponding to the particular pore sizes of interest, or continuously at a slow rate. The fall in the mercury column is measured up to the maximum required pressure. If required the pressure may be decreased either in stages or continuously at a slow rate to determine the mercury extrusion curve.

Corrections are made to take account of changes in the volume of the mercury, the penetrometer and other components of the volume detector system under elevated pressure. The extent of the corrections may be determined by means of blank measurements under the same conditions.

An experimentally determined volume-pressure curve is shown in Figure 2.9.32.-2.

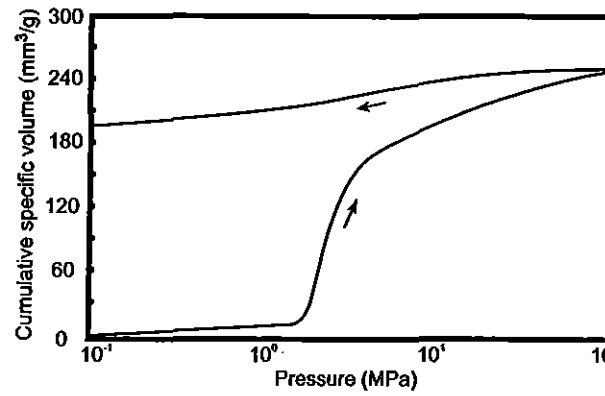


Figure 2.9.32.-2. – *Volume-pressure curve as semilogarithmic plot*

REPORTING OF RESULTS

The pressure readings can be converted to pore diameters by means of the Washburn equation or by another model.

The surface tension of mercury (σ) depends not only on the temperature, but also, in the case of markedly curved surfaces areas, on the radius of curvature. In general, values between 0.41 $N \cdot m^{-1}$ and 0.52 $N \cdot m^{-1}$ are measured at room temperature. If the value is not known, σ = 0.48 $N \cdot m^{-1}$ can be used.

The contact angle of mercury (θ) in most cases is more than 90°. It may be determined using a contact angle instrument. If the value is not known, θ = 130° can be used. The values of contact angle and surface tension and the model used in the calculation are reported.

Visualisation of the data can be done with several types of graphs. Frequently, in a graphical representation the pore diameter is plotted on the abscissa and the intruded volume

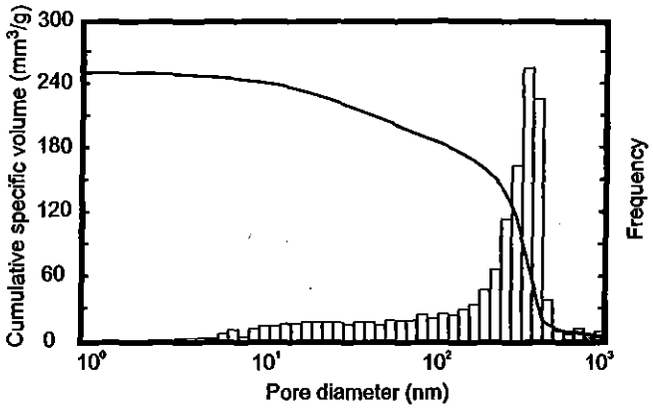


Figure 2.9.32.-3. – *Pore-size distribution as semilogarithmic plots of the cumulative and the normalised density distribution*

per sample mass on the ordinate to give the pore-size distribution. It is appropriate here to choose a logarithmic scale for the abscissa (see Figure 2.9.32.-3). The spaces between the particles of the solid sample are included as pores in the calculation. If the pores differ in size from the voids, the latter can be separated by choosing the appropriate pore-size range.

Extrusion curves may not be used for calculating the pore-size distribution (for hysteresis, see Figure 2.9.32.-2), because an intruded part of the mercury always remains in the pore system. The retention ratio may however be useful for the qualitative characterisation of pores that are only accessible via narrow openings ('ink-bottle pores').

Most common characteristic values, such as the total intruded specific volume and the mean and median pore diameters, are calculated from the pore-size distribution. Moreover, sufficient information must be documented about the sample, the sample preparation, the evacuation conditions and the instrument used.

CONTROL OF INSTRUMENT PERFORMANCE

As mercury porosimetry is considered to be used as a comparative test, no details are given in this chapter. However, it is recommended that a stable comparison material is tested on a regular basis to monitor instrument calibration and performance.

S. Bulk Density and Tapped Density of Powders[1]

(Ph. Eur. method 2.9.34)

BULK DENSITY

The bulk density of a powder is the ratio of the mass of an untapped powder sample to its volume, including the contribution of the interparticulate void volume. Hence, the bulk density depends on both the density of powder particles and the spatial arrangement of particles in the powder bed. The bulk density is expressed in grams per millilitre despite the International Unit being kilogram per cubic metre (1 g/mL = 1000 kg/m^3), because the measurements are made using cylinders. It may also be expressed in grams per cubic centimetre.

The bulking properties of a powder are dependent upon the preparation, treatment and storage of the sample, i.e. how it has been handled. The particles can be packed to have a range of bulk densities and, moreover, the slightest disturbance of the powder bed may result in a changed bulk density. Thus, the bulk density of a powder is often very difficult to measure with good reproducibility and, in reporting the results, it is essential to specify how the determination was made.

The bulk density of a powder is determined either by measuring the volume of a known mass of powder sample, which may have been passed through a sieve, in a graduated cylinder (Method 1), or by measuring the mass of a known volume of powder that has been passed through a volumeter into a cup (Method 2) or has been introduced into a measuring vessel (Method 3).

Methods 1 and 3 are favoured.

METHOD 1: MEASUREMENT IN A GRADUATED CYLINDER

Procedure Pass a quantity of powder sufficient to complete the test through a sieve with apertures greater than or equal to 1.0 mm, if necessary, to break up agglomerates that may have formed during storage; this must be done gently to avoid changing the nature of the material. Into a dry, graduated, 250 mL cylinder (readable to 2 mL), gently introduce, without compacting, approximately 100 g (m) of the test sample weighed with 0.1 per cent accuracy.

If necessary, carefully level the powder without compacting, and read the unsettled apparent volume (V_0) to the nearest graduated unit. Calculate the bulk density in grams per millilitre using the formula m/V_0. Generally, replicate determinations are desirable for the determination of this property.

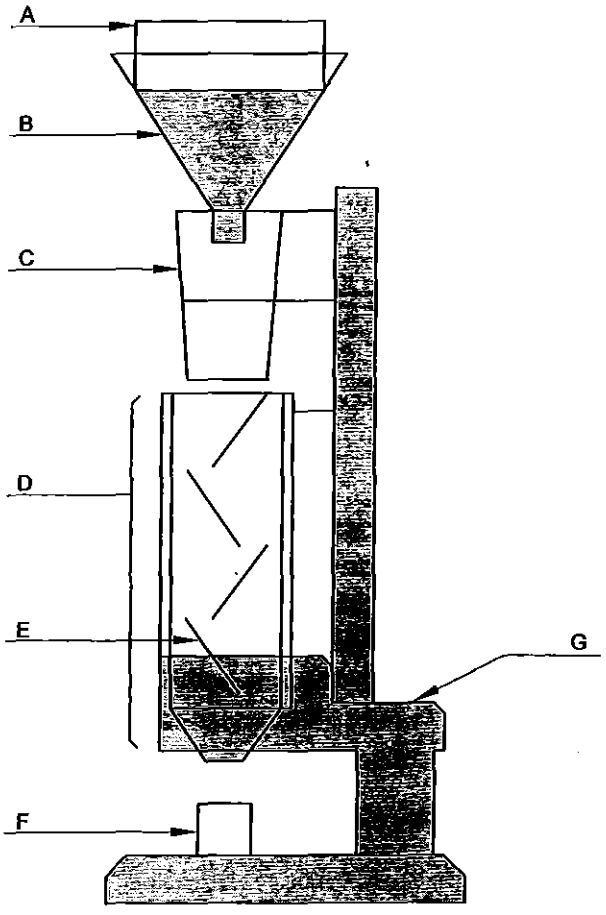
If the powder density is too low or too high, such that the test sample has an untapped apparent volume of more than 250 mL or less than 150 mL, it is not possible to use 100 g of powder sample. In this case, a different amount of powder is selected as the test sample, such that its untapped apparent volume is between 150 mL and 250 mL (apparent volume greater than or equal to 60 per cent of the total volume of the cylinder); the mass of the test sample is specified in the expression of results.

For test samples having an apparent volume between 50 mL and 100 mL, a 100 mL cylinder readable to 1 mL can be used; the volume of the cylinder is specified in the expression of results.

[1] *This chapter has undergone pharmacopoeial harmonisation. See chapter 5.8. Pharmacopoeial harmonisation.*

METHOD 2: MEASUREMENT IN A VOLUMETER

Apparatus The apparatus (Figure 2.9.34.-1) consists of a top funnel fitted with a 1.0 mm sieve, mounted over a baffle box containing 4 glass baffles over which the powder slides and bounces as it passes. At the bottom of the baffle box is a funnel that collects the powder and allows it to pour into a cup mounted directly below it. The cup may be cylindrical (25.00 ± 0.05 mL volume with an internal diameter of 30.00 ± 2.00 mm) or cubical (16.39 ± 0.20 mL volume with internal dimensions of 25.400 ± 0.076 mm).



A. 1.0 mm sieve
B. powder funnel
C. loading funnel
D. baffle box
E. glass baffle
F. cup
G. stand

Figure 2.9.34.-1. – *Volumeter*

Procedure Allow an excess of powder to flow through the apparatus into the sample receiving cup until it overflows, using a minimum of 25 cm^3 of powder with the cubical cup and 35 cm^3 of powder with the cylindrical cup. Carefully, scrape excess powder from the top of the cup by smoothly moving the edge of the blade of a spatula perpendicular to and in contact with the top surface of the cup, taking care to keep the spatula perpendicular to prevent packing or removal of powder from the cup. Remove any material from the side of the cup and determine the mass (M) of the powder to the nearest 0.1 per cent. Calculate the bulk density in grams per millilitre using the formula M/V_0 (where V_0 is the volume of the cup) and record the average of 3 determinations using 3 different powder samples.

METHOD 3: MEASUREMENT IN A VESSEL

Apparatus The apparatus consists of a 100 mL cylindrical vessel of stainless steel with dimensions as specified in Figure 2.9.34.-2.

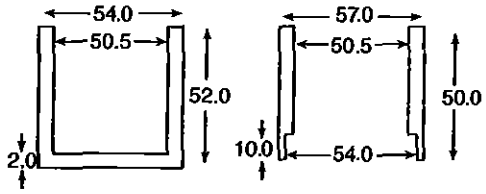


Figure 2.9.34.-2. – *Measuring vessel (left) and cap (right) Dimensions in millimetres*

Procedure Pass a quantity of powder sufficient to complete the test through a 1.0 mm sieve, if necessary, to break up agglomerates that may have formed during storage, and allow the obtained sample to flow freely into the measuring vessel until it overflows. Carefully scrape the excess powder from the top of the vessel as described under Method 2. Determine the mass (M_0) of the powder to the nearest 0.1 per cent by subtracting the previously determined mass of the empty measuring vessel. Calculate the bulk density in grams per millilitre using the formula $M_0/100$ and record the average of 3 determinations using 3 different powder samples.

TAPPED DENSITY

The tapped density is an increased bulk density attained after mechanically tapping a receptacle containing the powder sample.

The tapped density is obtained by mechanically tapping a graduated measuring cylinder or vessel containing the powder sample. After observing the initial powder volume or mass, the measuring cylinder or vessel is mechanically tapped, and volume or mass readings are taken until little further volume or mass change is observed. The mechanical tapping is achieved by raising the cylinder or vessel and allowing it to drop, under its own mass, a specified distance by one of 3 methods as described below. Devices that rotate the cylinder or vessel during tapping may be preferred to minimise any possible separation of the mass during tapping down.

METHOD 1

Apparatus The apparatus (Figure 2.9.34.-3) consists of the following:

— a 250 mL graduated cylinder (readable to 2 mL) with a mass of 220 ± 44 g;

— a settling apparatus capable of producing, per minute, either nominally 250 ± 15 taps from a height of 3 ± 0.2 mm, or nominally 300 ± 15 taps from a height of 14 ± 2 mm. The support for the graduated cylinder, with its holder, has a mass of 450 ± 10 g.

Procedure Proceed as described above for the determination of the bulk volume (V_0). Secure the cylinder in the support. Carry out 10, 500 and 1250 taps on the same powder sample and read the corresponding volumes V_{10}, V_{500} and V_{1250} to the nearest graduated unit. If the difference between V_{500} and V_{1250} is less than or equal to 2 mL, V_{1250} is the tapped volume. If the difference between V_{500} and V_{1250} exceeds 2 mL, repeat in increments of, for example, 1250 taps, until the difference between successive measurements is less than or equal to 2 mL. Fewer taps may be appropriate for some powders, when validated. Calculate

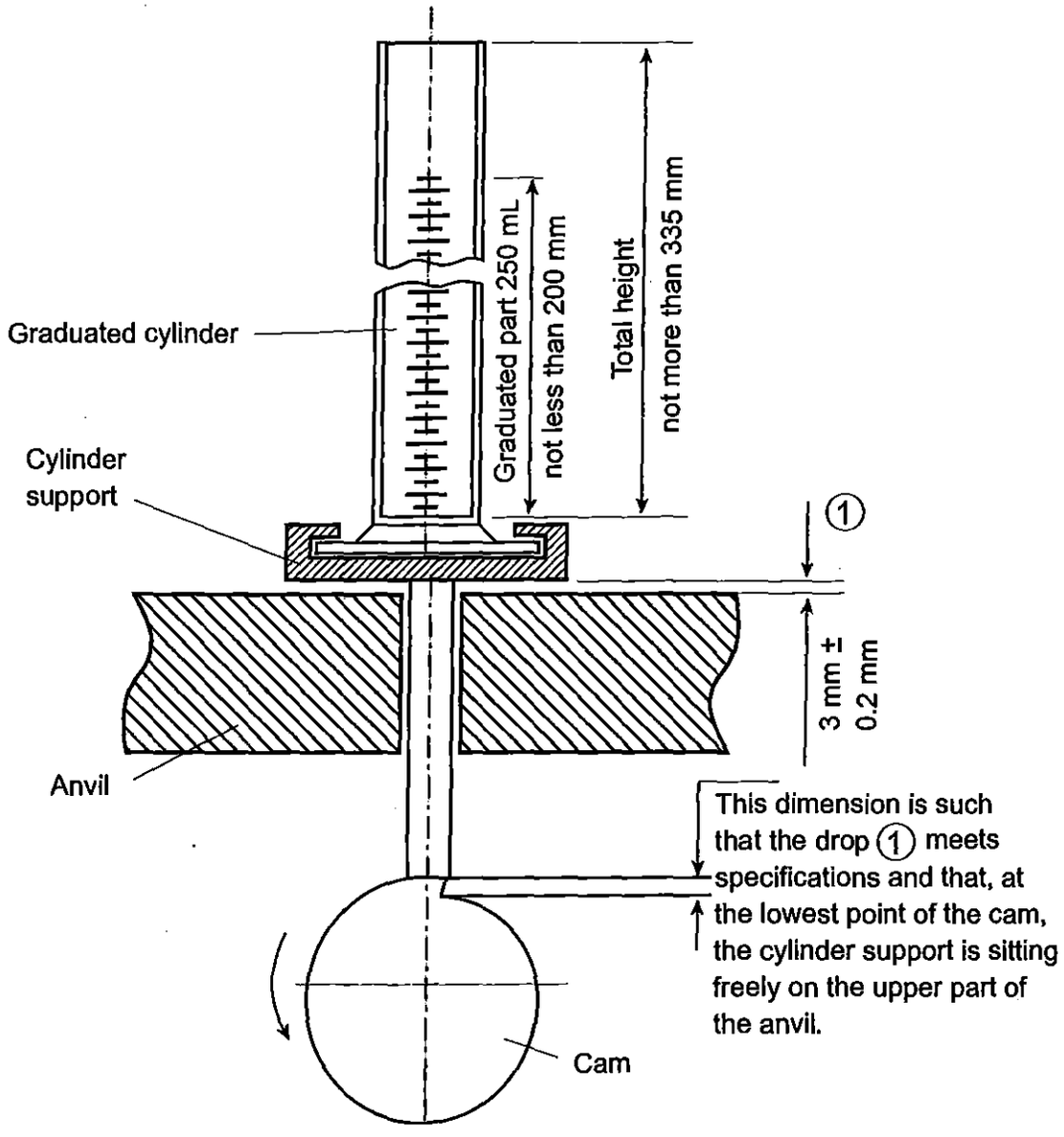


Figure 2.9.34.-3. – *Settling device for powder samples*
Dimensions in millimetres

the tapped density in grams per millilitre using the formula m/V_f (where V_f is the final tapped volume). Generally, replicate determinations are desirable for the determination of this property. Specify the drop height with the results.

If it is not possible to use a 100 g test sample, use a reduced amount and a suitable 100 mL graduated cylinder (readable to 1 mL) weighing 130 ± 16 g and mounted on a support weighing 240 ± 12 g. If the difference between V_{500} and V_{1250} is less than or equal to 1 mL, V_{1250} is the tapped volume. If the difference between V_{500} and V_{1250} exceeds 1 mL, repeat in increments of, for example, 1250 taps, until the difference between successive measurements is less than or equal to 1 mL. The modified test conditions are specified in the expression of the results.

METHOD 2

Procedure Proceed as directed under Method 1 except that the mechanical tester provides a fixed drop of 3 ± 0.2 mm at a nominal rate of 250 taps per minute.

METHOD 3

Procedure Proceed as described under Method 3 for measuring the bulk density, using the measuring vessel equipped with the cap shown in Figure 2.9.34.-2. The measuring vessel with the cap is lifted 50-60 times per minute by the use of a suitable tapped density tester. Carry out 200 taps, remove the cap and carefully scrape excess powder from the top of the measuring vessel as described under Method 3 for measuring the bulk density. Repeat the procedure using 400 taps. If the difference between the 2 masses obtained after 200 and 400 taps exceeds 2 per cent, repeat the test using 200 additional taps until the difference between successive measurements is less than 2 per cent. Calculate the tapped density in grams per millilitre using the formula $M_f/100$ (where M_f is the mass of powder in the measuring vessel). Record the average of 3 determinations using 3 different powder samples. The test conditions, including tapping height, are specified in the expression of the results.

MEASURES OF POWDER COMPRESSIBILITY

Because the interparticulate interactions influencing the bulking properties of a powder are also the interactions that interfere with powder flow, a comparison of the bulk and tapped densities can give a measure of the relative importance of these interactions in a given powder. Such a comparison is often used as an index of the ability of the powder to flow, for example the compressibility index or the Hausner ratio.

The compressibility index and Hausner ratio are measures of the propensity of a powder to be compressed as described above. As such, they are measures of the powder's ability to settle, and they permit an assessment of the relative importance of interparticulate interactions. In a free-flowing powder, such interactions are less significant, and the bulk and tapped densities will be closer in value. For more-poorly flowing materials, there are frequently greater interparticulate interactions, and a greater difference between the bulk and tapped densities will be observed. These differences are reflected in the compressibility index and the Hausner ratio.

Compressibility index:

$$\frac{100(V_0 - V_f)}{V_0}$$

V_0 = unsettled apparent volume;
V_f = final tapped volume.

Hausner Ratio:

$$\frac{V_0}{V_f}$$

Depending on the material, the compressibility index can be determined using V_{10} instead of V_0. If V_{10} is used, it is clearly stated with the results.

T. Wettability of Porous Solids Including Powders

(Ph. Eur. method 2.9.45)

INTRODUCTION

The wettability of solid surfaces is commonly characterised by direct or indirect contact angle measurements.

The contact angle (θ) between a liquid and a solid is the angle naturally formed when a drop of a liquid is placed on a solid surface. This is depicted in Figure 2.9.45.-1. For a given liquid, wettable solids show a low contact angle and non-wettable solids show a contact angle of 90° or more.

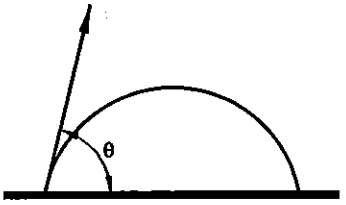


Figure 2.9.45.-1. – *Contact angle (θ) of a sessile drop observed on a non-porous surface*

2 methods for the determination of wettability are described below. The methods are capable of measuring the wettability of porous solids like powders or granules. Both methods express the wettability by a contact angle measurement between the porous solid and a given liquid.

The sessile drop method is based on direct measurement of a contact angle of a sessile drop on a compacted powder disc.

With the Washburn method the contact angle is indirectly measured. The method is based on the capillary effect of the powder pores. The effect (mass gain) is recorded by special electronic balances starting the moment when the powder sample touches the surface of a liquid, preferably not dissolving or poorly dissolving the sample. The measurement has very little or no effect on the state of the powder.

Any pre-treatment of the sample to be examined is disadvantageous, since the properties may be significantly altered. For example, the compaction of a powder as a disc may decrease the surface free energy when the crystalline state of the powder is changed (e.g. metastable forms), or may increase surface free energy by creating crystal defects (disadvantage of the sessile drop method since compacted powder discs are tested).

The methods are usually applied to examine the following parameters:

— batch-to-batch consistency of samples in terms of wettability;
— effect of liquid viscosity on wettability;
— effect of surface tension of a liquid on wettability;
— alteration of surface properties of samples.

SESSILE DROP METHOD

This method may be used to characterise directly the wettability of coatings and compacted formulations such as tablets. Moreover, it is sometimes possible to use the sessile drop instrument in a dynamic measurement (dynamic contact angle measurement, Figure 2.9.45.-2) of porous solid/liquid systems where the contact angle decreases. By taking several contact angle measurements as a function of time, the rate of spreading accompanied by penetration of a liquid droplet into a slightly porous solid may be studied.

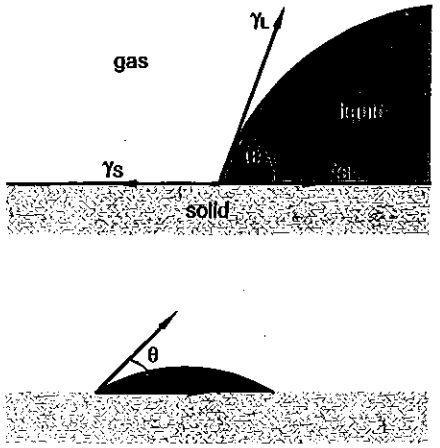


Figure 2.9.45.-2. – *Sessile drop determination with visual inspection of the droplet*

Under equilibrium conditions the contact angle of a sessile drop depends on 3 interrelated surface tensions and is determined using Young's equation (see Figure 2.9.45.-2, 1st part):

$$\gamma_S = \gamma_{SL} + \gamma_L \cos\theta$$

γ_S = surface tension of the solid with air;
γ_{SL} = interfacial tension of the solid with the liquid;
γ_L = surface tension of the liquid with air.

PROCEDURE

Since powders are unable to form a completely flat surface, the powder is usually compacted as a disc in an attempt to make the surface smoother. A liquid drop with a given volume is placed on the disc (see Figure 2.9.45.-2) allowing direct measurement of the contact angle using a goniometer fitted with an eyepiece protractor, or by geometric construction on a photomicrograph. Other physical and mathematical procedures of data analysis may also be appropriate. The drop volume may influence the result. Several determinations of the contact angle (θ) (n = 6) are usually carried out and the average is calculated.

WASHBURN METHOD

The Washburn method is able to measure the contact angle of porous solids with a contact angle in the range of 0-90°.

The tested material is the combination of the sample, the holder and the filter system. Therefore, an estimation or determination of the true value is not possible and only apparent values of the contact angle can be determined. However, the contact angle of the sample is the functional property on which the result is significantly dependent. The outcome of the test is a ranking order listing the wettability of different substances or formulations characterised by an apparent contact angle.

PRINCIPLE

If a porous solid is brought into contact with a liquid, such that the solid is not submerged in the liquid, but rather is just touching the liquid surface, then the rise of liquid into the pores of the solid due to capillary action will be governed by the following equations:

$$m^2 = \frac{t}{A} \qquad (1)$$

m = mass of liquid sucked into the solid;
t = time elapsed since the solid and the liquid were brought into contact;
A = constant, dependent on the properties of the liquid and the solid to be examined, calculated using the following equation:

$$A = \frac{\eta}{c \times \rho^2 \times \gamma \times \cos\theta} \qquad (2)$$

η = viscosity of the liquid;
ρ = density of the liquid;
γ = surface tension of the liquid;
θ = contact angle between the solid and the liquid;
c = material constant, dependent on the porous texture of the solid.

Equations (1) and (2) lead to equation (3):

$$\cos\theta = \frac{m^2}{t} \times \frac{\eta}{c \times \rho^2 \times \gamma} \qquad (3)$$

In setting up a Washburn determination, a liquid with known density (ρ), viscosity (η), and surface tension (γ) is used. Under these conditions, when the mass of liquid rising into the porous solid is monitored as a function of time (such that capillary penetration rate ($\frac{m^2}{t}$) is the experimental data), 2 unknowns remain according to equation (3): the contact angle (θ) of the liquid on the solid, and the solid material constant (c).

Determination of the material constant (c)

The material constant for a porous solid is determined by the following equation, considering cylindrical pores:

$$\frac{\pi^2 \times r^5 \times N^2}{2} \qquad (4)$$

r = average capillary radius within the porous solid;
N = number of capillaries per volumetric unit.

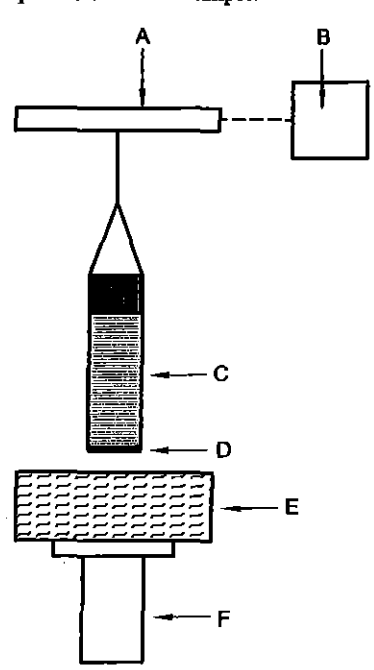
If a Washburn determination is performed with a liquid considered to have a contact angle of 0° (cos 0° = 1) on the solid, then the solid material constant (c) is the only remaining unknown in equation (3) and can thus be determined. *n*-Heptane is the liquid of choice for determining material constants because of its low surface tension (20.14 $mN \cdot m^{-1}$ at 25 °C). *n*-Hexane may also be used (18.43 $mN \cdot m^{-1}$ at 25 °C) but is more volatile. If the powder dissolves too quickly in these liquids, hexamethyldisiloxane may be used instead (15.9 $mN \cdot m^{-1}$ at 25 °C). Replicate determinations are performed (n = 6) and the average value calculated.

Once the material constant (c) has been determined for the solid to be examined, a sample of the solid can be tested for wettability by another liquid. The material constant determined by the *n*-heptane test is used in the Washburn equation, in combination with the capillary penetration rate ($\frac{m^2}{t}$) data obtained while testing the substance to be examined in the prescribed liquid. This allows calculation of the contact angle.

NOTE: if a series of liquids (at least 2 liquids in addition to the liquid used to determine the material constant) is tested against a given solid then the resultant contact angle data can be used to calculate the surface energy of the porous solid.

APPARATUS

Figure 2.9.45.-3 shows the principal components of the apparatus. The main device is an electronic balance with a suitable processor ensuring a suitable resolution in force measurement and a suitable resolution in lifting up the immersion liquid towards the sample.



A. electronic balance
B. computer
C. sample holder
D. filter
E. immersion liquid
F. lift

Figure 2.9.45.-3. – *Apparatus for contact angle measurement by the Washburn method*

Table 2.9.45.-1 indicates parameters of the electronic balance that are generally considered suitable.

Table 2.9.45.-1. – *Technical parameters of the electronic balance*

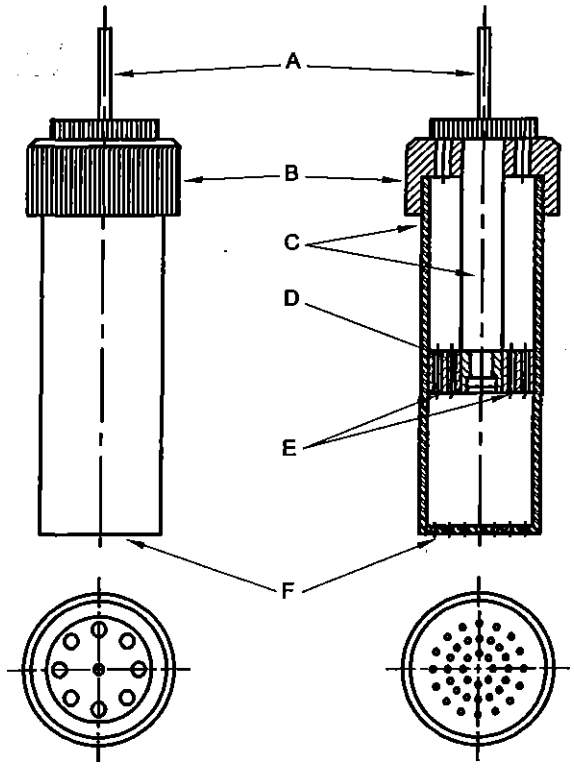
	Lift	Mass measurement
Range	> 110 mm	0 - 210 g
Resolution	0.1 µm	10 µg
Speed	0.099 - 500 mm/min	-

Sample holders

The sample holder may be a small glass cylinder with a sintered-glass filter at one end.

Powder material holders (see Figure 2.9.45-4) may also be made of aluminium; they are less fragile than those made of glass and have small holes in the bottom that render them easier to clean than a sintered-glass filter. The cover for the cell is equipped with 2 screw threads. One connects it with the sample chamber while the other allows the user to guide a piston down onto the sample itself and compact it.

The apparatus is similar to an automatic tensiometer, except for the sample holder.



A. fixing C. thread E. capillary holes
B. cover D. plunger F. capillary holes

Figure 2.9.45.-4. – *Example of sample holder with plunger for compaction of a powder*

PROCEDURE

Filling of the sample holder

Place a disc of filter paper in the bottom of the aluminium or glass sample holder. This prevents powder from leaking out of the bottom of the cell. The filter does not have to be made of paper, but it must be a material that is easily wetted by the liquid to be tested. A black-band filter (used for reverse osmosis) is recommended because of its high porosity and minimum flow resistance.

Place a known amount of powder into the cell.

The reproducibility of material constants and contact angles will depend on the ability to weigh out the same amount of powder for each test when a sufficient and adjusted amount of powder is compacted in a uniform way (i.e. tapping/compaction of the powder).

For most powders, a correct amount is in the range of a few grams, typically filling about 2/3 of the capacity of the holder. Place a second piece of filter paper on top of the powder in the cell. This will prevent powder from rising through the holes in the piston during the compaction process and/or during the determination.

Tapping/compaction of the powder

A bulk powder bed is very porous and thus very sensitive to small influences that can easily alter the porosity and consequently the *c*-constant. Therefore a tapped powder may be advantageous and will show more reproducible results. The appropriate number of taps must first be evaluated: 50-100 taps are usually appropriate.

If the aluminium sample holder is used then it may be mounted in the cylinder of a stamp volumeter, which can run the evaluated number of taps.

If tapping is not appropriate, the powder bed is compacted by screwing the piston of the aluminium sample holder applying a specified pressure.

A further possibility is centrifugation under defined conditions. Where applicable, a compacted disc of the powder sample may also be mounted on the electronic balance. A sample holder is omitted in this case.

After connecting to the balance, the sample holder is positioned with the porous solid just above the surface of the liquid (see Figure 2.9.45.-3), using the lift.

The liquid is raised further until it just touches the bottom of the porous sample. Mass-versus-time data is then collected as liquid penetrates into the solid. Data can be presented in either graphical or tabular format. The apparatus may perform the whole determination automatically.

CRITICAL PARAMETERS

The following points must be considered.

Sample properties:

— water content of the sample;
— crystalline or solid-state properties of the sample (polymorphic form, type of solvate).

Sample preparation:

— homogeneity of any powder blend to be examined;
— particle-size distribution; before testing it is sometimes advisable to sieve the sample (e.g. using a 250 µm sieve);
— the optimal compaction parameters (amount of sample, number of taps or piston mass) must be determined;
— the compaction state of the different powder samples must be uniform;
— the sample holder or, if used, the glass frit must be carefully cleaned;
— uniformity of the results is improved by using a sample holder made of aluminium.

Immersion liquid:

— specifications of the immersion liquid must be indicated.

U. Crystallinity

(Ph. Eur. general texts 5.16)

This chapter provides general information on crystallinity and refers to the various techniques described in the European Pharmacopoeia that are used for its determination.

INTRODUCTION - THE CONCEPT OF CRYSTALLINITY

Most organic and inorganic compounds of pharmaceutical relevance exist as a solid material, which can be characterised by a structure located between a perfectly ordered crystal and an amorphous material.

Real crystals lie somewhere between an ideal crystal state and the amorphous state. The position of a crystal on a scale bounded by these 2 extremes is termed its crystallinity.

A perfectly ordered crystal is an ideal state that is seldom, if ever, achieved. The structural units of a crystal, termed unit cells, are repeated regularly and indefinitely in 3 dimensions in space. The unit cell has a definite orientation and shape defined by the translational vectors *a*, *b* and *c*, and the angles α, β and γ, and hence has a definite volume, *V*, that contains the atoms and molecules necessary for forming the crystal. A crystalline system is defined by 3 long-range order symmetry operators (translational, orientational and conformational); the various mesophases (liquid crystals, crystals and plastic crystals) have 1 or 2 of the long-range symmetry operators and the ideal amorphous state is defined by the absence of all 3 operators.

Each crystal can be classified as a member of one of 7 possible crystal systems that are defined by the relationships between the individual dimensions *a*, *b* and *c* and between the individual angles α, β and γ of the unit cell. The structure of a given crystal may be classified according to one of the 7 crystal systems, to one of the 14 Bravais lattices and to one of the 230 space groups. All the 230 possible space groups, their symmetries and the symmetries of their diffraction patterns are compiled in the International Tables for Crystallography.

Many substances are capable of crystallising in more than one type of crystal lattice, which is known as polymorphism. The occurrence of polymorphism is a common phenomenon among organic molecules, giving rise to different physico-chemical properties. Crystalline polymorphs have the same chemical composition but different internal crystal structures and, therefore, possess different physico-chemical properties. The different crystal structures in polymorphs are due to different atomic packing arrangements and/or different conformations of the molecules (see chapter *5.9. Polymorphism*).

The other extreme of a crystal state is the ideal or true amorphous state, where all long-range order is lost. For most organic systems certain short-range order remains, but this is not expected to extend over distances much larger than nearest neighbour (NN) or next nearest neighbour (NNN) interactions, which are typically less than 2-2.5 nm for small organic molecules.

Amorphous material is characterised by the absence of distinct reflections in the X-ray powder diffraction (XRPD) pattern (*2.9.33*).

The crystallinity of a real powder can be considered by 2 models of crystallinity. In the 1-state model all particles will be of the same crystallinity whereas in the 2-state model each particle can be either crystalline or amorphous, such that the actual crystallinity of the powder is the weighted average of these 2 extreme crystallinities. Such a powder is obtained when pure crystalline and amorphous phases are physically mixed. In reality, a powder probably contains particles with different degrees of crystallinity, just as it may contain particles with different sizes and shapes.

The extent of disorder in a crystalline solid can affect many physico-chemical properties of substances for pharmaceutical use. Because of the great relevance of these properties, it is important to be able to assess the extent of disorder or the crystallinity of a solid by a suitable quantitative method.

METHODS FOR MONITORING AND DETERMINING CRYSTALLINITY

Various methods are available for determining the crystallinity of a solid. Many techniques cannot detect or quantify these properties independently; for this reason, it is useful to combine several of the methods described below. Such methods often do not give accurate results and limits of quantitation are usually much greater than those for chemical impurities. In addition, certain assumptions have to be made about the relationship between standards used for calibration, which are typically mixtures of crystalline and amorphous particles (2-state model), and the samples to be analysed that are likely to have at least a small component of material exhibiting 1-state model behaviour. Finally, the lack of well-defined standards for 100 per cent crystalline or 100 per cent amorphous material complicates the validation of such methods. As explained above, it is obvious that different amorphous or non-crystalline phases exist and even co-exist in a solid powder. These different non-crystalline forms of a solid can give different responses depending on the techniques used for determining the degree of crystallinity.

X-ray powder diffraction (*2.9.33*)

XRPD is still the most commonly used method for determining the degree of crystallinity, although this method suffers from some limitations due to peak broadening, amorphous halo and preferred orientation, which make interpretation and quantitation difficult.

XRPD alone is often insufficient to distinguish between the different non-crystalline phases. The X-ray diffraction pattern of a purely amorphous and nanocrystalline phase is characteristic of a broad diffuse halo. In-depth analysis of the X-ray diffraction patterns will show that the diffuse halo in the pattern of nanocrystalline material shows some correlation to the pattern of the parent crystalline phase, while in the case of a pure amorphous phase such a correlation does not exist. Additional techniques may be required to establish the true nature of X-ray amorphous materials.

Thermal analysis

Thermal analysis (*2.2.34*) of crystalline materials exhibits a melting transition that is often accompanied by decomposition or evaporation of solvents. In the case of true amorphous materials, thermal analysis reveals a glass transition, whereas only a melt would be expected for a nanocrystalline material.

Microcalorimetry (*2.2.61*)

It is a highly sensitive technique which allows the determination of the rate and extent of chemical reactions, changes of phase or changes of structure. Amorphous parts of a substance can recrystallise by subjecting the sample to higher relative humidity or an atmosphere containing organic vapour. The measurement of the heat of recrystallisation enables the amorphous content to be determined from the enthalpy of recrystallisation. By relating the output from the microcalorimeter for a sample to that obtained for an

amorphous standard, it is possible to quantify the amorphous content of the sample. The range of amorphous content covered by this method depends on the individual substance to be tested; in favourable cases limits of detection below 1 per cent can be reached.

Solution calorimetry (*2.2.61*)
Solution calorimetry provides a means of determining enthalpy of solution for a solid substance. The crystallinity of the solid sample to be examined is given by the enthalpy of solution of the solid sample ($\Delta H_x^!$) minus the enthalpy of solution of the chosen reference standard of the same substance ($\Delta H_r^!$) when determined under the same conditions. Because the reference standard is usually chosen for its perceived high crystallinity, its enthalpy of solution is usually algebraically greater (more endothermic or less exothermic) than that of the solid sample to be examined in the same solvent. Consequently, the crystallinity determined is a negative quantity with the SI units kJ/mol or J/g (J/kg is avoided because of its unwieldiness and potential for error). The preference for a negative value with respect to a highly crystalline reference standard recognises the fact that most samples have a lower crystallinity than this reference standard.

Near-infrared (NIR) spectroscopy
Near-infrared (NIR) spectroscopy (*2.2.40*) is another technique used to measure the degree of crystallinity, and has also been proven to be useful in studies of polymorphism. The NIR spectrum of a sample contains both physical and chemical information. Being non-invasive, non-destructive and operable at room temperature, the method is a valuable tool to assess changes in the amorphous and crystalline state.

Infrared absorption spectrophotometry and Raman spectroscopy
Infrared absorption spectrophotometry (*2.2.24*) and Raman spectroscopy (*2.2.48*) are other techniques used to measure the degree of crystallinity, and have also been proven to be useful in studies of polymorphism. The IR spectrum and Raman spectrum of a sample contain both physical and chemical information.

Solid-state NMR
Solid-state nuclear magnetic resonance spectrometry (ss NMR) (*2.2.33*) can be used to provide information about polymorphism and related relative molecular conformations. However, some caution has to be exercised in the interpretation of results, since it is not always simple to distinguish between samples that comprise a mixture of different physical forms (2-state model) and those that comprise crystals having disorder with exchange that is slow on the NMR timescale. Similarly, samples that contain defects arising from different molecular conformations or slightly different packing arrangements (1-state model) may show additional signals in the spectra. Solid-state NMR may be quite sensitive to this, even if lattice parameters are hardly affected and, consequently, little or no change is observed by XRPD. It is evident that the crystallinity of substances for pharmaceutical use can be complex, and both crystalline defects and amorphous material may co-exist.

Optical microscopy
A method to detect whether or not particles are crystalline is to use a polarising microscope (*2.9.37*), where particles show birefringence and extinction positions when the microscope stage is revolved.

V. Characterisation of Crystalline Solids by Microcalorimetry and Solution Calorimetry

(Ph. Eur. method 2.2.61)

For the purpose of this chapter, crystalline material, partially crystalline material and amorphous material are considered as solids.

INTRODUCTION - THE CONCEPT OF CRYSTALLINITY

The perfectly ordered crystal lattice with every molecule in its expected lattice position is an ideal that is seldom, if ever, achieved. The other extreme is the amorphous state, in which a solid contains the maximum possible density of imperfections (defects of various dimensionalities), such that all long-range order is lost while only the short-range order, imposed by its nearest neighbours, remains. Real crystals lie somewhere between these 2 extremes. A crystal's position on a scale bounded by these 2 extremes is termed *crystallinity*.

All real crystals, even in the pure state, possess some lattice imperfections or defects, which increase both the energy (enthalpy under conditions of constant atmospheric pressure) and the disorder (expressed as the entropy) of the crystal lattice. A crystal with a relatively low density of imperfections is said to be highly crystalline and to possess a high crystallinity. By contrast, a particle with a relatively high density of imperfections is said to be partially amorphous and to possess a low crystallinity. In ideal terms, a totally amorphous particle corresponds to zero crystallinity. Amorphous particles may contain somewhat ordered domains that can act as nuclei for crystallisation; such so-called amorphous particles are said to possess a low-level but finite crystallinity.

The ability to detect and to quantify the amount of amorphous material within a highly crystalline substance is of great importance during the development and subsequent manufacture of a pharmaceutical preparation.

In reality, a powder probably contains particles with different degrees of crystallinity, just as it may contain particles with varying sizes and shapes. The lower the crystallinity of a solid, the greater its enthalpy and entropy. The increase in enthalpy is never totally compensated for by the increase in entropy; therefore, the Gibbs free energy, which reflects the balance between them, actually increases. Hence, the lower the crystallinity of a material (powder), and consequently the greater its amorphous character, the greater its apparent intrinsic solubility and dissolution rate, but the lower its thermodynamic stability. Because of the great relevance of these properties, crystallinity is also an important property and requires measurement by a suitable method.

In the following chapter, the crystallinity or the content of amorphous parts of a powder are measured by calorimetric methods such as microcalorimetry or solution calorimetry, although other methods could be used (e.g. see general chapter *2.9.33. Characterisation of crystalline and partially crystalline solids by X-ray powder diffraction (XRPD)*).

Many substances are capable of crystallising in more than one type of crystal lattice, which is known as polymorphism. If water or a solvent is incorporated in the crystal lattice the crystals are termed hydrates or solvates. Because of the different crystal packing, and/or molecular conformation and lattice energy, they usually exhibit different physical properties. For simplicity, calorimetry measurements for degree of crystallinity determination discussed here assume

only one solid crystalline form present in the material of interest. The theory and experimental technique can be easily expanded to polymorphic systems with proper consideration of the enthalpy differences among the polymorphs.

METHOD 1 - MICROCALORIMETRY (DETERMINATION OF AMORPHOUS CONTENT)

Most chemical, physical and biological processes are associated with the exchange of heat. Microcalorimetry is a highly sensitive technique to monitor and quantify both exothermic (heat producing) and endothermic (heat absorbing) changes associated with those processes.
The technique allows the determination of the rate and extent of chemical reactions, changes of phase or changes of structure.

Thermal events producing only a fraction of a microwatt can be observed using microcalorimetry. This means that temperature differences less than 10^{-6} K must be detectable. Microcalorimetry typically uses the heat flow (heat leakage) principle, where the heat produced (or absorbed) in a thermally defined vessel flows away (or into) in an effort to re-establish thermal equilibrium with its surroundings. Exceptional thermal stability with its surrounding has to be achieved either by a heat sink or an electronically regulated surrounding.

Heat energy from an active sample in the reaction vessel is channelled typically through Peltier elements; they act as thermoelectric generators using the Seebeck effect. The heat energy is converted into a voltage signal proportional to the heat flow.

Results are typically presented as a measure of the thermal energy produced per unit of time (Watt) as a function of time.

APPARATUS

Microcalorimeters are typically designed as twin systems with a measuring vessel and a reference vessel. Vessels are typically made of glass or stainless steel. For certain applications specially designed vessels which allow the addition of a gas, a liquid or a solid material may be used.

CALIBRATION

The microcalorimeter is calibrated for heat flow (energy per time unit) using either calibrated external or internal electrical heat sources or a suitable standard reaction.

SENSITIVITY

The sensitivity of the microcalorimetric method can be assessed based on an appropriate standard sample analysed according to the corresponding method in conjunction with the determination of the instrument baseline noise.

PROCEDURE

Weigh in a suitable vessel an appropriate quantity of the substance to be examined. Close the vessel carefully to avoid any evaporation of solvents and place the vessel in the sample holder. If appropriate, allow the vessel to equilibrate at the temperature of the measurement before placing it in the measuring position.

Begin the analysis and record the heat flow, with the time on the abscissa and the heat flow on the ordinate (specify the direction of exothermic or endothermic heat flow).

DETECTION AND QUANTIFICATION OF AMORPHOUS CONTENT IN POWDERS

The amorphous state is metastable with respect to the crystalline state; recrystallisation may therefore occur.
The measurement of the heat of recrystallisation enables the amorphous content to be determined by the area of the recrystallisation peak. By relating the output from the microcalorimeter for a sample to that obtained from an amorphous standard, it is possible to quantify the amorphous content of the sample. The range of amorphous content covered by this method depends on the individual substance to be tested; in favourable cases limits of detection below 1 per cent can be reached.

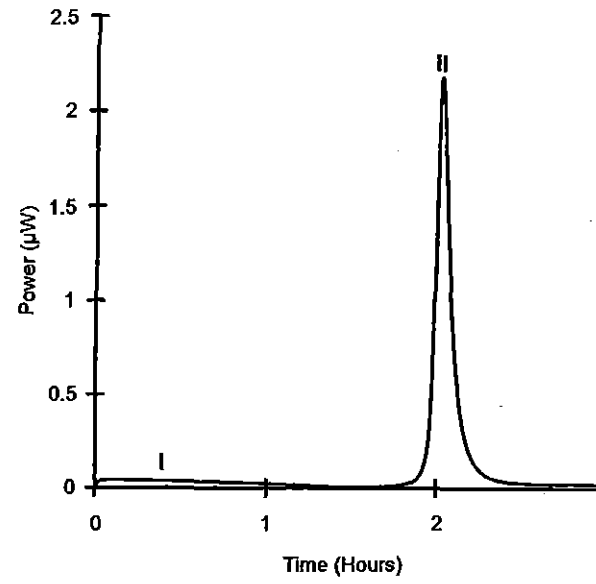


Figure 2.2.61.-1. – *Typical microcalorimetric output of power (in µW) as a function of time (in hours): amorphous collapse peak (I) and crystallisation peak (II) for mainly amorphous lactose at 25 °C and 75 per cent relative humidity*

Recrystallisation can be initiated by subjecting the sample to higher relative humidity or an atmosphere containing organic vapour. The sample is typically placed in an ampoule which also contains a small test-tube containing an aqueous saturated salt solution, an organic solvent, or a solvent mixture.

The heat of recrystallisation is typically measured using a fixed sample mass placed in a glass or steel vessel. The test-tube containing a saturated salt solution or an organic solvent is chosen to be large enough to allow a full saturation of the atmosphere above the sample. The mass of the sample and the nature of the vapour atmosphere above the sample are chosen so that recrystallisation occurs in such a way that a distinct peak is observed, clearly separated from initial thermal events caused by introduction of the sample.

The conditions under which the transition of the amorphous phase to a thermodynamically more stable crystalline state occurs will have a significant impact on the time of recrystallisation. In particular, physical mixtures of purely amorphous and crystalline material will behave differently from a partially crystalline material. These effects should be considered when developing a method.

A typical response for the recrystallisation of a mainly amorphous material is shown in Figure 2.2.61.-1. The first part of the curve represents several concurrent processes taking place simultaneously, such as the absorption of water vapour into the amorphous parts of the powder and the generation of water vapour from the test-tube. After this initial response there is a large exothermic response caused by the recrystallisation of the amorphous material. Also included, but not seen, are the expulsion of excess water from the recrystallised parts and its condensation. Thus, the

area under this exothermic recrystallisation response is proportional to the heat of recrystallisation.

METHOD 2 - SOLUTION CALORIMETRY (DETERMINATION OF CRYSTALLINITY)

Solution calorimetry provides a means of determining enthalpy of solution (i.e. heat of solution under constant atmospheric pressure) of a substance. Enthalpy of solution is defined as the enthalpy of the substance dissolved in the solution to a defined concentration minus the enthalpy of the original substance. The solvent for the dissolution process must be such that the mass of solid dissolves within a time frame that matches the response time of the calorimeter, as discussed below. The enthalpy of solution is proportional to the amount of solid being dissolved. This amount may be defined as 1 mol for molar enthalpy or as 1 g for specific enthalpy. If the substance possesses adequate purity (as determined by the degree of accuracy required) and if its molecular mass is known, the molar enthalpy is preferred, otherwise the specific enthalpy must be used. The enthalpy of solution is weakly dependent on both the temperature, which is usually 25.0 °C, and the final concentration of the dissolved solute.

It is usually preferred to express the crystallinity, P_c, of a substance on a percentage scale. This procedure requires 2 reference standards, namely a highly crystalline sample assuming 100 per cent crystallinity and having a measured enthalpy of solution of ΔH_c^s, and an amorphous sample assuming 0 per cent crystallinity and having a measured enthalpy of solution of ΔH_a^s. From these values and from the measured enthalpy of solution, ΔH_s^s, of the solid under study, the percentage crystallinity of the solid, P_c, may be calculated as follows:

$$P_c(\%) = 100(\Delta H_s^s - \Delta H_a^s)/(\Delta H_c^s - \Delta H_a^s)$$

Clearly, crystallinity expressed on a percentage scale depends on 3 measured values and the enthalpies of solution may be replaced by other corresponding physical quantities that depend on crystallinity. The value of the percentage crystallinity of a sample, however, depends not only on the nature and method of preparation of the 2 reference standards, but also on the choice of the physical quantity that is measured.

The enthalpy of solution is measured either by an isoperibol (constant perimeter, i.e. jacket) solution calorimeter or by an isothermal (constant temperature) solution calorimeter. Typically, at least 3 measurements are made with each sample. The mean of these values is then calculated. The exact requirements will depend upon the equipment capability and degree of accuracy needed.

ISOPERIBOL SOLUTION CALORIMETRY

In the isoperibol solution calorimeter, the heat change during the solution process causes a corresponding change in temperature of the solvent-solute system (i.e. solution). This temperature change is measured by a temperature sensor, which is wired to an electrical circuit that records an electrical signal corresponding to the temperature change. Typically, this temperature change in an electronic form is measured at precisely defined time intervals to produce temperature-time data that are collected, analysed by a computer, and then plotted. A blank run without addition of the solid solute to the solvent normally shows no discernible change in the slope of the temperature-time plot.

For isoperibol solution calorimeters, response is fairly rapid, but corrections must be made for any heat losses to or heat gains from the bath. Therefore, isoperibol solution calorimeters are more advantageous than isothermal solution calorimeters when the solution process is relatively fast.

For all measurements of enthalpy of solution using isoperibol solution calorimeters, the choice of solvent is critical. The nature and mass of the solvent and the mass of sample allow the total heat change, corresponding to total dissolution of the solid, to proceed to completion within 5 min under vigorous stirring at a constant rotational speed within the range of 400-600 r/min.

The effective heat capacity of the calorimeter cell and its contents is determined for every calorimeter run. This determination is accomplished by electrical heating of the contents of the calorimeter cell. The effective heat capacity is determined according to 1 of 2 protocols: either by making 1 determination after ampoule breakage or by making 1 determination before and a 2nd determination after ampoule breakage and then averaging the 2 results. The accuracy and reliability of the electrical heating are established by the accuracy and reliability of the aforementioned chemical calibrations.

ISOTHERMAL SOLUTION CALORIMETRY

In the isothermal (constant temperature) solution calorimeter, the heat change during the solution process is compensated for by an equal but opposite energy change, such that the temperature of the solvent-solute system (i.e. solution) remains essentially constant. This equal but opposite energy change is measured and, when its sign is reversed, provides the enthalpy of solution. For isothermal calorimeters, response is relatively slow, but the compensation process eliminates the effects of heat losses to or heat gains from the bath. Therefore, isothermal solution calorimeters are more advantageous than isoperibol solution calorimeters when the solution process is relatively slow.

SOLUTION CALORIMETER CALIBRATION

To ensure the accuracy of the calorimeter, chemical calibrations must be performed on a regular basis. For an endothermic solution process, the calibration of the calorimeter is checked by measuring the heat absorbed during the dissolution of potassium chloride in distilled water at 298.15 K (25.0 °C). The established enthalpy change in this endothermic process is 235.5 J/g (17.56 kJ/mol). For an exothermic solution process, the calorimeter is checked by measuring the heat evolved during the dissolution of 5 g per litre of tromethamine [tris(hydroxymethyl)aminomethane, THAM] in a 0.1 mol/L aqueous hydrochloric acid solution at 298.15 K (25.0 °C). The established heat for the aforementioned process is −246.0 J/g (−29.80 kJ/mol).

SAMPLE HANDLING

The chemical and physical stability of solids may decrease with decreasing crystallinity. In particular, solids of low crystallinity, especially amorphous solids, tend to sorb water vapour from the atmosphere, leading to crystallisation and a corresponding gain in crystallinity. For these reasons, anhydrous samples whose crystallinity is to be determined must be stored at zero humidity or below critical humidity levels in sealed chambers containing a desiccant, preferably containing an indicator of effectiveness. If crystallinity-humidity studies are to be carried out, the sample is stored in a sealed chamber containing a saturated salt solution to provide a defined relative humidity.

Appendix XVIII

Methods of Sterilisation

Methods of Sterilisation

(Methods of Preparation of Sterile Products, Ph. Eur. general texts 5.1.1)

GENERAL INTRODUCTION

Sterility is the absence of viable micro-organisms, as defined by a sterility assurance level equal to or less than 10^{-6}. Sterility is a critical quality attribute for a wide variety of human and veterinary preparations, including but not restricted to:

- preparations required to be sterile due to their route of administration, such as parenteral, ophthalmic and intramammary preparations, and some inhalation, irrigation and intrauterine preparations;
- preparations applied to severely injured skin, such as semi-solid preparations for cutaneous application.

The achievement of sterility for any one item in a population of items submitted to a sterilisation process can neither be guaranteed nor demonstrated. It is essential to study the effect of the chosen sterilisation procedure on the product (including its final container) to ensure its effectiveness and the integrity of the product, and to validate the procedure before it is applied in practice. Failure to follow meticulously a validated process introduces the risk of a non-sterile and/or deteriorated product.

Sterile products are prepared under appropriate conditions and are packed in suitable containers. It is recommended that the choice of container permits application of the optimum sterilisation process for the product. The container and closure system are required to maintain the sterility of the product throughout its shelf life.

Sterilisation process conditions are chosen to achieve the highest level of sterility assurance compatible with the drug product and, wherever possible, a process in which the product is sterilised in its final container (terminal sterilisation) is chosen. When a fully validated terminal sterilisation method by steam (moist heat), dry heat or ionising sterilisation is used, parametric release (i.e. the release of a batch of sterilised items based on process data rather than submission of a sample of the items to sterility testing) may be carried out, subject to the approval of the competent authority. If terminal sterilisation is not possible, aseptic assembly or filtration through a bacterial retentive filter is used. Wherever possible, an appropriate additional treatment (e.g. heating) of the product in its final container is applied to further ensure the sterility assurance level.

Requirements for the use of biological indicators for validation of sterilisation processes are given in general chapter *5.1.2.*

The present general chapter provides guidance on conditions, validation and control of sterilisation processes. The methods described here apply mainly to the inactivation or removal of bacteria, yeasts and moulds. For biological products of animal or human origin, or in cases where such material has been used in the production process, it is necessary to demonstrate during validation that the process is capable of the removal or inactivation of any relevant viral contamination. Further guidance is provided in general chapter *5.1.7. Viral safety.*

The efficacy of a sterilisation process is dependent on its nature, the processing conditions (e.g. time, temperature, moisture), the pre-sterilisation microbial contamination and the formulation of the product. The inactivation of micro-organisms by physical or chemical means follows an exponential law and hence there is always a non-zero probability that a micro-organism may survive the sterilisation process.

Sterility assurance level (SAL)

In the methods described, reference is made to a sterility assurance level (SAL) where appropriate. The SAL for a given sterilisation process is expressed as the probability of micro-organisms surviving in a product item after exposure to the process. An SAL of 10^{-6}, for example, denotes a probability of not more than 1 non-sterile item in 1×10^6 sterilised items of the final product. The SAL of a process for a given product is established by appropriate validation studies. Microbial contamination may be described by the number, type and resistance of any micro-organisms present. Microbiological monitoring and setting of suitable limits is therefore essential for all components of sterile preparations. Steps designed to reduce microbial contamination, such as filtration prior to sterilisation, will contribute significantly to sterility assurance. The composition of a product can affect the behaviour of micro-organisms present in the product, which in turn can affect the efficacy of the sterilisation process. The water activity (A_w), the pH and the presence of compounds with antimicrobial activity are examples of factors that can influence the resistance of any micro-organisms present. The water activity or the product formulation (including the presence of nutrients) can affect the number of micro-organisms, which in turn can affect the efficacy of the membrane-filtration process.

METHODS AND CONDITIONS OF STERILISATION

Sterilisation may be carried out by one of the methods described hereafter. Modifications to, or combinations of, these methods may be used, provided that the chosen procedure is validated with respect both to its effectiveness and to the integrity of the product including its container. For all sterilisation methods, the critical parameters of the procedure are monitored in order to confirm that any previously determined requirements or conditions are respected throughout the batch during the entire sterilisation process. This applies in all cases, including those where the reference conditions are used. Guidance concerning validation of a steam sterilisation process using the F_0 concept is described in general chapter *5.1.5.* Biological indicators of sterilisation are used to develop and validate sterilisation processes and also to monitor gas sterilisation processes. Guidance on the use of biological indicators is provided in general chapter *5.1.2.*

Precautions shall be taken to prevent contamination of the sterilised articles after the sterilisation phase.

STEAM STERILISATION

Principle

Steam sterilisation is achieved by heat transfer during condensation of water from a saturated vapour phase on the surfaces of the sterilised items. Where items (open or wrapped) are sterilised in direct contact with steam, the hydrating effect of the condensate adds to the sterilising effect. For direct steam exposure, it is essential that the items are fully penetrated by saturated steam, i.e. free of air and other non-condensable gases. Where items are sterilised in closed-containers, the chamber of the steriliser serves as a steam jacket. Condensation on the surface of the containers

still serves as a highly effective mechanism for energy transfer, but has no additional sterilising effect on its own. In closed-container sterilisation, the sterilising effect is determined by the conditions reached within the closed containers, where sterilisation must be achieved in the product itself and in the head-space.

Equipment

Steam sterilisation is performed in autoclaves, i.e. pressure vessels designed to admit or generate steam continuously and to remove condensate from the chamber to maintain the pressure and temperature at controlled levels.

For equipment used to perform direct steam exposure cycles, the supply of saturated steam, free of non-condensable gases, is assured. In autoclaves intended for the sterilisation of closed containers, steam-air mixtures or a superheated water spray can be used to achieve heat transfer. Suitable autoclaves are qualified to achieve homogeneous conditions within the chamber and the load. The principles of operation are appropriate for the items to be sterilised and the loading configuration. The suitability of the equipment for the items to be sterilised and its performance in the chosen cycle is demonstrated in autoclave performance qualification studies. Temperature profiles in the slowest-to-heat items are recorded.

Suitable autoclaves are equipped with temperature and pressure sensors of appropriate sensitivity that are placed in relevant positions to ensure effective process control. Chamber temperature and pressure profiles are recorded for each cycle. There is at least 1 independent thermal probe that controls the load temperature at the slowest-to-heat position or in the slowest-to-heat closed container of the load.

Cooling water sprayed into the chamber at the end of a sterilisation process for closed containers is of sufficient quality not to impact negatively the sterility of the sterilised items.

Sterilisation cycle

Suitable sterilisation cycles are chosen to be compatible with the items to be sterilised and the loading configuration. Where air is displaced from the chamber by gravity, the items to be autoclaved are designed to allow the removal of air and are arranged within the autoclave to prevent the formation of inaccessible air pockets. Where air is removed by vacuum cycles followed by steam pulses, it is assured that the items are not affected by the evacuation process. For pressure-sensitive products in closed containers, saturated steam sterilisation may not be possible. Steam-air mixtures may be applied to the chamber in order to balance pressure conditions inside the closed containers. Steam penetration is assured by choosing suitable cycles to remove air from porous loads or hollow bodies. Steam penetration is verified during cycle development by, for example, the use of physical/chemical indicators, while the biological effectiveness of the cycle is verified by the use of biological indicators (*5.1.2*). Appropriate loading patterns are specified.

Cycle effectiveness

The reference cycle for steam sterilisation is 15 min at 121 °C in saturated steam determined in the coldest position of the chamber. Product- and load-specific cycles, e.g. applying another combination of time and temperature, may be adopted based on cycle development and validation. The minimum temperature acceptable for a steam sterilisation process is 110 °C. The minimum F_0, calculated in the slowest-to-heat position of the load is not less than 8 min. The calculation of sterilisation effectiveness by the F_0 concept is performed according to general chapter *5.1.5*.

Calculated effectiveness from physical parameters (F_{phys}) is correlated with biological effectiveness (F_{bio}). F_{bio} expresses the lethality, in minutes, provided by the process in terms of destruction of the biological indicators used. F_{bio} is calculated by the following equation:

$$F_{bio} = D_{121}(\log_{10} N_0 - \log_{10} N)$$

D_{121} is the *D*-value of the biological indicator at an exposure temperature of 121 °C, N_0 is the number of viable micro-organisms in the biological indicator before exposure, and N is the number of viable micro-organisms in the biological indicator after exposure.

In cycle validation, the relevant positions in the load that are the most difficult to sterilise are determined and adequate biological effectiveness is verified by exposure of biological indicators (*5.1.2*) in these positions or products, whichever is relevant. Protection of spores from the sterilising effect (e.g. by physical occlusion of steam or by the protective properties of the product) are suitably addressed. The F_{bio} determined for the most-difficult-to-sterilise position is used to define the parameters necessary to achieve reliably the required SAL equal to or less than 10^{-6} for the chosen cycle.

Routine control

Autoclave cycles are monitored by physical determination of chamber pressure and temperature profiles, at a minimum, in the coldest position of the chamber. For each cycle, pressure, time and temperature are recorded and, if possible, F_0 is calculated and recorded.

DRY HEAT STERILISATION

Principle

Dry heat sterilisation is a terminal sterilisation method based on the transfer of heat to the articles to be sterilised. Heat may be transferred by means of convection, radiation or direct transfer.

Equipment

Dry heat sterilisation is carried out in an oven with forced air circulation or using other equipment specifically designed for this purpose, e.g. a tunnel.

Sterilisation cycle

The steriliser is loaded in such a way that the specified or required temperature is achieved throughout the load. Knowledge of the temperature within the steriliser during the sterilisation cycle is obtained by means of temperature-sensing elements suitably placed in or on representative items situated in the coolest part (as previously established) of the loaded steriliser. The time and temperature throughout each cycle is suitably recorded.

Cycle effectiveness

The reference conditions for this method of sterilisation are a minimum of 160 °C for at least 2 h. Other combinations of time and temperature may be used if it has been satisfactorily demonstrated that the process chosen delivers an adequate and reproducible level of lethality when operated within the established tolerances. The procedures and precautions employed are such as to achieve an SAL equal to or less than 10^{-6}. Dry heat sterilisation processes are validated using a combination of temperature mapping and biological indicator studies (*5.1.2*).

Dry heat at temperatures greater than 220 °C, for a validated time, is frequently used for depyrogenation of glassware. In this case, demonstration of a 3 $\log_{10}$ reduction in heat-

resistant endotoxin can be used as validation criteria and biological indicators will not be needed.

Routine control
Dry heat sterilisation cycles are monitored by determination of temperature profiles, at a minimum, in the coldest position of the chamber. Time and temperature are recorded for each cycle.

IONISING RADIATION STERILISATION

Principle
Sterilisation by irradiation is achieved by exposure of the product to ionising radiation in the form of either gamma rays from a suitable isotopic source (such as cobalt 60), a beam of electrons energised by a suitable electron accelerator, or X-rays resulting from bombarding a suitable target with energised electrons. Ionising radiation may be used for the terminal sterilisation of finished dosage forms, the microbial inactivation of tissues and cells, or the sterilisation of materials or containers to be employed in aseptic processing. Low-energy electrons may be used for the surface sterilisation of materials upon entry to isolators used in the preparation of sterile products.

Cycle effectiveness
For this method of sterilisation, the reference absorbed dose is 25 kGy. Other doses may be used if, during validation of the sterilising dose, it has been satisfactorily demonstrated that the dose chosen delivers an adequate and reproducible level of lethality when the process is operated routinely within the established tolerances. The procedures and precautions employed are such as to achieve an SAL equal to or less than 10^{-6}. Biological indicators may be required for the development and validation of the sterilisation of tissues and cell products. They may also be required for products with a potential to prevent spore inactivation.

Routine control
During the sterilisation process, the sterilisation dose delivered is monitored using a dosimetry system, measurements from which are traceable to national standards.

GAS STERILISATION (VAPOR PHASE STERILISATION)

Principle
Gas sterilisation of surfaces may be used for the sterilisation of primary packaging materials, equipment and some pharmaceuticals.

It is essential that penetration by gas and moisture into the material to be sterilised is ensured, and that it is followed by a process whereby the gas is eliminated under conditions that have been previously established as sufficient to ensure that any residues of gas or related transformation by-products are below concentrations that could give rise to toxic effects during product use.

Sterilising agents
There are 2 main categories of gaseous sterilising agents as distinguished by their antimicrobial action: alkylating agents and oxidising agents.

Alkylating agents Alkylating agents are highly reactive compounds and interact with many components, such as amino, sulfhydryl and hydroxyl groups in proteins and purine bases in nucleic acids.

Ethylene oxide is an alkylating agent that is associated with cytotoxic, carcinogenic and mutagenic effects.

Oxidising agents Oxidising agents are highly reactive, toxic compounds. Such compounds currently used as sterilising agents include hydrogen peroxide and peracetic acid.

Development and validation of sterilisation processes
Gas sterilisation is performed by exposure of the product to the sterilising agent in a leak-proof chamber under specified conditions.

A typical gas sterilisation process consists of 3 phases: (pre) conditioning, sterilisation and aeration. The parameters necessary for these phases to produce the required SAL are established during process development. A combination of physical and biological methods is used to determine the optimum sterilisation conditions. The cycle shall not compromise the functionality of either product or the container.

Sterilisation cycle
Specialised equipment may be required for the monitoring of temperature, humidity and gas concentration during both validation and routine operation.

Cycle effectiveness
Validation of microbiological performance shall confirm the effectiveness of the defined process for the product/load combination in the steriliser. The lethality of the cycle may be determined by using an appropriate approach: after time-graded exposures, the rate of inactivation (*D*-value) of the test organisms can be established by construction of a survivor curve or by using a fraction-negative method.

Biological indicators shall be shown to be, at a minimum, as resistant to the sterilising agent as the microbiological contaminants of the product to be sterilised. They shall be placed within the product at locations where sterilising conditions are most difficult to achieve.

The effectiveness of the process is dependent on a number of parameters, including gas concentration, temperature, humidity, exposure time, load configuration and characteristics of the product and its packaging materials. The effect on the process effectiveness of any change in one or more of these parameters shall be investigated.

Routine control
The relevant cycle process parameters (including the results of the biological indicator test) are recorded.

MEMBRANE FILTRATION

Principle
Membrane filtration is used for reduction of viable and non-viable particles in gases and fluid products that are not amenable to sterilisation by heat or irradiation. In contrast to other sterilisation methods, the principle of membrane filtration is not inactivation but removal of microorganisms from the product. Removal is achieved by a combination of sieving and surface interaction.

Equipment
Membrane filters are available as flat stock (discs) in appropriate holders or as cartridges. Pore size ratings are based on the correlation between microbial retention and diffusion characteristics or bubble-point measurement. Many factors contribute to the effectiveness of the filtration process, e.g. shape, pore size, structure, surface properties, the structure and arrangement of the filter unit, interaction of the filter matrix with the product, applied pressure, flow and duration of the process. Filter characteristics have to be determined in a product-specific validation. Suitable integrity test procedures (e.g. diffusive flow measurement, bubble-point determination or water-intrusion testing) are employed, as recommended by filter manufacturers. Chemical and physical compatibility of the membranes with the product to

be filtered and the conditions of the filtration process are demonstrated in development studies. The filter size is suitable for the volume of the product to be filtered and the bioburden.

For sterilisation of process gases, an appropriate frequency for physical integrity testing is established.

Filtration effectiveness

Microbial challenge tests with a suitable model system shall demonstrate the effectiveness of the filtration process. Where testing with the product is not possible (e.g. due to the antimicrobial properties of the product), a fluid that is representative of the product shall be used, or the test conditions are modified.

It is recommended that the filtration process is carried out as close as possible to the filling point.

Sterilisation of membrane filters

Membrane filters may be sterilised off-line or in-line.
If sterilisation is off-line, steam penetration is verified and the filter is suitably protected against contamination.
The sterilised filter is aseptically assembled in the production line by means of a validated procedure. For in-line sterilisation, steam penetration throughout the filtration equipment is assured and the pressure difference across the membrane is controlled to prevent damage to the membrane itself.

Filtration process

Sterilisation by membrane filtration is performed by passage of the product through a microporous membrane with a nominal pore size not greater than 0.22 µm.

The pre-sterilisation microbial contamination is determined for each batch of product and process parameters are applied as established and validated in the development of the filtration process.

Where multiple bioburden-reduction filters are used to increase the efficacy of the filtration process, the filter closest to the filling point in the final container is characterised as the sterilising filter.

The sterility and integrity of the equipment downstream from the point of filtration, the qualified environmental conditions and the validated aseptic procedures applied in the handling of the filtered product all contribute to preventing recontamination of the product. This is addressed in the section on aseptic assembly.

Routine control

Filtration processes are monitored by physical and microbiological determination of parameters established during validation studies. These parameters include the following: pre-sterilisation microbial contamination, pre-filtration integrity test results, duration of filtration, volume filtered, differential pressure and post-filtration integrity test results.

ASEPTIC ASSEMBLY

Principle

The objective of aseptic assembly is to maintain the sterility of a product that is assembled from components, each of which has been sterilised by one of the above methods. This is achieved by using conditions and facilities designed to prevent microbial contamination.

Aseptic processing may include aseptic filling of products into container/closure systems, freeze-drying under aseptic conditions, aseptic blending of formulations followed by aseptic filling, and aseptic packaging.

Development and validation of aseptic assembly

In order to maintain the sterility of the components and the product during assembly, careful attention needs to be given to the following:

- environment;
- personnel;
- critical surfaces;
- container/closure sterilisation and transfer procedures;
- the maximum holding period of the product before filling into the final container.

Process validation includes appropriate checks on all of the above and also regular checks on the process, which are carried out by means of process simulation tests using microbial growth media that are then incubated and examined for microbial contamination (media fill tests).
In addition, a suitable sample of each batch of any product that is aseptically processed is tested for sterility (*2.6.1*).

Biological Indicators of Sterilisation

(Biological Indicators and Related Microbial Preparations used in the Manufacture of Sterile Products, Ph. Eur. general texts 5.1.2)

1 INTRODUCTION

The use of biological indicators in this general chapter is intended to cover the sterilisation of finished products and relevant related sterilisation processes i.e. sterilisation processes for items coming into direct contact with the final sterilised product. Other uses of biological indicators to validate the sterilisation of other non-terminal units is outside the scope of this general chapter.

Biological indicators are test systems containing viable micro-organisms (usually spores of bacteria) that provide a defined challenge to verify the required effectiveness of a specified sterilisation process.

Biological indicators are intended for the development and validation of the sterilisation processes and not for routine monitoring unless otherwise stated in this general chapter.

The validity of the sterilisation process and the validity of the biological indicators can be assured by the use of reduced sterilisation process conditions, whereby a small proportion of the micro-organisms within the biological indicator will be shown to survive. However, when the validated sterilisation process is used, there will be no surviving viable micro-organisms (see section 3-1-2).

Bacterial spores are resistant forms of life, they can be produced and standardised, and may be stored for long periods of time under appropriate conditions.

Commercially available biological indicators typically contain a standardised population of spores of a suitable bacterium. In cases where no suitable commercial biological indicators are available to characterise the sterilising effect in the product or at a position difficult to penetrate by the sterilant, custom-made biological indicators may be used. Such biological indicators can be prepared by inoculating a standardised spore suspension onto or into the item or product to be sterilised, such action may change the characteristics of the biological indicator.

A suspension of vegetative bacterial cells is used to validate the bacterial retention capability of sterilising grade filters when applied as a sterilisation step in an aseptic production process.

2 BIOLOGICAL INDICATORS FOR STERILISATION PROCESSES

In addition to the physical sterilisation parameters, the effectiveness of a sterilisation process as described in general chapter *5.1.1* is dependent on a large number of variables,

which may include, but is not necessarily restricted to, the number and resistance of contaminating micro-organisms, penetration of the sterilant, time, temperature, concentration, pH, moisture content, and the chemical composition of the product or item being sterilised.

To validate a sterilisation process, physical conditions are chosen that are expected to sterilise the items in the load to achieve a sterility assurance level (SAL) equal to or less than 10^{-6} as described in general chapter *5.1.1*. In a physical validation process it is demonstrated that these conditions are delivered homogeneously to all parts and positions of the load. It is the aim of biological validation to demonstrate the correlation between the predicted effect of the physical conditions applied during the process and the observed biological effect on biological indicators. Using process parameters that have been demonstrated to deliver the required biological effect will ensure sterility of the resulting product in routine processing.

The selection of the type of biological indicator used will depend on:

— the nature of the sterilising agent (e.g. heat, gas or radiation);
— the expected effectiveness of the treatment (e.g. the F_{phys} calculated from the process parameters);
— the process conditions (e.g. temperature, time, relative humidity, gas concentration, radiation dose);
— the characteristics of the pharmaceutical product or item (e.g. product in final container, packaging material, utensils such as tubes or pumps) to be sterilised.

In the development of a sterilisation process, the load and the product should be assessed to determine the most difficult position to sterilise (e.g. cold spots, vial-stopper interface, difficult to penetrate areas). When choosing the optimum biological challenge to a sterilisation process, the conditions in the most difficult position to sterilise in the load and the product should be simulated as closely as possible.

Spores inoculated into a product or onto surfaces are known to react differently to sterilising conditions as compared to biological indicator units. In these cases, commercially available biological indicator units may not be suitable to test sterilisation effectiveness and an inoculated test product/item prepared from a well-characterised spore suspension may be a better model to evaluate the effectiveness of the sterilisation cycle.

2-1 DESCRIPTION OF BIOLOGICAL INDICATORS FOR STERILISATION PROCESSES

Depending on the process to be characterised, a suitable biological challenge may consist of biological indicators presented as test micro-organism suspensions, inoculated carriers, or self-contained biological indicators. The user must establish a high level of confidence in the manufacturer's compliance to quality standards for the biological indicator (e.g. by means of auditing) in order to rely on the characteristics stated by the manufacturer (see section 2-2). Alternatively, the labelled characteristics of biological indicators shall be verified by the user or by an independent, contract laboratory that is formally approved by the user. For custom-made biological indicators (see section 2-1-4), the characteristics shall be verified by the user or by a contract laboratory.

2-1-1 Inoculated carriers

Inoculated carriers consist of a defined population of bacterial spores inoculated into or onto a suitable carrier, and in most cases, in a protective envelope. The type of carrier (and the envelope if used) may influence the resistance of the bacterial spores and must be compatible with the chosen sterilisation process (e.g. strips of filter paper in glassine envelopes are frequently used for steam and ethylene oxide, while metal discs packaged in non-woven fibre envelopes are used for hydrogen peroxide vapour). After exposure to the sterilisation process, the carrier is aseptically handled according to the manufacturer's instructions, transferred to a suitable culture medium and incubated for a sufficient period of time at the appropriate temperature.

2-1-2 Self-contained biological indicators

A self-contained biological indicator may be, for example:

— a system consisting of an inoculated carrier and a container (e.g. ampoule) with a nutrient medium suitable for the test micro-organism used; the system is designed in such a way that the sterilising agent comes into contact with the inoculated carrier (e.g. through a tortuous path or a filter) while the growth-promoting properties of the nutrient medium are not adversely affected by the sterilisation process. After sterilisation, the carrier is brought into contact with the nutrient medium by simple manipulation. This type of biological indicator system may be used to characterise moist heat sterilisation processes including assurance of the penetration of steam into the system;
— a container (e.g. ampoule) of a population of the test micro-organism in an appropriate nutrient medium. After sterilisation, the container is incubated without further manipulation. This type of biological indicator is sensitive only to an exposure time and temperature and may be used primarily to monitor sterilisation of aqueous fluids. In order to facilitate detection of growth, the medium may contain an indicator (e.g. a pH indicator).

Self-contained biological indicators might not be suitable for the validation of certain sterilisation processes.

2-1-3 Characterised spore suspensions

Characterised spore suspensions consist of a defined population of bacterial spores, prepared from a clearly characterised and suitably maintained strain of a spore-forming bacterial species (e.g. of the genera *Bacillus* or *Clostridium*) in a stable suspension.

2-1-4 Custom-made biological indicators

Custom-made biological indicators are test items (e.g. rubber stoppers), or products, inoculated with a suitable test micro-organism, usually from a characterised spore suspension but also from spore suspensions prepared from isolates from environmental monitoring or other microbiological testing using a well-defined procedure designed to give satisfactory sporulation. The *D*-value (time for 90 per cent reduction of micro-organisms under the stated conditions) and, when appropriate, the *z*-value (see section 3-1-1) of the spore suspension must be determined. Also the *D*-value and *z*-value (if appropriate) of the spores of the inoculated test items/ products must be determined as this may be different from the spores in suspension.

After exposure to the sterilisation cycle, the custom-made biological indicator is enumerated or tested for the presence/absence of surviving test micro-organisms using a validated, appropriate microbiological technique.

2-2 QUALITY REQUIREMENTS FOR BIOLOGICAL INDICATORS

The following are required to be known by the user per delivery of each batch:

— genus and species of the micro-organism (including the type culture collection number where applicable);
— unique reference (e.g. batch number);

— logarithm of the viable spore count expressed to 1 decimal place in scientific notation;
— recovery method used;
— type of carrier;
— type of packaging (e.g. envelope);
— composition of the recovery medium, if needed (e.g. in case of self-contained biological indicators);
— type of indicator (e.g. pH indicator) for growth, if relevant;
— type of sterilisation process(es) and the conditions for which the biological indicator has been characterised;
— resistance (*D*-value) per batch of finished biological indicator against the specified sterilisation processes throughout the labelled shelf-life; the *D*-value should be stated in applicable units (e.g. time or dose) and expressed to 1 decimal place, together with a 95 per cent confidence interval if feasible;
— method (inactivation kinetics or fraction negative method) used to determine the resistance (*D*-value); parameters to verify e.g. exposure conditions, number of replicates tested, medium and incubation conditions used for recovery after exposure, etc.;
— the *z*-value (where relevant) for the biological indicator stated in temperature units expressed to 1 decimal place in scientific notation, including the range of temperatures used to determine the *z*-value;
— the storage conditions and the expiry date.

2-2-1 User requirement specification (URS)

The particular sterilisation process (moist heat, dry heat, gas, or ionising radiation) is considered as the basis for the choice of the biological indicator. This choice includes the selection of the test micro-organism, the type of biological indicator (inoculated carrier, self-contained, or custom-made), the *D*-value, and the initial spore count. Moreover, the resistance of the test strain is suitable for the particular sterilisation method and is great compared to the resistance of micro-organisms potentially contaminating the product.

2-2-2 Quality control

Quality control for biological indicators consists of testing for purity, identity and estimation of the number of viable cells. The biological indicator should be compliant with the URS. Users employing biological indicators outside of the manufacturer's labelled recommendations should thoroughly characterise the biological indicators for the particular sterilisation process.

Purity

Examination of the micro-organisms on a suitable culture medium incubated under appropriate conditions shall not show any evidence of contamination.

Identification

Colony morphology and homogeneity of the population are verified, as appropriate.

Viable count

The viable count is performed according to the manufacturer's instructions or by any other validated method.

2-2-3 Suitability for purpose

The user shall ensure that the biological indicator is inactivated to the expected survival rate by the particular range of sterilisation conditions used.

3 BIOLOGICAL INDICATORS FOR HEAT STERILISATION

3-1 PARAMETERS OF BIOLOGICAL INDICATORS FOR HEAT STERILISATION

3-1-1 *z*-Value

Sterilisation processes can be operated at temperatures lower than the standard 121 °C (for longer exposure times) or at higher temperatures (for shorter exposure times). The *z*-value (the temperature difference that leads to a 10-fold change in the *D*-value of the biological indicator) is used to compare the efficacy of 2 cycles operated at different temperatures. For a *z*-value determination, the *D*-value must be determined at 3 or more temperatures. The intended process temperature should be within the range of the 3 temperatures. The $\log_{10}$ of the *D*-value is plotted against the temperature in degrees Celsius. The *z*-value is equal to the negative reciprocal of the slope of the best-fit linear curve as determined by $\log_{10}$-linear regression analysis.

3-1-2 Establishment of validation cycle

The characteristics of the sterilisation process (e.g. time-temperature combination, level of sterility assurance or F_0 required) are the basis for the choice of the biological indicator (type of biological indicator, test micro-organism, and initial viable count).

Inactivation of micro-organisms under sterilising conditions can be described by lethality kinetics and statistical probabilities. For a number of biological indicator units with an initial population of N_0 micro-organisms per unit and a given *D*-value, the exposure time in minutes where all units are expected to carry survivors (average of 100 surviving spores per unit) is calculated by equation (1).

$$t_s = D \times (\log_{10} N_0 - 2) \qquad (1)$$

t_s = survival time

The exposure time in minutes where all units are expected to be inactivated (average of 10^{-4} surviving spores per unit) is calculated by equation (2).

$$t_k = D \times (\log_{10} N_0 + 4) \qquad (2)$$

t_k = kill time

The objective of a validation study is to demonstrate that the sterilisation effectiveness anticipated from the physical process parameters is equivalent to the biological sterilisation effectiveness. As part of that objective, the exposure time during validation t_{vb} shall not exceed t_k. If a too high t_{vl} is chosen, even a relatively large increase in the *D*-value would still result in biological indicator units with no surviving micro-organisms. In this case, the suboptimal sterilising conditions would not be detected. It is considered reasonable to choose a t_{vl} not higher than required to expect 1 in 1000 biological indicator units having surviving micro-organisms. However, too short a t_{vl} shall not be chosen. If a t_{vl} is chosen such that 50 per cent of the biological indicator units have surviving micro-organisms, changes in the sterilising conditions (e.g. time, temperature) could still result in 100 per cent of the biological indicator units having surviving micro-organisms, and the test would be meaningless. For these reasons, a t_{vl} is chosen such that a theoretical survival rate between 10^{-1} and 10^{-3} is expected, thus:

$$D \times (\log_{10} N_0 + 1) \leq t_{el} \leq D \times (\log_{10} N_0 + 3) \qquad (3)$$

In general, biological indicators are subjected to the intended sterilisation process. However, for highly effective sterilisation processes, the calculated effectiveness of the cycle may be such that the t_k is exceeded by a wide margin. In such instances, biological validation is carried out with reduced sterilisation cycles. Such reduced cycles may be shorter in time (e.g. half cycle) or be performed at a lower temperature. In the latter case the z-value for the test micro-organism under the actual sterilising conditions shall be known.
A reduced cycle is chosen such that the temperature is not more than 1 z-value below the reference sterilisation process temperature. Biological indicators of an appropriate resistance for that cycle show an expected micro-organism survival rate within a window between the lower t_{el} and the t_k (see equation (3)). A decision not to perform this test must be justified.

Depending on the D-value of the test micro-organism and the t_{el} chosen, biological indicators having surviving micro-organisms can be expected with a low frequency (not more than 1 in 10). If it can be demonstrated that the frequency of biological indicators having surviving micro-organisms is within the expected range and is not due to inappropriate sterilising conditions, the process can be accepted.

Following a full sterilisation cycle, all biological indicators in a validation study must be inactivated, thereby proving at least a 10^6 reduction in micro-organisms. It can then be concluded, from the resistance of the spore preparation used, that the process has delivered sufficient lethality to achieve the required sterility assurance level.

3-2 BIOLOGICAL INDICATORS FOR MOIST HEAT STERILISATION

3-2-1 Test micro-organisms

Geobacillus stearothermophilus is the most widely accepted biological indicator micro-organism for moist heat sterilisation processes. Reported $D_{121°C}$-values for its spores are in the range of 1.5 min to about 4.5 min, depending on sporulation conditions, the carrier material on which the spores are inoculated, the primary package surrounding the inoculated carrier, and the environment during sterilisation. Strains ATCC 7953, NCTC 10007, CIP 52.81, NCIMB 8157 and ATCC 12980 (equivalent to NRRL B-4419) have been found to be suitable. Other strains may be used, provided equivalent performance has been demonstrated. It is recognised that a 10^5 or 10^6 population of *Geobacillus stearothermophilus* may not be suitable for sterilisation processes delivering an F_0 between 8 and 15, therefore a lower spore number (i.e. 10^3 or 10^4) or a different test micro-organism may be used. Where a test micro-organism other than *Geobacillus stearothermophilus* (e.g. *Bacillus subtilis* ATCC 35021) is used, the resistance of the test micro-organism is evaluated to ensure its suitability for the process.

3-3 BIOLOGICAL INDICATORS FOR DRY HEAT STERILISATION

The reference conditions are stated in general chapter *5.1.1.* Heat transfer is less effective with dry heat than with steam, and temperature distribution in dry heat sterilisers is less homogeneous compared to steam sterilisers.

For example, biological indicators available for dry heat sterilisation have $D_{160°C}$-values within a range of 1 to 5 min. When exposed to the reference cycle of 2 h at 160 °C, a biological indicator with a $D_{160°C}$-value of 2.5 min would be inactivated by 48 $\log_{10}$ scales. For dry-heat sterilisation processes, z-values of about 20 °C are typically assumed in calculations of equivalence of cycle effectiveness (F_H-calculations). F_H is the equivalent time in minutes at a temperature of 160 °C delivered by the sterilisation process to the product in its final container. For a biological indicator with a $D_{160°C}$-value of 5 min, the $D_{150°C}$-value would be about 16 min, and inactivation in the reference cycle would be 7.5 $\log_{10}$ scales. The use of a sterilisation process at a temperature reduced from the target temperature by 10 °C would give an expected 1 in 30 biological indicator units having surviving micro-organisms.

3-3-1 Test micro-organisms

Spores of *Bacillus atrophaeus* (e.g. ATCC 9372, NCIMB 8058, NRRL B-4418, or CIP 77.18) have been found to be suitable for use as biological indicators for dry heat sterilisation processes performed at temperatures between 160 °C and 180 °C. Where a test micro-organism other than *Bacillus atrophaeus* is used, to ensure its suitability, the resistance of the test micro-organism for the sterilisation process is evaluated as described in section 3-1-2

4 BIOLOGICAL INDICATORS FOR GAS STERILISATION

The use of biological indicators is necessary for the development, validation and monitoring of all gaseous sterilisation processes. Gas sterilisation is a multi-factorial process: gas concentration, humidity, temperature, time, surface characteristics interact in a complex manner.
A number of gas sterilisation processes are currently used, including ethylene oxide, hydrogen peroxide and peracetic acid or combinations of the latter.

Gas surface disinfection is widely used for medical devices, isolators, chambers, etc. Use for such purposes is outside the scope of the European Pharmacopoeia but the use of biological indicators as described in this general chapter may assist in the validation of such disinfection processes.

4-1 TEST MICRO-ORGANISMS

4-1-1 Ethylene oxide sterilisation

The use of spores of *Bacillus atrophaeus* (e.g. ATCC 9372, NCIMB 8058, NRRL B-4418, or CIP 77.18), or other strains of micro-organism having demonstrated equivalent performance, is recommended for ethylene oxide sterilisation. The number of viable spores is greater than or equal to 10^6 per carrier. Test micro-organisms shall have D-values relevant to the process to be validated. These biological indicators are used routinely during each sterilisation cycle thus allowing the effectiveness of the process to be checked.

4-1-2 Other processes

It is the responsibility of the user to define the sterilisation cycle and the suitability of any biological indicator used. *Geobacillus stearothermophilus* has been found suitable for vaporised hydrogen peroxide processes.

5 BIOLOGICAL INDICATORS FOR IONISING RADIATION STERILISATION

Unless otherwise indicated, biological indicators are not generally considered necessary for validation of the sterilising dose for radiation sterilisation. The use of biological indicators may however be required for the development and validation of ionising radiation sterilisation e.g. of tissues, cell preparations or other specific cases (e.g. products with a potential for spore protection).

5-1 TEST MICRO-ORGANISMS

Spores of *Bacillus pumilus* (e.g. ATCC 27142, NCTC 10327, NCIMB 10692 or CIP 77.25) or other strains of micro-

organisms having demonstrated equivalent or better performance are recommended.

6 MICROBIAL PREPARATIONS FOR STERILISATION GRADE FILTRATION

As stated in general chapter *5.1.1*, certain products that cannot be sterilised in their final container may be sterilised by a filtration process. In contrast to the biological indicators discussed in the previous sections, which assess kill-based sterilisation, the biological challenge assesses the retention of micro-organisms by the filters.

To validate the sterilisation process, it must be demonstrated that the filtration process (usually in a scaled-down model) is capable of completely retaining a microbial challenge of at least 10^7 CFU per square centimetre of effective filter surface using a suitable test micro-organism. This test should mimic the actual filtration process as closely as possible. Where feasible, the test is carried out in the product using the specified filtration conditions. If this is not possible, e.g. due to the antimicrobial properties of the product, a medium as similar as possible to the product must be used in the test.

6-1 TEST MICRO-ORGANISMS

For processes using a filtration system with a nominal pore size not greater than 0.22 µm, a suspension of *Brevundimonas diminuta* (ATCC 19146, NCIMB 11091 or CIP 103020) is recommended. The *Brevundimonas diminuta* suspension must be prepared in order to achieve predominantly single cells of the smallest possible size. Other micro-organisms, for example natural flora isolated from the product or process in question, may be used if presenting a stronger challenge to the sterile filtration system than *Brevundimonas diminuta*. For filtration systems with a nominal pore size of 0.1 µm or less, a suspension of *Acholeplasma laidlawii* (ATCC 23206) may be used.

Methods of Sterilisation

(Application of the F Concepts to Heat Sterilisation Processes, Ph. Eur. general texts 5.1.5)

The following chapter is published for information.

INTRODUCTION

Heat sterilisation can be differentiated into 2 types: moist heat sterilisation using saturated steam or water heated to the sterilisation temperature; and dry heat sterilisation using hot air with a moisture content so low as to have an insignificant biological activity.

DEFINITIONS

The *D*-value (or decimal reduction value) is the time in minutes required at a defined temperature to reduce the number of viable test micro-organisms by 90 per cent. It is only of significance under precisely defined experimental conditions.

The *z*-value is the change in temperature in degrees Celsius required to alter the *D*-value by a factor of 10 (the *z*-value relates the resistance of a micro-organism to changes in temperature). The *z*-value is calculated using the following equation:

$$z = \frac{T_2 - T_1}{log_{10}D_1 - log_{10}D_2}$$

D_1 = *D*-value of the micro-organism at temperature T_1;
D_2 = *D*-value of the micro-organism at temperature T_2;
T = temperature.

The *F*-value of a heat sterilisation process (F_0 for moist heat sterilisation or F_H for dry heat sterilisation) is the lethality expressed in terms of the equivalent time in minutes at the reference temperature delivered by the process to the sterilisation load, with reference to micro-organisms possessing the relevant theoretical *z*-value in Table 5.1.5-1.

The total *F* of a process takes into account the heating and cooling phases of a cycle and can be calculated by integration of lethal rates with respect to time at discrete temperature intervals above the minimum temperature specified in Table 5.1.5-1.

The following mathematical relationships apply:

$$F_0 = D_{121}(\log_{10} N_0 - \log_{10} N)$$

$$F_H = D_{160}(\log_{10} N_0 - \log_{10} N)$$

D_{121} = *D*-value of the reference spores (*5.1.2*) at 121 °C;
D_{160} = *D*-value of the reference spores (*5.1.2*) at 160 °C;
N_0 = initial number of viable micro-organisms;
N = final number of viable micro-organisms.

Table 5.1.5.-1 – *Parameters for moist heat and dry heat sterilisation*

Sterilisation	*F*	Theoretical *z*-value (°C)	Reference temperature (°C)	Minimum temperature (°C)
Moist heat	F_0	10	121	110
Dry heat	F_H	20	160	140

For both dry and moist heat sterilisation cycles, the relevant *F*-value is used to demonstrate that the required sterility assurance level of equal to or less than 10^{-6} is consistently achieved.

Appendix XIX

A. Containers Introduction

(Ph. Eur. general texts 3.2)

This Appendix provides requirements, guidance and information on containers for pharmaceutical use. Additional guidance is provided in a number of British Standards. Attention is drawn in particular to British Standards 1679-5: 1973, 1679-6: 1994, 1679-7: 1968 and 1679-8: 1992. The expression 'tamper-evident container' means a closed container fitted with a device that reveals irreversibly whether the container has been opened, whereas, the expression 'tamper-proof container' means a closed container in which access to the contents is prevented under normal conditions of use. The two terms are considered to be synonymous by the European Pharmacopoeia Commission.

A container for pharmaceutical use is an article that contains or is intended to contain a product and is, or may be, in direct contact with it. The closure is a part of the container.

The container (see General Notices section 1.3) is so designed that the contents may be removed in a manner appropriate to the intended use of the preparation.
It provides a varying degree of protection depending on the nature of the product and the hazards of the environment, and minimises the loss of constituents. The container does not interact physically or chemically with the contents in a way that alters their quality beyond the limits tolerated by official requirements.

Single-dose container A single-dose container holds a quantity of the preparation intended for total or partial use on 1 occasion only.

Multidose container A multidose container holds a quantity of the preparation suitable for 2 or more doses.

Well-closed container A well-closed container protects the contents from contamination with extraneous solids and liquids and from loss of contents under ordinary conditions of handling, storage and transport.

Airtight container An airtight container is impermeable to solids, liquids and gases under ordinary conditions of handling, storage and transport. If the container is intended to be opened on more than 1 occasion, it must be so designed that it remains airtight after re-closure.

Sealed container A sealed container is a container closed by fusion of the material of the container.

Tamper-evident container A tamper-evident container is a closed container fitted with a device that reveals irreversibly whether the container has been opened.

Child-proof container A container that is fitted with a closure that prevents opening by children.

B. Glass Containers for Pharmaceutical Use

(Ph. Eur. method 3.2.1)

Glass containers for pharmaceutical use are glass articles intended to come into direct contact with pharmaceutical preparations.

Colourless glass Is highly transparent in the visible spectrum.

Coloured glass Is obtained by the addition of small amounts of metal oxides, chosen according to the desired spectral absorbance.

Neutral glass Is a borosilicate glass containing significant amounts of boric oxide, aluminium oxide, alkali metal oxides and/or alkaline earth oxides in the glass network. Due to its composition, neutral glass has a high hydrolytic resistance and a high thermal shock resistance.

Soda-lime-silica glass Is a silica glass containing alkali metal oxides, mainly sodium oxide, and alkaline earth oxides, mainly calcium oxide, in the glass network. Due to its composition, soda-lime-silica glass has only a moderate hydrolytic resistance.

The hydrolytic stability of glass containers for pharmaceutical use is expressed by the resistance to the release of soluble mineral substances into water under the prescribed conditions of contact between the inner surface of the container or glass grains and water. The hydrolytic resistance is evaluated by titrating released alkali reacting ions.
According to their hydrolytic resistance, glass containers are classified as follows:

— type I glass containers: neutral glass, with a high hydrolytic resistance due to the chemical composition of the glass itself;
— type II glass containers: usually of soda-lime-silica glass with a high hydrolytic resistance resulting from suitable treatment of the inner surface;
— type III glass containers: usually of soda-lime-silica glass with only moderate hydrolytic resistance.

The following italicised statements constitute general recommendations concerning the type of glass container that may be used for different types of pharmaceutical preparations. The manufacturer of a pharmaceutical product is responsible for ensuring the suitability of the chosen container.

Type I glass containers are suitable for most preparations whether or not for parenteral administration.

Type II glass containers are suitable for most acidic and neutral, aqueous preparations whether or not for parenteral administration.

Type III glass containers are in general suitable for non-aqueous preparations for parenteral administration, for powders for parenteral administration (except for freeze-dried preparations) and for preparations not for parenteral administration.

Glass containers with a hydrolytic resistance higher than that recommended above for a particular type of preparation may generally also be used.

The container chosen for a given preparation shall be such that the glass material does not release substances in quantities sufficient to affect the stability of the preparation or to present a risk of toxicity. In justified cases, further detailed information may be necessary to assess the impact on chronic use and for vulnerable patient groups.

Preparations for parenteral administration are normally presented in colourless glass, but coloured glass may be used for substances known to be light-sensitive. Colourless or coloured glass is used for the other pharmaceutical preparations. It is recommended that all glass containers for liquid preparations and for powders for parenteral administration permit the visual inspection of the contents.

The inner surface of glass containers may be specially treated to improve hydrolytic resistance, to confer water-repellancy, etc. The outer surface may also be treated, for example to reduce friction and to improve resistance to abrasion.

The outer treatment is such that it does not contaminate the inner surface of the container.

Except for type I glass containers, glass containers for pharmaceutical preparations are not to be re-used. Containers for human blood and blood components must not be re-used.

PRODUCTION

When glass containers for pharmaceutical use are manufactured under stressed conditions (e.g. temperature-time profile) and/or are placed in contact with particularly aggressive pharmaceutical preparations, they may undergo delamination, i.e the separation of the inner glass surface into thin layers called lamellae or flakes. Glass delamination may be the result of a chemical attack that occurs according to well-known glass corrosion mechanisms, such as dissolution by hydrolysis and ion exchange (leaching) as a function of the pH. The process of interaction between the glass surface and the pharmaceutical preparation requires incubation time, and flaking may only become visible a number of months after filling.

Several risk factors are known to increase the propensity of a glass to delaminate. The chemical composition of the pharmaceutical preparation, the presence of buffers like citrate or phosphate, which are known to corrode glass, and the ionic strength of the liquid medium may all strongly favour delamination. The manufacturing process of the container, chemical treatments of the inner surface, and terminal sterilisation and processing at the pharmaceutical filling lines are other important risk factors to be considered. It is recommended that the user of the container assesses the compatibility of the glass container and the pharmaceutical preparation on a case-by-case basis, considering for example the dosage form, properties of the formulation and glass quality.

The propensity to delamination of glass containers from different sources can be assessed and ranked by exposing the container to accelerated degradation testing, carried out at specified temperatures for a short time and using the solutions associated with the actual pharmaceutical preparation as extractants. The presence of particles in the extraction solution, the occurrence of phase separation on the inner surface, and the steep increase of silica concentration in the extraction solution are all indicators of a potential propensity for delamination. Accelerated degradation testing can be used as a predictive tool to select the most appropriate container for the intended preparation, but the full compatibility of the active substance with the glass leachate can only be assessed by a stability test under normal conditions of use.

TESTS

Glass containers for pharmaceutical use comply with the relevant test or tests for hydrolytic resistance. When glass containers have non-glass components, the tests apply only to the glass part of the container.

To define the quality of glass containers according to the intended use, one or more of the following tests are necessary.

Tests for hydrolytic resistance are carried out to define the type of glass (I, II or III) and to control its hydrolytic resistance.

In addition, containers for aqueous parenteral preparations are tested for arsenic release and coloured glass containers are tested for spectral transmission.

HYDROLYTIC RESISTANCE

Table 3.2.1.-1. – *Types of glass*

Type of container	Test to be performed
Type I and type II glass containers (to distinguish from type III glass containers)	Test A (surface test)
Type I glass containers (to distinguish from type II and type III glass containers)	Test B (glass grains test) or test C (etching test)
Type I and type II glass containers (if there are doubts whether the high hydrolytic resistance is due to the chemical composition or to the surface treatment)	Tests A and B, or tests A and C

The test is carried out by titration of the extraction solutions obtained under the conditions described for tests A, B and C. Test C is performed if there are uncertainties whether the container is type I or type II.

EQUIPMENT

— An autoclave or steam steriliser capable of withstanding a pressure of 2.5×10^5 N/m^2 (equivalent to 0.25 MPa = 2.5 bar) or more and capable of carrying out the heating cycle described under Autoclaving process. Preferably it is equipped with a constant-pressure regulator or other suitable means in order to maintain the temperature at 121 ± 1 °C. The autoclave vessel is equipped with a heating device, a thermometer integrated in the autoclave, a pressure gauge, a vent cock (for manually operated autoclaves only) and a tray of sufficient capacity to accommodate, above the water level, the number of containers needed to carry out the test. The autoclave has the possibility to connect a calibrated resistance thermometer or a calibrated thermocouple from the inner chamber to an external measuring device to allow a temperature measurement independent from the autoclave system.

The autoclave vessel and all ancillary equipment must be washed thoroughly with water R before use.

— A calibrated resistance thermometer or calibrated thermocouple connected to a suitable temperature measuring device.
— Burettes with a suitable capacity.
— One-mark volumetric flasks, with a capacity of 1000 mL.
— Pipettes and beakers.
— Conical flasks with capacities of 100 mL and 250 mL.
— A water-bath.
— Metal foil (e.g. aluminium, stainless steel).

Flasks and beakers must already have been used for the test or have been filled with *water R* and kept in an autoclave at 121 °C for at least 1 h before being used.

DETERMINATION OF THE FILLING VOLUME

The filling volume is the volume of water to be introduced into the container for the purpose of the test. For vials and bottles the filling volume is 90 per cent of the brimful capacity. For ampoules it is the volume up to the height of the shoulder.

Vials and bottles

Select, at random, 6 containers from the sample lot, or 3 if their capacity exceeds 100 mL, and remove any debris or dust. Weigh the empty containers with an accuracy of 0.1 g. Place the containers on a horizontal surface and fill them with *water R* until about the rim edge, avoiding overflow and introduction of air bubbles. Adjust the liquid levels to the brimful line. Weigh the filled containers to obtain the mass of

the water expressed to 2 decimal places for containers having a nominal volume less than or equal to 30 mL, and expressed to 1 decimal place for containers having a nominal volume greater than 30 mL. Calculate the mean value of the brimful capacity in millilitres and multiply it by 0.9. This volume, expressed to 1 decimal place, is the filling volume for the particular container lot.

Ampoules

Place at least 6 dry ampoules on a flat, horizontal surface and fill them with *water R* from a burette, until the water reaches point A, where the body of the ampoule declines to the shoulder (see Figure 3.2.1.-1). Read the capacities (expressed to 2 decimal places) and calculate the mean value. This volume, expressed to 1 decimal place, is the filling volume for the particular ampoule lot. The filling volume may also be determined by weighing.

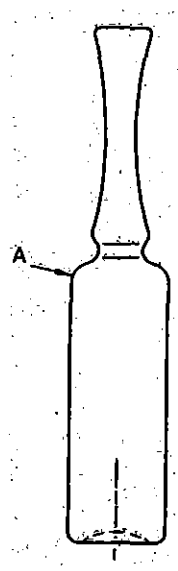


Figure 3.2.1.-1. – *Filling volume of ampoules (up to point A)*

Syringes and cartridges

Select 6 syringes or cartridges. Close the small opening (mouth of cartridges and needle and/or Luer cone of syringes) using an inert material (e.g. a tip cap) or any other suitable means to prevent water leakage. Determine the mean brimful volume in accordance with the procedure described under Vials and bottles and multiply it by 0.9. This volume, expressed to 1 decimal place, is the filling volume for the particular container lot.

TEST A. HYDROLYTIC RESISTANCE OF THE INNER SURFACES OF GLASS CONTAINERS (SURFACE TEST)

The determination is carried out on unused containers. The volumes of the test liquid necessary are indicated in Table 3.2.1.-2.

Table 3.2.1.-2. – *Volume of test liquid and number of titrations*

Filling volume (mL)	Volume of test liquid for 1 titration (mL)	Number of titrations
Up to 3	25.0	1
Above 3 and up to 30	50.0	2
Above 30 and up to 100	100.0	2
Above 100	100.0	3

Cleaning

Remove any debris or dust. Shortly before the test, fill each container to the brim with *water R* and allow to stand, filled with water, for 20 ± 5 min. Empty the containers, carefully rinse twice with *water R* and once with *water R1* and allow to drain.

Closed ampoules are not rinsed before testing. Closed ampoules may be warmed on a water-bath or in an oven at about 40 °C for approximately 2 min before opening to avoid underpressure when opening.

Filling

Fill the containers with *water R1* up to the filling volume.

Loosely cap each container with an inert material, for example with inverted beakers of such a size that the bottoms of the beakers fit snugly down on the rims of the sample. Ampoules and vials capped with clean aluminium foil are further examples. Place syringes and cartridges in a beaker and cover the beaker with clean aluminium foil.

Containers of a volume of 2 mL or less, in which the water is not sufficiently retained during the autoclaving process, may be closed in a suitable way, e.g. with a stopper or plug of inert material, such as silicone, and fixed using a plunger or a stable fixing or clamping device.

Place the samples, gathered in groups in glass dishes or in beakers or other suitable holders, on the rack in the autoclave containing *water R* at room temperature. Ensure that they are held above the level of the water in the autoclave.

Autoclaving process

Reference thermal cycle

The autoclave is run in such a way that the temperature in the containers to be tested follows a thermal cycle with the following characteristics: temperature raised from room temperature to 100 °C within 20-30 min; temperature maintained at 100 ± 1 °C for 10 ± 1 min; temperature in the containers raised from 100 °C to 121 °C within 20-22 min; temperature maintained at 121 ± 1 °C for 60 ± 1 min; temperature cooled to 100 °C within 40-44 min.

Autoclave calibration

Before being used for the first time, the autoclave and the temperature measuring system are calibrated to ensure that the autoclave settings are suitable to guarantee that the temperature inside the containers is 121 ± 1 °C.

NOTE: significant differences may be observed between the temperature measured in the autoclave chamber and inside the containers.

Take a set of containers of intermediate capacity (10 mL for instance) and fill them with *water R1*. Select a sufficient number of containers to fill completely the tray within the autoclave chamber. Insert the end of the calibrated resistance thermometer or calibrated thermocouple into one of the containers through a hole in the closure having approximately the same diameter as the probe and connect it to the external measuring device. If the container is too small to insert a thermocouple, place the thermocouple in a similar container of suitable size filled with *water R1*. Close the autoclave door or lid securely and run the autoclave to achieve the target thermal cycle in the containers. Where a manual autoclave is run, leave the vent cock open. Heat the autoclave at a regular rate so that steam issues vigorously from the vent cock after 20-30 min, and maintain a vigorous evolution of steam for a further 10 min.

Close the vent cock, follow the temperature increase on the calibrated thermocouple measuring device by comparison with readings taken from the autoclave thermometer and adjust the autoclave settings accordingly in order to match

the target thermal cycle. Keep the temperature ramp as smooth as possible.

Using the calibrated thermocouple measuring device, ensure that deviations from the holding temperature of 121 ± 1 °C are within the tolerance. When cooling down, vent to prevent the formation of a vacuum. For safety reasons (boiling retardation) do not open the autoclave before the water in the containers has reached a temperature of 95 °C. Remove the hot samples from the autoclave and cool cautiously to room temperature within 30 min.

Record the autoclave settings used to carry out the thermal cycle and use these settings for routine autoclave runs.

At regular intervals verify the validation of the calibration. Establish a re-calibration plan based on quality assurance criteria, recalibrate as appropriate and keep records.

Routine autoclave runs

Use the autoclave settings established during the calibration stage and follow the same thermal cycle described above. Container sets of different capacity can be tested during the same run. Keep the glass load very similar in size and mass to the load used during the calibration stage. The use of the calibrated thermocouple is no longer necessary provided the calibration is proved to be valid over a defined time span.

At the end of the cycle, remove the hot samples from the autoclave and cool them cautiously to room temperature within 30 min.

NOTE: depending on the type or size of the autoclave the heat transfer and thus the resulting thermal cycle in the containers may vary with the total load of the autoclave. It may therefore be necessary to adjust the autoclave load.

Method

Carry out the titration within 1 h of removal of the containers from the autoclave. Combine the liquids obtained from the containers and mix. Introduce the prescribed volume (Table 3.2.1.-2) into a conical flask (test solution). Place the same volume of *water R1* into a 2nd similar flask as a blank. Add to each flask 0.05 mL of *methyl red solution R* for each 25 mL of liquid. Titrate the blank with *0.01 M hydrochloric acid*. Titrate the test solution with the same acid until the colour of the resulting solution is the same as that obtained for the blank. Subtract the value found for the blank titration from that found for the test solution and express the results in millilitres of *0.01 M hydrochloric acid* per 100 mL. Express titration values of less than 1.0 mL to 2 decimal places and titration values of more than or equal to 1.0 mL to 1 decimal place.

Limits

The results, or the average of the results if more than 1 titration is performed, is not greater than the values stated in Table 3.2.1.-3.

Table 3.2.1.-3. – *Limit values in the test for surface hydrolytic resistance*

	Maximum volume of 0.01 M HCl per 100 mL of test solution (mL)	
Filling volume (mL)	Types I and II glass containers	Type III glass containers
Up to 0.5	3.0	30.0
Above 0.5 and up to 1	2.0	20.0
Above 1 and up to 2	1.8	17.6
Above 2 and up to 3	1.6	16.1
Above 3 and up to 5	1.3	13.2
Above 5 and up to 10	1.0	10.2
Above 10 and up to 20	0.80	8.1
Above 20 and up to 50	0.60	6.1
Above 50 and up to 100	0.50	4.8
Above 100 and up to 200	0.40	3.8
Above 200 and up to 500	0.30	2.9
Above 500	0.20	2.2

TEST B. HYDROLYTIC RESISTANCE OF GLASS GRAINS (GLASS GRAINS TEST)

Check that the articles as received have been annealed to a commercially acceptable quality.

The test may be performed on the canes used for the manufacture of tubing glass containers or on the containers.

Equipment

- a mortar, pestle (see Figure 3.2.1.-2) and hammer made of tempered, magnetic steel;
- as an alternative to the mortar, pestle and hammer, a ball mill can be used; the ball mill is made of agate, zirconia or stainless steel with a volume of 250 mL; 2 balls with a diameter of 40 mm or 3 balls with a diameter of 30 mm are suitable;
- a set of 3 square-mesh sieves of stainless steel, mounted on frames of the same material and consisting of the following:

(a) sieve no. 710;

(b) sieve no. 425;

(c) sieve no. 300;

- a mechanical sieve-shaker or a sieving machine may be used to sieve the grains;
- a permanent magnet;
- metal foil (e.g. aluminium, stainless steel);
- a hot-air oven, capable of maintaining a temperature of 140 ± 5 °C;
- a balance, capable of weighing up to 500 g with an accuracy of 0.005 g;
- a desiccator;
- an ultrasonic bath.

Method

Rinse the containers to be tested with *water R* and dry in the oven. Wrap at least 3 of the glass articles in clean paper and crush to produce 2 samples of about 100 g each, in pieces not more than 30 mm across.

Where a mortar, pestle and hammer are used, place in the mortar 30-40 g of the pieces 10-30 mm across taken from 1 of the samples, insert the pestle and strike it heavily, once only, with the hammer. Transfer the contents of the mortar to sieve (a), the coarsest of the set. Repeat the operation until all fragments have been transferred to the sieve. Shake the set of sieves for a short time by hand and remove the glass that remains on sieves (a) and (b). Submit these portions to further fracture, repeating the operation until about 10 g of glass remains on sieve (a). Reject this portion and the portion that passes through sieve (c). Reassemble the set of sieves and shake for 5 min. Transfer to a weighing bottle those glass grains that pass through sieve (b) and are retained on sieve (c).

Where a ball mill is used, place in the ball mill beaker about 50 g of the pieces 10-30 mm across taken from 1 of the samples, add the balls and crush thin-walled glass (wall thickness up to 1.5 mm) for up to 2 min and thick-walled glass (wall thickness greater than 1.5 mm) for up to 5 min. Transfer the grains to sieve (a), sieve for about 30 s and collect the grains retained on sieve (c). Transfer the glass from sieves (a) and (b) into the ball mill and crush and sieve again as indicated above. Combine the grains retained on sieve (c).

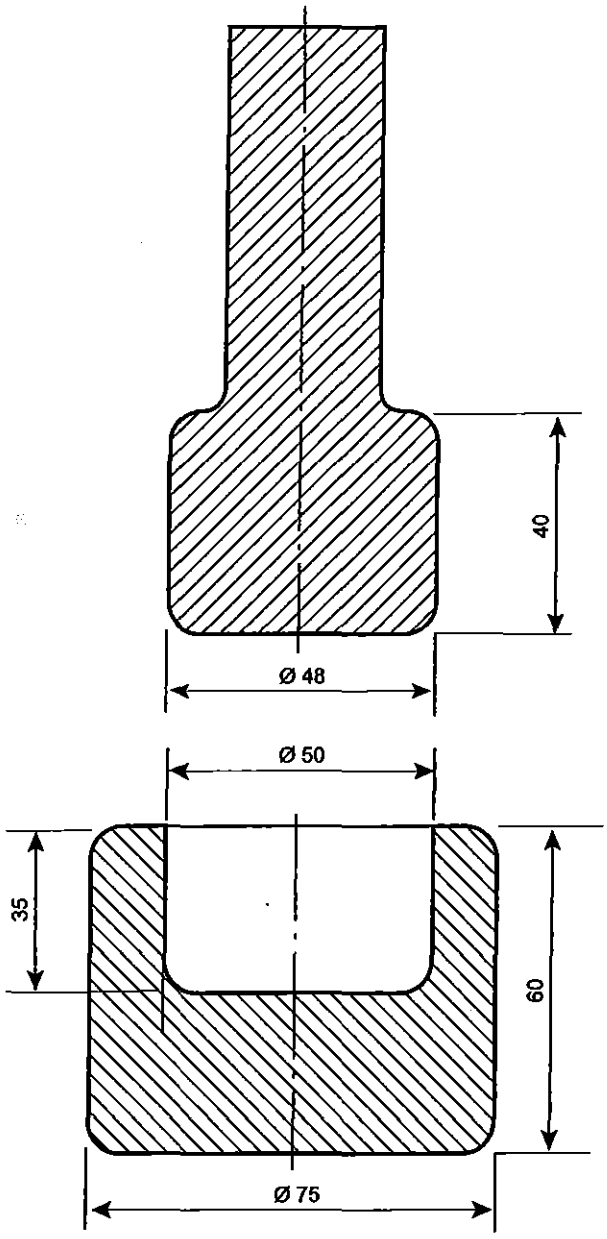


Figure 3.2.1.-2. – *Mortar and pestle apparatus for glass grains method (dimensions in millimetres)*

Repeat the crushing and sieving procedure with the other glass sample and thus 2 samples of grains, each of which shall be in excess of 10 g, are obtained. Spread each sample on a piece of clean glazed paper and remove any iron particles by passing the magnet over them. Transfer each sample into a beaker for cleaning. Add to the grains in each beaker 30 mL of *acetone R* and scour the grains by suitable means, such as a rubber- or plastic-coated glass rod. After scouring the grains, allow to settle and decant as much acetone as possible. Add another 30 mL of *acetone R*, swirl, allow to settle and decant again, and add 30 mL of *acetone R*.

Fill the bath of the ultrasonic vessel with water at room temperature, then place the beaker in the rack and immerse it until the level of the acetone is at the level of the water; apply the ultrasound for 1 min. Swirl the beaker, allow to settle and decant the acetone as completely as possible, add 30 mL of *acetone R* and repeat the ultrasonic cleaning operation. If a fine turbidity persists, repeat the ultrasonic cleaning and acetone washing until the solution remains clear. Swirl and decant the acetone then dry the grains, first by putting the beaker on a warm plate to remove excess acetone and then by heating at 140 °C for 20 min in the drying oven. Transfer the dried grains from each beaker into separate weighing bottles, insert the stoppers and cool in the desiccator. Weigh 10.00 g of the cleaned and dried grains into 2 separate conical flasks. Add 50 mL of *water R1* into each by means of a pipette (test solutions). Pipette 50 mL of *water R1* into a 3rd conical flask as a blank. Distribute the grains evenly over the flat bases of the flasks by gentle shaking. Close the flasks with neutral glass dishes or aluminium foil rinsed with *water R*, or with inverted beakers so that the inner surface of the beakers fit snugly down onto the top rims of the flasks. Place all 3 flasks in the rack in the autoclave containing the water at room temperature, and ensure that they are held above the level of the water in the vessel. Carry out the autoclaving procedure in a similar manner to that described under test A, but maintain the temperature of 121 ± 1 °C only for 30 ± 1 min. Do not open the autoclave until it has cooled to 95 °C. Remove the hot samples from the autoclave and cool the flasks in running tap water as soon as possible, avoiding thermal shock.

To each of the 3 flasks add 0.05 mL of *methyl red solution R*. Titrate the blank solution immediately with *0.02 M hydrochloric acid* then titrate the test solutions with the same acid until the colour matches that obtained with the blank solution. Subtract the titration volume for the blank solution from that for the test solution.

NOTE: where necessary to obtain a sharp end-point, the clear solution is to be decanted into a separate 250 mL flask. Rinse the grains with 3 quantities, each of 15 mL, of water R1 by swirling and add the washings to the main solution. Add 0.05 mL of methyl red solution R. Titrate and calculate as described below. In this case also add 45 mL of water R1 and 0.05 mL of methyl red solution R to the blank solution.

Calculate the mean value of the results in millilitres of *0.02 M hydrochloric acid* per gram of the sample and if required its equivalent in alkali extracted, calculated as micrograms of sodium oxide per gram of glass grains.

1 mL of *0.02 M hydrochloric acid* is equivalent to 620 µg of sodium oxide.

Repeat the test if the highest and lowest observed values differ by more than 20 per cent.

Limits

Type I glass containers require not more than 0.1 mL of *0.02 M hydrochloric acid* per gram of glass, type II and type III glass containers require not more than 0.85 mL of *0.02 M hydrochloric acid* per gram of glass.

TEST C. TO DETERMINE WHETHER THE CONTAINERS HAVE BEEN SURFACE-TREATED (ETCHING TEST)

If there are uncertainties whether a container has been surface-treated, and/or to distinguish between type I and type II glass containers, test C is used in addition to test A. Alternatively, tests A and B may be used. Test C may be carried out either on unused samples or on samples previously used in test A.

Vials and bottles

The volumes of test liquid required are shown in Table 3.2.1.-2.

Rinse the containers twice with *water R*, fill to the brimful point with a mixture of 1 volume of *hydrofluoric acid R* and 9 volumes of *hydrochloric acid R* and allow to stand for 10 min. Empty the containers and rinse carefully 5 times with *water R*. Immediately before the test, rinse once again with *water R*. Submit the containers thus prepared to the

same autoclaving and determination procedure as described in Test A for surface hydrolytic resistance. If the results are considerably higher than those obtained from the original surfaces (by about a factor of 5 to 10), the samples have been surface-treated.

Ampoules, cartridges and syringes

NOTE: ampoules, cartridges and syringes made from glass tubing are not normally subjected to internal surface treatment because their high chemical resistance is dependent upon the chemical composition of the glass as a material.

Apply the test method as described above for vials and bottles. If the ampoules are not surface-treated, the new values are slightly lower than those obtained in previous tests.

Distinction between type I and type II glass containers

The results obtained in Test C are compared to those obtained in Test A. The interpretation of the result is shown in Table 3.2.1.-4.

Table 3.2.1.-4. – *Distinction between type I and type II glass containers*

Type I	Type II
The values are closely similar to those found in the test for surface hydrolytic resistance for type I glass containers.	The values greatly exceed those found in the test for surface hydrolytic resistance and are similar to but not larger than those for type III glass containers.

ARSENIC

The test applies to glass containers for aqueous parenteral preparations.

Hydride generation atomic absorption spectrometry (*2.2.23, Method I*).

Test solution Use the extraction solution obtained from containers of types I and II, after autoclaving at 121 °C for 1 h as described under Test A for surface hydrolytic resistance. Transfer 10.0 mL to a 100 mL volumetric flask. Add 10 mL of *hydrochloric acid R* and 5 mL of a 200 g/L solution of *potassium iodide R*. Heat on a water-bath at 80 °C for 20 min, allow to cool and dilute to 100.0 mL with *water R*.

Reference solutions Prepare the reference solutions using *arsenic standard solution (1 ppm As) R*. Add 10 mL of *hydrochloric acid R* and 5 mL of a 200 g/L solution of *potassium iodide R*. Heat on a water-bath at 80 °C for 20 min, allow to cool and dilute to 100.0 mL with *water R*. The concentration range of the reference solutions is typically 0.005-0.015 ppm of As.

Acid reservoir *Hydrochloric acid R.*

Reducing reservoir *Sodium tetrahydroborate reducing solution R.*

Use a hydride generation device to introduce the test solution into the cuvette of the spectrometer. Establish and standardise instrumental operating conditions according to the manufacturer's instructions, optimise the uptake rate of the peristaltic pump, then connect it to the acid reservoir, the reducing reservoir and the test solution.

Source Hollow-cathode lamp.

Wavelength 193.7 nm.

Atomisation device Air-acetylene flame.

Limit Maximum 0.1 ppm of As.

SPECTRAL TRANSMISSION FOR COLOURED GLASS CONTAINERS

Equipment

A UV-Vis spectrophotometer, equipped with a photodiode detector or equipped with a photomultiplier tube coupled with an integrating sphere.

Preparation of the specimen

Break the glass container or cut it with a circular saw fitted with a wet abrasive wheel, such as a carborundum or a bonded-diamond wheel. Select sections representative of the wall thickness and trim them as suitable for mounting in a spectrophotometer. If the specimen is too small to cover the opening in the specimen holder, mask the uncovered portion with opaque paper or tape, provided that the length of the specimen is greater than that of the slit. Before placing in the holder, wash, dry and wipe the specimen with a lens tissue. Mount the specimen with the aid of wax, or by other convenient means, taking care to avoid leaving fingerprints or other marks.

Method

Place the specimen in the spectrophotometer with its cylindrical axis parallel to the slit and in such a way that the light beam is perpendicular to the surface of the section and that the losses due to reflection are at a minimum. Measure the transmission of the specimen with reference to air in the spectral region of 290-450 nm, continuously or at intervals of 20 nm.

Limits

The observed spectral transmission for coloured glass containers for preparations that are not for parenteral administration does not exceed 10 per cent at any wavelength in the range of 290-450 nm, irrespective of the type and the capacity of the glass container. The observed spectral transmission in coloured glass containers for parenteral preparations does not exceed the limits given in Table 3.2.1.-5.

Table 3.2.1.-5. – *Limits of spectral transmission for coloured glass containers for parenteral preparations*

	Maximum percentage of spectral transmission at any wavelength between 290 nm and 450 nm	
Nominal volume (mL)	Flame-sealed containers	Containers with closures
Up to 1	50	25
Above 1 and up to 2	45	20
Above 2 and up to 5	40	15
Above 5 and up to 10	35	13
Above 10 and up to 20	30	12
Above 20	25	10

ANNEX – TEST FOR SURFACE HYDROLYTIC RESISTANCE – DETERMINATION BY FLAME SPECTROMETRY

The surface hydrolytic resistance of glass of types I and II may be determined by analysis of the leaching solution by flame spectrometry. A number of elements that, when present as oxides in glass, contribute to the alkalinity of the solution, are determined and used to express an alkali equivalent. The spectrometric method has the advantage of allowing the use of a much smaller sample of extract so that it can be applied to small individual containers. This enables an evaluation of the uniformity of the containers in a

given batch where this is critical. The results of this measurement are not equivalent to those of titrimetry and the 2 methods cannot be considered interchangeable. A correlation between the 2 is dependent on the type of glass and the size and shape of the container. The titrimetric method is the reference method of the Pharmacopoeia; the spectrometric method may be used in justified and authorised cases.

A method suitable for this type of analysis is shown below.

The determination is carried out on unused containers. The number of containers to be examined is indicated in Table 3.2.1.-6.

Table 3.2.1.-6. – *Number of containers to be examined for the spectrometric method*

Filling volume (mL)	Number of containers to be measured separately	Additional containers for preliminary measurements
Up to 2	20	2
Above 2 and up to 5	15	2
Above 5 and up to 30	10	2
Above 30 and up to 100	5	1
Above 100	3	1

Instructions on determination of the filling volume, cleaning of the containers, filling and heating are given above under Hydrolytic resistance and Test A.

SOLUTIONS

Spectrochemical buffer solution Dissolve 80 g of *caesium chloride R* in about 300 mL of *water R1*, add 10mL of *6 M hydrochloric acid R*, dilute to 1.0 L with *water R1* and mix.

Stock solutions:

— sodium oxide, $c(Na_2O)$ = 1 mg/mL;
— potassium oxide, $c(K_2O)$ = 1 mg/mL;
— calcium oxide, $c(CaO)$ = 1 mg/mL.

Commercially available stock solutions may also be used.

Standard solutions Prepare standard solutions by diluting the stock solutions with *water R1* to obtain concentrations suitable for establishing the reference solutions in an appropriate manner, e.g. with concentrations of 20 µg/mL of sodium oxide, potassium oxide and calcium oxide, respectively. Commercially available standard solutions may also be used.

Reference solutions Prepare the reference solutions for establishing the calibration graph (set of calibration solutions) by diluting suitable concentrated standard solutions with *water R1*, so that the normal working ranges of the specific elements are covered, taking into account the instrument used for the measurement. Typical concentration ranges of the reference solutions are:

— for determination by atomic emission spectrometry of sodium oxide and potassium oxide: up to 10 µg/mL;
— for determination by atomic absorption spectrometry of sodium oxide and potassium oxide: up to 3 µg/mL;
— for determination by atomic absorption spectrometry of calcium oxide: up to 7 µg/mL.

Use reference solutions containing 5 per cent *V/V* of the spectrochemical buffer solution.

METHOD

Carry out preliminary measurements of the potassium oxide and calcium oxide concentrations on one of the extraction solutions. If, for one container type, the concentration of potassium oxide is less than 0.2 µg/mL and the concentration of calcium oxide is less than 0.1 µg/mL, the remaining extraction solutions of this container type need not be analysed for these ions. Aspirate the extraction solution from each sample directly into the flame of the atomic absorption or atomic emission instrument and determine the approximate concentrations of sodium oxide (and potassium oxide and calcium oxide, if present) by reference to calibration graphs produced from the reference solutions of suitable concentration.

FINAL ANALYSIS

If dilution is unnecessary, add to each container a volume of the spectrochemical buffer solution equivalent to 5 per cent of the filling volume, mix well and determine sodium oxide, calcium oxide and potassium oxide, by reference to calibration graphs. For the determination of the calcium oxide concentration by flame spectrometry, a nitrous oxide/acetylene flame is used.

If dilution is necessary, determine sodium oxide, calcium oxide and potassium oxide, if present, following the procedures as described above. The solutions shall contain 5 per cent *V/V* of the spectrochemical buffer solution. Concentration values less than 1.0 µg/mL are expressed to 2 decimal places, values greater than or equal to 1.0 µg/mL to 1 decimal place. Correct the result for the buffer addition and for any dilution.

DETERMINATION

Determine the mean value of the concentration of individual oxides found in the samples tested, in micrograms of the oxide per millilitre of the extraction solution, and calculate the sum of the individual oxides, expressed as micrograms of sodium oxide per millilitre of the extraction solution, using the following mass conversion factors:

— 1 µg of potassium oxide corresponds to 0.658 µg of sodium oxide;
— 1 µg of calcium oxide corresponds to 1.105 µg of sodium oxide.

Limits

The mean value is not greater than the value given in Table 3.2.1.-7.

Table 3.2.1.-7. – *Limit values in the test for surface hydrolytic resistance by flame spectrometry, for type I and type II glass containers*

Filling volume (mL)	Limit values for the concentration of oxides, expressed as sodium oxide (µg/mL)
Up to 0.5	7.50
Above 0.5 and up to 1	5.00
Above 1 and up to 2	4.50
Above 2 and up to 3	4.10
Above 3 and up to 5	3.20
Above 5 and up to 10	2.50
Above 10 and up to 20	2.00
Above 20 and up to 50	1.50
Above 50 and up to 100	1.20
Above 100 and up to 200	1.00
Above 200 and up to 500	0.75
Above 500	0.50

C. Plastic Containers and Closures

(Plastic Containers and Closures for Pharmaceutical Use, Ph. Eur. method 3.2.2)

A plastic container for pharmaceutical use is a plastic article which contains or is intended to contain a pharmaceutical product and is, or may be, in direct contact with it. The closure is a part of the container.

Plastic containers and closures for pharmaceutical use are made of materials in which may be included certain additives; these materials do not include in their composition any substance that can be extracted by the contents in such quantities as to alter the efficacy or the stability of the product or to present a risk of toxicity.

The most commonly used polymers are polyethylene (with and without additives), polypropylene, poly(vinyl chloride), poly(ethylene terephthalate) and poly(ethylene-vinyl acetate).

The nature and amount of the additives are determined by the type of the polymer, the process used to convert the polymer into the container and the intended purpose of the container. Additives may consist of antioxidants, stabilisers, plasticisers, lubricants, colouring matter and impact modifiers. Antistatic agents and mould-release agents may be used only for containers for preparations for oral use or for external use for which they are authorised. Acceptable additives are indicated in the type specification for each material described in the Pharmacopoeia. Other additives may be used provided they are approved in each case by the competent authority responsible for the licensing for sale of the preparation.

For selection of a suitable plastic container, it is necessary to know the full manufacturing formula of the plastic, including all materials added during formation of the container so that the potential hazards can be assessed. In justified cases, further detailed information may be necessary to assess the impact on chronic use and for vulnerable patient groups. The plastic container chosen for any particular preparation should be such that:

— the ingredients of the preparation in contact with the plastic material are not significantly adsorbed on its surface and do not significantly migrate into or through the plastic,
— the plastic material does not release substances in quantities sufficient to affect the stability of the preparation or to present a risk of toxicity.

Using material or materials selected to satisfy these criteria, a number of identical type samples of the container are made by a well-defined procedure and submitted to practical testing in conditions that reproduce those of the intended use, including, where appropriate, sterilisation. In order to confirm the compatibility of the container and the contents and to ensure that there are no changes detrimental to the quality of the preparation, various tests are carried out such as verification of the absence of changes in physical characteristics, assessment of any loss or gain through permeation, detection of pH changes, assessment of changes caused by light, chemical tests and, where appropriate, biological tests.

The method of manufacture is such as to ensure reproducibility for subsequent bulk manufacture and the conditions of manufacture are chosen so as to preclude the possibility of contamination with other plastic materials or their ingredients. The manufacturer of the product must ensure that containers made in production are similar in every respect to the type samples.

For the results of the testing on type samples to remain valid, it is important that:

— there is no change in the composition of the material as defined for the type samples,
— there is no change in the manufacturing process as defined for the type samples, especially as regards the temperatures to which the plastic material is exposed during conversion or subsequent procedures such as sterilisation,
— scrap material is not used.

Recycling of excess material of well-defined nature and proportions may be permitted after appropriate validation.

Subject to satisfactory testing for compatibility of each different combination of container and contents, the materials described in the Pharmacopoeia are recognised as being suitable for the specific purposes indicated, as defined above.

Plastic Containers for Aqueous Solutions for Parenteral Infusions

(Plastic Containers for Aqueous Solutions for Infusion, Ph. Eur. method 3.2.2.1)

DEFINITION

Plastic containers for aqueous solutions for infusion are manufactured from one or more polymers, if necessary with additives. The containers described in this section are not necessarily suitable for emulsions. The polymers most commonly used are polyethylene, polypropylene and poly(vinyl chloride). The specifications of this text are to be read in conjunction with section *3.2.2. Plastic containers and closures for pharmaceutical use.*

The containers may be bags or bottles. They have a site suitable for the attachment of an infusion set designed to ensure a secure connection. They may have a site that allows an injection to be made at the time of use. They usually have a part that allows them to be suspended and which will withstand the tension occurring during use. The containers must withstand the sterilisation conditions to which they will be submitted. The design of the container and the method of sterilisation chosen are such that all parts of the containers that may be in contact with the infusion are sterilised. The containers are impermeable to micro-organisms after closure. The containers are such that after filling they are resistant to damage from accidental freezing which may occur during transport of the final preparation. The containers are and remain sufficiently transparent to allow the appearance of the contents to be examined at any time, unless otherwise justified and authorised.

The empty containers display no defects that may lead to leakage and the filled and closed containers show no leakage.

For satisfactory storage of some preparations, the container has to be enclosed in a protective envelope. The initial evaluation of storage has then to be carried out using the container enclosed in the envelope.

TESTS

Solution S

Use solution S within 4 h of preparation Fill a container to its nominal capacity with *water R* and close it, if possible using the usual means of closure; otherwise close using a sheet of pure aluminium. Heat in an autoclave so that a temperature of 121 ± 2 °C is reached within 20 min to 30 min and maintain at this temperature for 30 min. If heating at 121 °C leads to deterioration of the container, heat at 100 °C for 2 h.

Blank Prepare a blank by heating *water R* in a borosilicate-glass flask closed by a sheet of pure aluminium at the temperature and for the time used for the preparation of solution S.

Appearance of solution S
Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

Acidity or alkalinity
To a volume of solution S corresponding to 4 per cent of the nominal capacity of the container add 0.1 mL of *phenolphthalein solution R*. The solution is colourless. Add 0.4 mL of *0.01 M sodium hydroxide*. The solution is pink. Add 0.8 mL of *0.01 M hydrochloric acid* and 0.1 mL of *methyl red solution R*. The solution is orange-red or red.

Absorbance (*2.2.25*)
Measure the absorbance of solution S from 230 nm to 360 nm, using the blank (see solution S) as the compensation liquid. At these wavelengths, the absorbance is not greater than 0.20.

Reducing substances
To 20.0 mL of solution S add 1 mL of *dilute sulfuric acid R* and 20.0 mL of *0.002 M potassium permanganate*. Boil for 3 min. Cool immediately. Add 1 g of *potassium iodide R* and titrate immediately with *0.01 M sodium thiosulfate*, using 0.25 mL of *starch solution R* as indicator. Carry out a titration using 20.0 mL of the blank. The difference between the titration volumes is not greater than 1.5 mL.

Transparency
Fill a container previously used for the preparation of solution S with a volume equal to the nominal capacity of the primary opalescent suspension (*2.2.1*) diluted 1 in 200 for a container made from polyethylene or polypropylene and 1 in 400 for other containers. The cloudiness of the suspension is perceptible when viewed through the container and compared with a similar container filled with *water R*.

LABELLING
The label accompanying a batch of empty containers includes a statement of:
— the name and address of the manufacturer,
— a batch number which enables the history of the container and of the plastic material of which it is manufactured to be traced.

D. Containers for Blood and Blood Components

1. Sterile Plastic Containers for Human Blood and Blood Components

(Ph. Eur. method 3.3.4)

Plastic containers for the collection, storage, processing and administration of blood and its components are manufactured from one or more polymers, if necessary with additives. The composition and the conditions of manufacture of the containers are registered by the appropriate competent authorities in accordance with the relevant national legislation and international agreements.

When the composition of the materials of the different parts of the containers corresponds to the appropriate specifications, their quality is controlled by the methods indicated in those specifications (see *3.1. Materials used for the manufacture of containers* and subsections and *3.3. Containers for human blood and blood components, and materials used in their manufacture; transfusion sets and materials used in their manufacture; syringes* and subsections).

Materials other than those described in the Pharmacopoeia may be used provided that their composition is authorised by the competent authority and that the containers manufactured from them comply with the requirements prescribed in this general chapter.

In normal conditions of use the materials do not release monomers, or other substances, in amounts likely to be harmful nor do they lead to any abnormal modifications of the blood.

Components of inks, glues or adhesives used for marking or on the labels do not migrate into the contents of the container in quantities sufficient to affect the stability of the contents or to present a risk of toxicity.

The containers may contain anticoagulant solutions, depending on their intended use, and are supplied sterile.

Each container is fitted with attachments suitable for the intended use. The container may be in the form of a single unit or the collecting container may be connected by one or more tubes to one or more secondary containers to allow separation of the blood components to be effected within a closed system.

The outlets are of a shape and size allowing for adequate connection of the container with the blood-giving equipment. The protective coverings on the blood-taking needle and on the appendages must be such as to ensure the maintenance of sterility. They must be easily removable but must be tamper-evident.

The capacity of the containers is related to the nominal capacity prescribed by the national authorities and to the appropriate volume of anticoagulant solution. The nominal capacity is the volume of blood to be collected in the container. The containers are of a shape such that when filled they may be centrifuged.

The containers are fitted with a suitable device for suspending or fixing which does not hinder the collection, storage, processing or administration of the blood.

CHARACTERS
The container is sufficiently transparent to allow adequate visual examination of its contents before and after the taking of the blood and is sufficiently flexible to offer minimum resistance during filling and emptying under normal conditions of use. The container contains not more than 5 mL of air.

TESTS

Solution S_1
Fill the container with 100 mL of a sterile, pyrogen-free 9 g/L solution of *sodium chloride R*. Close the container and heat it in an autoclave so that the contents are maintained at 110 °C for 30 min.

If the container to be examined contains an anticoagulant solution, first empty it, rinse the container with 250 mL of *water R* at 20 ± 1 °C and discard the rinsings.

Solution S_2
Introduce into the container a volume of *water R* corresponding to the intended volume of anticoagulant solution. Close the container and heat it in an autoclave so that the temperature of the water is maintained at 110 °C for 30 min. After cooling, add sufficient *water R* to fill the container to its nominal capacity.

If the container to be examined contains an anticoagulant solution, first empty it and rinse it as indicated above.

Resistance to centrifugation
Introduce into the container a volume of *water R*, acidified by the addition of 1 mL of *dilute hydrochloric acid R*, sufficient to fill it to its nominal capacity. Envelop the container with absorbent paper impregnated with a 1 in 5 dilution of *bromophenol blue solution R1* or other suitable indicator and then dried. Centrifuge at 5000 *g* for 10 min. No leakage perceptible on the indicator paper and no permanent distortion occur.

Resistance to stretch
Introduce into the container a volume of *water R*, acidified by the addition of 1 mL of *dilute hydrochloric acid R*, sufficient to fill it to its nominal capacity. Suspend the container by the suspending device at the opposite end from the blood-taking tube and apply along the axis of this tube an immediate force of 20 N (2.05 kgf). Maintain the traction for 5 s. Repeat the test with the force applied to each of the parts for filling and emptying. No break and no deterioration occur.

Leakage
Place the container which has been submitted to the stretch test between two plates covered with absorbent paper impregnated with a 1 in 5 dilution of *bromophenol blue solution R1* or other suitable indicator and then dried. Progressively apply force to the plates to press the container so that its internal pressure (i.e. the difference between the applied pressure and atmospheric pressure) reaches 67 kPa within 1 min. Maintain the pressure for 10 min. No signs of leakage are detectable on the indicator paper or at any point of attachment (seals, joints, etc.).

Vapour permeability
For a container containing an anticoagulant solution, fill with a volume of a 9 g/L solution of *sodium chloride R* equal to the volume of blood for which the container is intended.

For an empty container, fill with the same mixture of anticoagulant solution and sodium chloride solution. Close the container, weigh it and store it at 5 ± 1 °C in an atmosphere with a relative humidity of (50 ± 5) per cent for 21 days. At the end of this period the loss in mass is not greater than 1 per cent.

Emptying under pressure
Fill the container with a volume of *water R* at 5 ± 1 °C equal to the nominal capacity. Attach a transfusion set without an intravenous cannula to one of the connectors. Compress the container so as to maintain throughout the emptying an internal pressure (i.e the difference between the applied pressure and atmospheric pressure) of 40 kPa. The container empties in less than 2 min.

Speed of filling
Attach the container by means of the blood-taking tube fitted with the needle to a reservoir containing a suitable solution having a viscosity equal to that of blood, such as a 335 g/L solution of *sucrose R* at 37 °C. Maintain the internal pressure of the reservoir (i.e. the difference between the applied pressure and atmospheric pressure) at 9.3 kPa with the base of the reservoir and the upper part of the container at the same level. The volume of liquid which flows into the container in 8 min is not less than the nominal capacity of the container.

Resistance to temperature variations
Place the container in a suitable chamber having an initial temperature of 20-23 °C. Cool it rapidly in a deep-freeze to −80 °C and maintain it at this temperature for 24 h. Raise the temperature to 50 °C and maintain for 12 h. Allow to cool to room temperature. The container complies with the tests for resistance to centrifugation, resistance to stretch, leakage, vapour permeability emptying under pressure and speed of filling prescribed above.

Transparency
Fill the empty container with a volume equal to its nominal capacity of the primary opalescent suspension (*2.2.1*) diluted so as to have an absorbance (*2.2.25*) at 640 nm of 0.37 to 0.43 (dilution factor about 1 in 16). The cloudiness of the suspension must be perceptible when viewed through the bag, as compared with a similar container filled with *water R*.

Extractable matter
Tests are carried out by methods designed to simulate as far as possible the conditions of contact between the container and its contents which occur in conditions of use.

The conditions of contact and the tests to be carried out on the eluates are prescribed, according to the nature of the constituent materials, in the particular requirements for each type of container.

Haemolytic effects in buffered systems
Stock buffer solution Dissolve 90.0 g of *sodium chloride R*, 34.6 g of *disodium hydrogen phosphate dodecahydrate R* and 2.43 g of *sodium dihydrogen phosphate R* in *water R* and dilute to 1000 mL with the same solvent.

Buffer solution A_0 To 30.0 mL of stock buffer solution add 10.0 mL of *water R*.

Buffer solution B_0 To 30.0 mL of stock buffer solution add 20.0 mL of *water R*.

Buffer solution C_0 To 15.0 mL of stock buffer solution add 85.0 mL of *water R*.

Introduce 1.4 mL of solution S_2 into each of three centrifuge tubes. To tube I add 0.1 mL of buffer solution A_0, to tube II add 0.1 mL of buffer solution B_0 and to tube III add 0.1 mL of buffer solution C_0. To each tube add 0.02 mL of fresh, heparinised human blood, mix well and warm on a water-bath at 30 ± 1 °C for 40 min. Use blood collected less than 3 h previously or blood collected into an anticoagulant citrate-phosphate-dextrose solution (CPD) less than 24 h previously.

Prepare three solutions containing, respectively:

3.0 mL of buffer solution A_0 and 12.0 mL of *water R* (solution A_1),

4.0 mL of buffer solution B_0 and 11.0 mL of *water R* (solution B_1),

4.75 mL of buffer solution B_0 and 10.25 mL of *water R* (solution C_1).

To tubes I, II and III add, respectively, 1.5 mL of solution A_1, 1.5 mL of solution B_1 and 1.5 mL of solution C_1. At the same time and in the same manner, prepare three other tubes, replacing solution S_2 by *water R*. Centrifuge simultaneously the tubes to be examined and the control tubes at exactly 2500 *g* in the same horizontal centrifuge for 5 min. After centrifuging, measure the absorbances (*2.2.25*) of the liquids at 540 nm using the stock buffer solution as compensation liquid. Calculate the haemolytic value as a percentage from the expression:

$$\frac{A_{exp}}{A_{100}} \times 100$$

A_{100} = absorbance of tube III;
A_{exp} = absorbance of tube I or II or of the corresponding control tubes.

The solution in tube I gives a haemolytic value not greater than 10 per cent and the haemolytic value of the solution in

tube II does not differ by more than 10 per cent from that of the corresponding control tube.

Sterility (*2.6.1*)
The containers comply with the test for sterility. Introduce aseptically into the container 100 mL of a sterile 9 g/L solution of sodium chloride and shake the container to ensure that the internal surfaces have been entirely wetted. Filter the contents of the container through a membrane filter and place the membrane in the appropriate culture medium, as prescribed in the test for sterility.

Pyrogens (*2.6.8*)
Solution S_1 complies with the test for pyrogens. Inject 10 mL of the solution per kilogram of the rabbit's mass.

STORAGE
Containers are enclosed in sealed, protective envelopes.

Protective envelopes are sealed in such a manner that they cannot be opened and re-closed without leaving visible traces that the seal has been broken.

LABELLING
The labelling complies with the relevant national legislation and international agreements.

2. Empty Sterile Containers of Plasticised Poly(vinyl chloride) for Human Blood and Blood Components

(Ph. Eur. method 3.3.5)

This general chapter is published for information.

DEFINITION
Unless otherwise authorised as described in general chapter *3.3.4. Sterile plastic containers for human blood and blood components*, the nature and composition of the material from which the containers are made comply with the requirements in general chapter *3.3.2. Materials based on plasticised poly(vinyl chloride) for containers for human blood and blood components.*

TESTS
They comply with the tests prescribed in general chapter *3.3.4. Sterile plastic containers for human blood and blood components* and with the following tests.

Reference solution
Heat *water R* in a borosilicate-glass flask in an autoclave at 110 °C for 30 min.

Acidity or alkalinity
To a volume of solution S_2 (see *3.3.4*) corresponding to 4 per cent of the nominal value of the container add 0.1 mL of *phenolphthalein solution R*. The solution remains colourless. Add 0.4 mL of *0.01 M sodium hydroxide*. The solution is pink. Add 0.8 mL of *0.01 M hydrochloric acid* and 0.1 mL of *methyl red solution R*. The solution is orange-red or red.

Absorbance (*2.2.25*)
Maximum 0.30, determined between wavelengths of 230 nm and 250 nm on solution S_2 (see *3.3.4*); maximum 0.10, determined between wavelengths of 251 nm and 360 nm on solution S_2. Use the reference solution as the compensation liquid.

Reducing substances
Immediately after preparation of solution S_2 (see *3.3.4*), transfer to a borosilicate-glass flask a volume corresponding to 8 per cent of the nominal value of the container. At the same time, prepare a blank using an equal volume of the freshly prepared reference solution in another borosilicate-glass flask. To each solution add 20.0 mL of *0.002 M potassium permanganate* and 1 mL of *dilute sulfuric acid R*. Allow to stand protected from light for 15 min. To each solution add 0.1 g of *potassium iodide R*. Allow to stand protected from light for 5 min and titrate immediately with *0.01 M sodium thiosulfate*, using 0.25 mL of *starch solution R* as indicator. The difference between the 2 titrations is not greater than 2.0 mL.

Plastic additives 01, 24, 25, 26 and 27
Gas chromatography (*2.2.28*) coupled with mass spectrometry (*2.2.43*).

Internal standard solution S3 1 mg/mL solution of *di-n-octyl phthalate R* in *tetrahydrofuran for chromatography R.*

Internal standard solution S4 5 µg/mL solution of *di-n-octyl phthalate R* in *anhydrous ethanol R.*

Test solution Cut 0.2 g of the material to be examined into pieces about 0.5 cm in length. Dissolve the pieces in 12.5 mL of internal standard solution S3 using a polytetrafluoroethylene magnetic stirring bar. Complete dissolution of the material to be examined is obtained after stirring for about 20-30 min. The poly(vinyl chloride) is precipitated as a white powder by adding dropwise 37.5 mL of *anhydrous ethanol R*. Centrifuge, then dilute 1.0 mL of the supernatant to 50.0 mL with *anhydrous ethanol R*. The final concentration of the internal standard in the test solution is 5 µg/mL.

The stock solutions may be stored at 4 °C for up to 2 weeks.

Stock solution (a) Dissolve 20.0 mg of *plastic additive 01 CRS* in internal standard solution S4 and dilute to 20.0 mL with internal standard solution S4.

Stock solution (b) Dissolve 20.0 mg of *plastic additive 24 CRS* in internal standard solution S4 and dilute to 20.0 mL with internal standard solution S4.

Stock solution (c) Dissolve 20.0 mg of *plastic additive 25 CRS* in internal standard solution S4 and dilute to 20.0 mL with internal standard solution S4.

Stock solution (d) Dissolve 20.0 mg of *plastic additive 26 CRS* in internal standard solution S4 and dilute to 20.0 mL with internal standard solution S4.

Stock solution (e) Dissolve 20.0 mg of *plastic additive 27 CRS* in internal standard solution S4 and dilute to 20.0 mL with internal standard solution S4.

Reference solutions (a1)-(a5) Dilute stock solution (a) with internal standard solution S4 to obtain 5 reference solutions containing 10-40 µg/mL of *plastic additive 01 CRS.*

Reference solutions (b1)-(b5) Dilute stock solution (b) with internal standard solution S4 to obtain 5 reference solutions containing 10-40 µg/mL of *plastic additive 24 CRS.*

Reference solutions (c1)-(c5) Dilute stock solution (c) with internal standard solution S4 to obtain 5 reference solutions containing 10-40 µg/mL of *plastic additive 25 CRS.*

Reference solutions (d1)-(d5) Dilute stock solution (d) with internal standard solution S4 to obtain 5 reference solutions containing 10-40 µg/mL of *plastic additive 26 CRS.*

Reference solutions (e1)-(e5) Dilute stock solution (e) with internal standard solution S4 to obtain 5 reference solutions containing 10-40 µg/mL of *plastic additive 27 CRS.*

Column:
- *material*: fused silica;
- *size*: *l* = 30 m, Ø = 0.25 mm;
- *stationary phase*: *phenyl(5)methyl(95)polysiloxane R* (film thickness 0.25 µm).

Carrier gas *helium for chromatography R.*

Flow rate 1 mL/min.

Split ratio 1:20.

Temperature:

	Time (min)	Temperature (°C)
Column	0 - 3.3	100 → 200
	3.3 - 20	200 → 250
	20 - 22.5	250
	22.5 - 23	250 → 270
	23 - 25	270
	25 - 25.6	270 → 320
	25.6 - 30.6	320
Injection port		300

Detection Mass spectrometer as described below; adjust the detector settings so as to comply with the system suitability criteria:
- quadrupole mass spectrometer equipped with an electron impact ionisation mode (70 eV);
- *ion source temperature*: 230 °C;
- *acquisition system*: performed on full-scan (*m/z* = 40-350) and on single-ion monitoring (SIM) modes;
- *solvent delay*: 2.5 min;
- mass spectrometer parameters for the fragmentometric mode (SIM) set as follows:

Substance	Ion 1 [*m/z*]	Ion 2 [*m/z*]	Ion 3 [*m/z*]
Plastic additive 01	149	167	279
Plastic additive 24	155	127	299
Plastic additive 25	71	213	315
Plastic additive 26	305	193	323
Plastic additive 27	261	149	167
DnOP (internal standard)	149	279	167

Injection 1 µL.

Relative retention With reference to di-*n*-octyl phthalate (retention time = about 22 min): plastic additive 01 = about 0.80; plastic additive 24 = about 0.95-1.09; plastic additive 27 = about 1.02; plastic additive 25 = about 1.14; plastic additive 26 = about 1.34.

The specificity of the detection is checked by monitoring 3 different ions for each substance using a mass spectrometer in SIM mode. Ion ratios are determined from the peak areas after the injection of a standard solution. The ratios in the table below are given for information.

Substance	Ion 1 [*m/z*]	Ion 2 [*m/z*]	Ion 3 [*m/z*]	Ion ratio 2/1 (%)	Ion ratio 3/1 (%)
Plastic additive 01	149	167	279	50	30
Plastic additive 24	155	127	299	30	13
Plastic additive 25	71	213	315	45	20
Plastic additive 26	305	193	323	55	20
Plastic additive 27	261	149	167	130	85
DnOP (internal standard)	149	279	167	/	/

System suitability:
- *resolution*: if plastic additive 27 is tested, minimum 1.5 between the peaks due to the internal standard and plastic additive 27;
- *repeatability*: maximum relative standard deviation of 1.0 per cent for the retention time of the peak due to the plastic additive, determined on 6 injections of a reference solution of each plastic additive tested situated in the middle of the calibration range (e.g. 20 µg/mL); maximum relative standard deviation of 3.0 per cent for the ratio of the area of the peak due to the plastic additive to that due to the internal standard, determined on 6 injections of a reference solution of each plastic additive tested situated in the middle of the calibration range (e.g. 20 µg/mL).

From the calibration curve obtained with the reference solutions, calculate the percentage content of plastic additives in the material to be examined.

Limits:
- *plastic additive 01*: maximum 40 per cent;
- *plastic additive 24*: maximum 45 per cent;
- *plastic additive 25*: maximum 45 per cent;
- *plastic additive 26*: maximum 45 per cent;
- *plastic additive 27*: maximum 45 per cent.

Chlorides (*2.4.4*)

Maximum 0.4 ppm, determined with solution S_2 (see *3.3.4*).

Prepare the standard using a mixture of 1.2 mL of *chloride standard solution (5 ppm Cl) R* and 13.8 mL of *water R*.

Residue on evaporation

Evaporate to dryness 100 mL of solution S_2 (see *3.3.4*) in a borosilicate-glass beaker of appropriate capacity, previously heated to 105 °C. Evaporate to dryness in the same conditions 100 mL of the reference solution (blank test). Dry to constant mass at 100-105 °C. The residue from solution S_2 weighs a maximum of 3 mg, taking into account the blank test.

STORAGE

See general chapter *3.3.4. Sterile plastic containers for human blood and blood components.*

LABELLING

See general chapter *3.3.4. Sterile plastic containers for human blood and blood components.*

3. Sterile Containers of Plasticised Poly(vinyl chloride) for Human Blood Containing Anticoagulant Solution

(Ph. Eur. method 3.3.6)

This general chapter is published for information.

DEFINITION

Sterile plastic containers containing an anticoagulant solution complying with the monograph *Anticoagulant and preservative solutions for human blood (0209)* are used for the collection, storage and administration of blood. Before filling they comply with the description and characters given in general chapter *3.3.5. Empty sterile containers of plasticised poly(vinyl chloride) for human blood and blood components.*

Unless otherwise authorised as described in general chapter *3.3.4. Sterile plastic containers for human blood and blood components*, the nature and composition of the material from which the containers are made comply with the requirements prescribed in general chapter *3.3.2. Materials based on plasticised poly(vinyl chloride) for containers for human blood and blood components.*

TESTS

They comply with the tests prescribed in general chapter *3.3.4. Sterile plastic containers for human blood and blood components* and with the following tests.

Volume of anticoagulant solution
Empty the container, collecting the anticoagulant solution in a graduated cylinder. The volume does not differ by more than ± 10 per cent from the stated volume.

Absorbance (*2.2.25*)
Measure the absorbance of the anticoagulant solution removed from the container between 250 nm and 350 nm, using as the compensation liquid an anticoagulant solution of the same composition that has not been in contact with a plastic material. The absorbance at the maximum at 280 nm is not greater than 0.5.

Plastic additives 01, 24, 25, 26 and 27
Gas chromatography (*2.2.28*) coupled with mass spectrometry (*2.2.43*).

Carefully remove the anticoagulant solution by means of the flexible transfer tube. Using a funnel fitted to the tube, completely fill the container with *water R*, leave in contact for 1 min while squeezing the container gently, then empty completely. Repeat the rinsing. The container, emptied and rinsed in this manner, complies with the test for plastic additives 01, 24, 25, 26 and 27 prescribed in general chapter *3.3.5. Empty sterile containers of plasticised poly(vinyl chloride) for human blood and blood components.*

STORAGE
See general chapter *3.3.4. Sterile plastic containers for human blood and blood components.*

LABELLING
See general chapter *3.3.4. Sterile plastic containers for human blood and blood components.*

E. Rubber Closures for Containers for Aqueous Parenteral Preparations

(Rubber Closures for Containers for Aqueous Parenteral Preparations for Powders and for Freeze-dried Powders, Ph. Eur. method 3.2.9)

Rubber closures for containers for aqueous parenteral preparations, for powders and for freeze-dried powders are elastomers made of materials obtained by vulcanisation (cross-linking) of macromolecular organic substances, using appropriate additives. They cover all types of rubber closures including stoppers for vials, sealing disks and plunger stoppers for cartridges, as well as rubber tip caps, needle shields and plunger stoppers for syringes. The elastomers are polymers, obtained by chemical synthesis, or are polymers of natural origin. The choice of the principal components and of the various additives (for example, vulcanisers, accelerators, stabilisers, pigments) depends on the properties required for the finished article. The specifications apply to rubber closures made from rubber of 1 kind only, to coated closures, to bi-layer seals and to lubricated closures. Coated closures consist of a bulk of rubber, bearing on its surface or part of its surface a layer of a different polymer. Bi-layer seals are composed of 2 different layers of rubber, 1 of which exhibits a higher level of chemical purity and is intended for contact with a pharmaceutical preparation; the other layer exhibits a higher level of elasticity and is intended to improve self-sealing and fragmentation resistance of the seal. Lubricated rubber closures are closures treated with silicone oil (*3.1.8*) or other lubricants, for example materials chemically or mechanically bonded to the closures.

If closures are lubricated they comply in lubricated state with the requirements as defined in this general chapter.
The specifications do not apply to closures made from silicone elastomer (which are dealt with in general chapter *3.1.9. Silicone elastomer for closures and tubing*).

Rubber closures may be classified in 2 types: type I closures meet the strictest requirements and are preferred; type II closures have mechanical properties suitable for special uses (for example, multiple piercing) and cannot meet requirements as severe as for type I closures because of their chemical composition.

The closures chosen for use with a particular preparation are such that:
- the components of the preparation in contact with the closures are not adsorbed onto the surface of the closures and do not migrate into or through the closures to an extent sufficient to affect the preparation adversely;
- the closures do not release substances in quantities sufficient to affect the stability of the preparation or to present a risk of toxicity;
- the closures are compatible with the preparation for which they are used throughout its period of validity.

The manufacturer of the preparation must obtain from the supplier an assurance that the composition of the closure does not vary and that it is identical to that of the closure used during compatibility testing. If the supplier informs the manufacturer of the preparation that changes have been made to the composition, a risk assessment should be applied to determine the need to repeat the compatibility testing, totally or partly, depending on the nature of the changes.

The closures are washed and may be sterilised before use.

CHARACTERS
Rubber closures are elastic. They are translucent or opaque and have no characteristic colour, the latter depending on the additives used. They are practically insoluble in tetrahydrofuran, in which, however, a considerable reversible swelling may occur. They are homogeneous and practically free from flash and adventitious materials (for example, fibres, foreign particles, waste rubber).

IDENTIFICATION
Identification of the type of rubber used for the closures is not within the scope of this specification. The identification tests given below distinguish between closures made from rubber and those made from silicone elastomer and plastic materials but do not differentiate all types of rubber. Other identity tests may be carried out with the aim of detecting differences in a batch compared with the closures used for compatibility testing. One or more of the following analytical methods may be applied for this purpose: determination of relative density, determination of sulfated ash, determination of sulfur content, thin-layer chromatography carried out on an extract, ultraviolet absorption spectrophotometry of an extract, infrared absorption spectrophotometry of a pyrolysate or attenuated total reflectance (ATR).

A. Infrared absorption spectrophotometry (*2.2.24*).
Examine by attenuated total reflectance (ATR).

If necessary, cut the sample along an appropriate axis and examine the cut surface. For coated, bi-layer and lubricated closures, perform the test for each different part of the closure. This is not required for silicone oil used as lubricant. Identification of silicone oil can be performed prior to it being used.

Comparison Type sample.

If direct ATR measurement on the surface is not feasible (mainly rubber closures filled with carbon black), heat an

appropriate amount of rubber in a heat-resistant test-tube over an open flame to dry the sample and continue heating until pyrolysate vapours are condensed near the top edge of the test-tube. Examine the pyrolysate of the sample by ATR and compare the spectrum with that obtained with the pyrolysate of the type sample.

B. Total ash (*2.4.16*).

If the sample has not been subjected to steam sterilisation, drying at 100-105 °C can be omitted. Determine the percentage content of total ash in the sample to be examined and compare with the percentage content of total ash in the type sample (A_0). The total ash content falls within the following ranges depending on the total ash content of the type sample, or, if not available, in the range defined as the target for the specific rubber type.

Total ash in the type sample, A_0 (per cent)	Limit for total ash in the sample (per cent)
$A_0 \leq 5.0$	$(A_0 - 0.75)$ to $(A_0 + 0.75)$
$5.0 < A_0 \leq 10$	$(A_0 - 1.0)$ to $(A_0 + 1.0)$
$A_0 > 10$	$(A_0 - 2.0)$ to $(A_0 + 2.0)$

In addition to the use of platinum and silica crucibles described in general chapter *2.4.16*, porcelain crucibles may be used. The sample may be ignited using a microwave oven instead of a muffle furnace.

TESTS

Solution S

Place a number of uncut closures with a total surface area of about 100 cm^2 in a wide-necked flask (type I glass, *3.2.1*), add 200 mL of *water R* and weigh. Cover the mouth of the flask with a borosilicate-glass beaker. Heat in an autoclave so that a temperature of 121 ± 2 °C is reached within 20-30 min and maintain at this temperature for 30 min. Immerse the temperature probe for the autoclave programme-control in water in a container comparable to that used for the sample. Cool to room temperature over about 30 min. Make up to the original mass with *water R*. Shake and decant the solution immediately. Shake solution S before each test. If using a tightly closed flask (type I glass, *3.2.1*) with an inert closure instead of a wide-necked flask covered with a borosilicate-glass beaker, it is not necessary to make up to the original mass.

Blank solution Prepare a blank solution in the same manner using 200 mL of *water R*.

Appearance of solution S

Solution S is not more intensely coloured than reference solution GY_5 (*2.2.2, Method II*). For type I closures, solution S is not more opalescent than reference suspension II (*2.2.1*) and for type II closures, solution S is not more opalescent than reference suspension III. In case of nephelometric determination, the limit for type I closures is 6 NTU and the limit for type II closures is 18 NTU.

Acidity or alkalinity

To 20 mL of solution S add 0.1 mL of *bromothymol blue solution R1*. Carry out a titration using 20.0 mL of the blank (see solution S). Not more than 0.3 mL of *0.01 M sodium hydroxide* or 0.8 mL of *0.01 M hydrochloric acid* is required to change the colour of the indicator to blue or yellow, respectively. If after adding the indicator the solution is green, it is neutral and no titration is needed.

Absorbance

Carry out the test within 5 h of preparation of solution S. Filter solution S through a membrane filter (nominal pore size 0.45 µm), rejecting the first few millilitres of filtrate. Measure the absorbance (*2.2.25*) of the filtrate at wavelengths from 220-360 nm using the blank (see solution S) as compensation liquid: absorbance within the 220-360 nm range does not exceed 0.2 for type I closures or 4.0 for type II closures. If necessary, dilute the filtrate before measurement of the absorbance and correct the result for the dilution.

Reducing substances

Carry out the test within 4 h of preparation of solution S. To 20.0 mL of solution S add 1 mL of *dilute sulfuric acid R* and 20.0 mL of *0.002 M potassium permanganate*. Boil for 3 min. Cool. Add 1 g of *potassium iodide R* and titrate immediately with *0.01 M sodium thiosulfate*, using 0.25 mL of *starch solution R* as indicator. Carry out a titration using 20.0 mL of the blank (see solution S). The difference between the titration volumes is not greater than 3.0 mL for type I closures and 7.0 mL for type II closures.

Ammonium (*2.4.1, Method A*)

Maximum 2 ppm.

Dilute 5 mL of solution S to 14 mL with *water R*.

Extractable zinc

Maximum 5 µg of extractable Zn per millilitre of solution S.

Atomic absorption spectrometry (*2.2.23, Method I*).

Test solution Use solution S. If results are outside the calibration range, dilute 10.0 mL of solution S to an appropriate volume with *0.1 M hydrochloric acid*.

Reference solutions Prepare the reference solutions using *zinc standard solution (10 ppm Zn) R* diluted with *0.1 M hydrochloric acid*.

Source Zinc hollow-cathode lamp.

Wavelength 213.9 nm.

Atomisation device Air-acetylene flame.

Extractable heavy metals (*2.4.8*)

Maximum 2 ppm.

Solution S complies with test A. Prepare the reference solution using *lead standard solution (2 ppm Pb) R*.

Residue on evaporation

Evaporate 50.0 mL of solution S to dryness on a water-bath and dry at 100-105 °C. The residue weighs not more than 2.0 mg for type I closures and not more than 4.0 mg for type II closures.

Volatile sulfides

Place closures, cut if necessary, with a total surface area of 20 ± 2 cm^2 in a 100 mL conical flask and add 50 mL of a 20 g/L solution of *citric acid monohydrate R*. Place a piece of *lead acetate paper R* over the mouth of the flask and maintain the paper in position by placing over it an inverted weighing bottle. Heat in an autoclave at 121 ± 2 °C for 30 min. Any black stain on the paper is not more intense than that of a standard, treated in the same manner, prepared by mixing 50 mL of a 20 g/L solution of *citric acid monohydrate R* and 5.0 mL of a freshly prepared 0.0308 g/L solution of *sodium sulfide R*.

The tests for penetrability, fragmentation and self-sealing are performed on whole closures.

For the tests for penetrability, fragmentation and self-sealing, treat non-sterilised closures as described for the preparation of solution S and allow to dry. To perform these 3 tests, use for each closure a new, lubricated, long-bevel[1] (bevel angle 12 ± 2°) hypodermic needle with an external diameter of 0.8 mm and

[1] *See ISO 7864, Sterile hypodermic needles for single use.*

pierce the closures with the needle perpendicular to the surface without rotating the needle.

Penetrability
For closures intended to be pierced by a hypodermic needle, carry out the following test. Fill 10 suitable vials to the nominal volume with *water R*, fit the closures to be examined and secure with a cap. The force required for piercing, determined with an accuracy of ± 0.25 N, is not greater than 10 N for each closure.

Fragmentation
For closures intended to be pierced by a hypodermic needle, carry out the following test. If the closures are to be used for aqueous preparations, introduce in 12 clean vials a volume of *water R* corresponding to the nominal volume minus 4 mL, close the vials with the closures to be examined, secure with a cap and allow to stand for 16 h. If the closures are to be used with dry preparations, close 12 clean vials with the closures to be examined. Using a needle fitted to a clean syringe, inject into the vial 1 mL of *water R* and remove 1 mL of air; carry out this operation 4 times for each closure, piercing the closure each time at a different site. Use a new needle for each closure and check that the needle is not blunted during the test. Pass the liquid in the vials through a filter with a pore size of 0.5 µm. Count the fragments of rubber visible to the naked eye. The total number of fragments does not exceed 5. This limit is based on the assumption that fragments with a diameter equal to or greater than 50 µm are visible to the naked eye; in cases of doubt or dispute, the fragments are examined with a microscope to verify their nature and size.

Self-sealing test
For closures intended to be used with multidose containers, carry out the following test. Fill 10 vials matching the design of the stopper to the nominal volume with *water R*, fit the closures to be examined, secure with a cap and crimp tightly. Pierce each closure 10 times, piercing the closure each time at a different site. Immerse the vials upright in a 1 g/L solution of *methylene blue R* and reduce the external pressure by 27 kPa for 10 min. Restore atmospheric pressure and leave the vials immersed for 30 min. Rinse the outside of the vials. None of the vials contains any trace of coloured solution.

F. Sets for the Transfusion of Blood and Blood Components

(Ph. Eur. method 3.3.7)

This general chapter is published for information.

DEFINITION
Sets for the transfusion of blood and blood components consist principally of plastic tubing to which are fitted the parts necessary to enable the set to be used for transfusion in the appropriate manner. Sets include a closure-piercing device, a blood filter, a drip chamber, a flow regulator, a Luer connector and, usually, a site that allows an injection to be made at the time of use. When the sets are to be used with containers requiring an air filter, this may be incorporated in the closure-piercing device or a separate air-inlet device may be used. The chamber enclosing the blood filter, the drip chamber and the main tubing are transparent. The materials chosen and the design of the set are such as to ensure absence of haemolytic effects. The sets comply with current standards regarding dimensions and performance.

All parts of the set that may be in contact with blood and blood components are sterile and pyrogen-free. Each set is presented in an individual package that maintains the sterility of the contents. The sets are not to be re-sterilised or re-used.

Sets for the transfusion of blood and blood components are manufactured within the framework of a suitable quality system and in accordance with any relevant national regulations.

TESTS
Carry out the tests on sterilised sets.

Solution S
Make a closed circulation system from 3 sets and a 300 mL borosilicate-glass vessel. Fit to the vessel a suitable thermostat device that maintains the temperature of the liquid in the vessel at 37 ± 1 °C. Circulate 250 mL of *water R* through the system in the direction used for transfusion for 2 h at a rate of 1 L/h (for example using a peristaltic pump applied to as short a piece of suitable silicone elastomer tubing as possible). Collect the whole of the solution and allow to cool.

Appearance of solution
Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

Acidity or alkalinity
To 25 mL of solution S add 0.15 mL of *BRP indicator solution R*. Not more than 0.5 mL of *0.01 M sodium hydroxide* is required to change the colour of the indicator to blue.
To 25 mL of solution S add 0.2 mL of *methyl orange solution R*. Not more than 0.5 mL of *0.01 M hydrochloric acid* is required to reach the beginning of the colour change of the indicator.

Absorbance (*2.2.25*)
Maximum 0.30, determined between wavelengths of 230 nm and 250 nm on solution S; maximum 0.15, determined between wavelengths of 251 nm and 360 nm on solution S.

Reducing substances
Carry out the test within 4 h of preparation of solution S To 20.0 mL of solution S add 1 mL of *dilute sulfuric acid R* and 20.0 mL of *0.002 M potassium permanganate*. Boil under a reflux condenser for 3 min and cool immediately. Add 1 g of *potassium iodide R* and titrate with *0.01 M sodium thiosulfate* using 0.25 mL of *starch solution R* as indicator. Carry out a blank titration using 20.0 mL of *water R*. The difference between the 2 titration volumes is not greater than 2.0 mL.

Ethylene oxide
Head-space gas chromatography (*2.2.28*) coupled with mass spectrometry (*2.2.43*). *Carry out the test on each plastic part of the set for transfusion and prepare the reference solutions immediately before use.*

Test sample For hard plastic materials (e.g. cyclo-olefin polymers and copolymers), crush the sample into fine particles. For soft plastic materials (e.g. silicone or plasticised poly(vinyl chloride)), cut the sample into pieces not greater than 0.5 cm^2 in size. In both cases, weigh 0.10-3.00 g of the sample, depending on the amount of ethylene oxide residue expected in the plastic part tested, into a 20 mL injection vial and close the vial. For low ethylene-oxide-releasing plastic materials (e.g. cyclo-olefin polymers and copolymers), heat the vial in an oven at 120 °C for at least 15 h (pre-thermal extraction step) before the 1st injection.

Reference solution (a) Dilute 1.0 mL of *ethylene oxide stock solution R1* to 50.0 mL with *anhydrous ethanol R* (1000 µg of ethylene oxide per millilitre). *If the solution is prepared using commercial ethylene oxide standard from a previously opened container, it should be noted that some ethylene oxide may have been lost owing to its high volatility.*

Reference solution (b) Dilute 10 mL of reference solution (a) to 20 mL with *anhydrous ethanol R* (500 µg of ethylene oxide per millilitre).

Reference solution (c) Dilute 8.0 mL of reference solution (a) to 20.0 mL with *anhydrous ethanol R* (400 µg of ethylene oxide per millilitre). Transfer 20 µL of reference solution (c) into an injection vial and close immediately.

Reference solution (d) Dilute 6.0 mL of reference solution (a) to 20.0 mL with *anhydrous ethanol R* (300 µg of ethylene oxide per millilitre). Transfer 20 µL of reference solution (d) into an injection vial and close immediately.

Reference solution (e) Dilute 4.0 mL of reference solution (a) to 20.0 mL with *anhydrous ethanol R* (200 µg of ethylene oxide per millilitre). Transfer 20 µL of reference solution (e) into an injection vial and close immediately.

Reference solution (f) Dilute 2.0 mL of reference solution (a) to 20.0 mL with *anhydrous ethanol R* (100 µg of ethylene oxide per millilitre). Transfer 20 µL of reference solution (f) into an injection vial and close immediately.

Reference solution (g) Dilute 1.0 mL of reference solution (a) to 20.0 mL with *anhydrous ethanol R* (50 µg of ethylene oxide per millilitre). Transfer 20 µL of reference solution (g) into an injection vial and close immediately.

Reference solution (h) Dilute 0.5 mL of reference solution (a) to 20.0 mL with *anhydrous ethanol R* (25 µg of ethylene oxide per millilitre). Transfer 20 µL of reference solution (h) into an injection vial and close immediately.

Reference solution (i) Dilute 100 mg of *acetaldehyde R* to 100 mL with *anhydrous ethanol R* (1000 µg of *acetaldehyde R* per millilitre).

Reference solution (j) Transfer 10 µL of reference solution (i) and 20 µL of reference solution (b) into an injection vial, mix and close immediately.

Column:
- *material*: fused silica;
- *size*: *l* = 30 m, Ø = 0.32 mm;
- *stationary phase*: *porous silica for chromatography R* (film thickness 4 µm).

A particle trap may be used to prevent particles that are dislodged from the column from damaging the detector.

Carrier gas *helium for chromatography R.*

Flow rate 1.0 mL/min.

Split ratio 1:50.

Static head-space conditions that may be used:
- *equilibration temperature*: 80 °C for plasticised poly(vinyl chloride); 120 °C for cyclo-olefin polymers and copolymers, and polyurethane; 160 °C for silicone;
- *equilibration time*: 60 min;
- *transfer-line temperature*: 130 °C;
- *pressurisation time*: 0.5 min;
- *injection time*: 3 min;
- *shaking mode*: high agitation.

Temperature:

		Time (min)	Temperature (°C)
Column		0 - 2	100
		2 - 8.25	100 → 225
		8.25 - 13.25	225
Injection port			160
Detector:	transfer line		260
	source		230
	analyser		150

Detection Mass spectrometer; the following settings have been found to be suitable:
- quadrupole mass spectrometer equipped with an electron impact ionisation mode (70 eV);
- *acquisition system*: single-ion monitoring (SIM) for ethylene oxide quantification and complete spectrum mode (*m/z* = 10-350) for ethylene oxide identification;
- mass spectrometer parameters for the fragmentometric mode (SIM) set as follows: *m/z* = 44 as ethylene oxide quantitation ion; *m/z* = 29 and *m/z* =15 as ethylene oxide qualification ions.

Injection 1 mL of the test sample and reference solutions (c), (d), (e), (f), (g), (h) and (j).

After injection of the test sample, remove the cap from the vial in a fume cupboard and purge the vial with dry nitrogen for 30 s. Close the vial with the cap and a new septum and repeat the heating and injection to exhaustion. Exhaustion is achieved when the amount of ethylene oxide extracted is less than 10 per cent of the 1st extraction or when no analytically significant increase in the cumulative residue levels is detected.

System suitability:
- *resolution*: minimum 1.5 between the peaks due to ethylene oxide and acetaldehyde in the chromatogram obtained with reference solution (j);
- *signal-to-noise ratio*: minimum 10 for the peak due to ethylene oxide in the chromatogram obtained with reference solution (h).

Verify the absence of peaks interfering with the peak due to ethylene oxide by carrying out the test on an unsterilised sample.

Calculation of content Establish a calibration curve with the mass of ethylene oxide in each reference solution as the abscissa and the corresponding peak areas as the ordinate.

For sets for transfusion, the total amount of ethylene oxide is calculated using the following formula:

$$EO = \sum_{i=1}^{n} (mi \times Ci)/M$$

EO	=	total amount of ethylene oxide, in ppm;
mi	=	mass of each plastic part of the set for transfusion, in grams;
Ci	=	cumulative amount of ethylene oxide determined in the corresponding plastic part, in micrograms per gram;
M	=	mass of the set for transfusion, in grams.

Limit If the label states that ethylene oxide has been used for sterilisation:
- *total (sum of amounts of ethylene oxide quantified in each plastic part of the set for transfusion)*: maximum 10 ppm.

Extraneous particles

Fill the set via the normal inlet with a 0.1 g/L solution of *sodium laurilsulfate R*, previously filtered through a sintered-glass filter (16) (*2.1.2*) and heated to 37 °C. Collect the

liquid via the normal outlet. When examined under suitable conditions of visibility, the liquid is clear and practically free from visible particles and filaments (it is assumed that particles and filaments with a diameter equal to or greater than 50 µm are visible to the naked eye).

Flow rate
Pass through a complete set with the flow regulator fully open 50 mL of a solution having a viscosity of 3 mPa·s (3 cP) (for example a 33 g/L solution of *macrogol 4000 R* at 20 °C) under a static head of 1 m. The time required for passage of 50 mL of the solution is not greater than 90 s.

Resistance to pressure
Make tight the extremities of the set and any air-inlet device. Connect the set to a compressed air outlet fitted with a pressure regulator. Immerse the set in a tank of water at 20-23 °C. Apply progressively an excess pressure of 100 kPa and maintain for 1 min. No air bubble escapes from the set.

Transparency
Use as reference suspension the primary opalescent suspension (*2.2.1*) diluted 8-fold for sets having tubing with an external diameter less than 5 mm and diluted 16-fold for sets having tubing with an external diameter of 5 mm or greater. Circulate the reference suspension through the set and compare with a set from the same batch filled with *water R*. The opalescence and presence of bubbles are discernible.

Residue on evaporation
Evaporate 50.0 mL of solution S to dryness on a water-bath and dry to constant mass in an oven at 100-105 °C. Carry out a blank test using 50.0 mL of *water R*. The residue obtained with solution S weighs not more than 1.5 mg, taking into account the blank test.

Sterility (*2.6.1*)
The sets comply with the test for sterility. If the sets are stated to be sterile only internally, pass 50 mL of buffered sodium chloride-peptone solution pH 7.0 (*2.6.12*) through the set and use the solution to carry out the test by the membrane filtration method.

If the sets are stated to be sterile both internally and externally, open the package with the necessary aseptic precautions and:
- for the direct inoculation method, place the set or its components in a suitable container containing a sufficient quantity of the culture medium to ensure complete immersion;
- for the membrane filtration method, place the set or its components in a suitable container containing a sufficient quantity of buffered sodium chloride-peptone solution pH 7.0 (*2.6.12*) to allow total rinsing for 10 min.

Pyrogens (*2.6.8*)
Connect together 5 sets and pass through the assembly, at a flow rate not exceeding 10 mL/min, 250 mL of a sterile, pyrogen-free, 9 g/L solution of *sodium chloride R*. Collect the solution aseptically in a pyrogen-free container. The solution complies with the test for pyrogens. Inject per kilogram of the rabbit's mass 10 mL of the solution.

LABELLING
The label states, where applicable, that the set has been sterilised using ethylene oxide.

G. Sterile Single-use Plastic Syringes

(Ph. Eur. method 3.3.8)

This general chapter is published for information.

DEFINITION
Sterile single-use plastic syringes are medical devices intended for immediate use for the administration of injectable preparations. They are supplied sterile and bacterial endotoxin-free and are not to be re-sterilised or re-used. They consist of a syringe barrel and a piston that may have an elastomer sealing ring; they may be fitted with a needle that may be non-detachable. Each syringe is presented with individual protection for maintaining sterility.

The barrel of the syringe is sufficiently transparent to permit dosages to be read without difficulty and allow air bubbles and foreign particles to be discerned.

The plastics and elastomer materials of which the barrel and piston are made comply with appropriate specifications or with requirements of the competent authority. The most commonly used materials are polypropylene and polyethylene. The syringes comply with current standards regarding dimensions and performance.

Silicone oil (*3.1.8*) may be applied to the internal wall of the barrel, in which case there remains no excess capable of contaminating the contents at the time of use. The inks, glues and adhesives for the marking on the syringe or on the package and, where necessary, the assembly of the syringe and its package, do not migrate across the walls.

TESTS

Solution S
Prepare the solution in a manner that avoids contamination by foreign particles. Using a sufficient number of syringes to produce 50 mL of solution, fill the syringes to their nominal volume with *water R* and maintain at 37 °C for 24 h. Combine the contents of the syringes in a suitable borosilicate-glass container.

Appearance of solution
Solution S is clear (*2.2.1*) and colourless (*2.2.2, Method II*) and is practically free from foreign solid particles.

Acidity or alkalinity
To 20 mL of solution S add 0.1 mL of *bromothymol blue solution R1*. Not more than 0.3 mL of *0.01 M sodium hydroxide* or *0.01 M hydrochloric acid* is required to change the colour of the indicator.

Absorbance (*2.2.25*)
Maximum 0.40, determined between wavelengths of 220 nm and 360 nm on solution S.

Ethylene oxide
Head-space gas chromatography (*2.2.28*) coupled with mass spectrometry (*2.2.43*). *Prepare the reference solutions immediately before use.*

Test sample Crush the sample into fine particles. Weigh 0.10-3.00 g of the sample, depending on the amount of ethylene oxide residue expected, into a 20 mL injection vial and close the vial. For low ethylene-oxide-releasing plastic materials (e.g. cyclo-olefin polymers and copolymers), heat the vial in an oven at 120 °C for at least 15 h (pre-thermal extraction step) before the 1st injection.

Reference solution (a) Dilute 1.0 mL of *ethylene oxide stock solution R1* to 50.0 mL with *anhydrous ethanol R* (1000 µg of ethylene oxide per millilitre). *If the solution is prepared using commercial ethylene oxide standard from a previously opened container, it should be noted that some ethylene oxide may have been lost owing to its high volatility.*

Reference solution (b) Dilute 10 mL of reference solution (a) to 20 mL with *anhydrous ethanol R* (500 µg of ethylene oxide per millilitre).

Reference solution (c) Dilute 8.0 mL of reference solution (a) to 20.0 mL with *anhydrous ethanol R* (400 µg of ethylene oxide per millilitre). Transfer 20 µL of reference solution (c) into an injection vial and close immediately.

Reference solution (d) Dilute 6.0 mL of reference solution (a) to 20.0 mL with *anhydrous ethanol R* (300 µg of ethylene oxide per millilitre). Transfer 20 µL of reference solution (d) into an injection vial and close immediately.

Reference solution (e) Dilute 4.0 mL of reference solution (a) to 20.0 mL with *anhydrous ethanol R* (200 µg of ethylene oxide per millilitre). Transfer 20 µL of reference solution (e) into an injection vial and close immediately.

Reference solution (f) Dilute 2.0 mL of reference solution (a) to 20.0 mL with *anhydrous ethanol R* (100 µg of ethylene oxide per millilitre). Transfer 20 µL of reference solution (f) into an injection vial and close immediately.

Reference solution (g) Dilute 1.0 mL of reference solution (a) to 20.0 mL with *anhydrous ethanol R* (50 µg of ethylene oxide per millilitre). Transfer 20 µL of reference solution (g) into an injection vial and close immediately.

Reference solution (h) Dilute 0.5 mL of reference solution (a) to 20.0 mL with *anhydrous ethanol R* (25 µg of ethylene oxide per millilitre). Transfer 20 µL of reference solution (h) into an injection vial and close immediately.

Reference solution (i) Dilute 100 mg of *acetaldehyde R* to 100 mL with *anhydrous ethanol R* (1000 µg of acetaldehyde per millilitre).

Reference solution (j) Transfer 10 µL of reference solution (i) and 20 µL of reference solution (b) into an injection vial, mix and close immediately.

Column:
- *material*: fused silica;
- *size*: *l* = 30 m, Ø = 0.32 mm;
- *stationary phase*: *porous silica for chromatography R* (film thickness 4 µm).

A particle trap may be used to prevent particles that are dislodged from the column from damaging the detector.

Carrier gas *helium for chromatography R.*

Flow rate 1.0 mL/min.

Split ratio 1:50.

Static head-space conditions that may be used:
- *equilibration temperature*: 80 °C for plasticised poly(vinyl chloride); 120 °C for cyclo-olefin polymers and copolymers, and polyurethane; 160 °C for silicone;
- *equilibration time*: 60 min;
- *transfer-line temperature*: 130 °C;
- *pressurisation time*: 0.5 min;
- *injection time*: 3 min;
- *shaking mode*: high agitation.

Temperature:

		Time (min)	Temperature (°C)
Column		0 - 2	100
		2 - 8.25	100 → 225
		8.25 - 13.25	225
Injection port			160
Detector:	transfer line		260
	source		230
	analyser		150

Detection Mass spectrometer; the following settings have been found to be suitable:
- quadrupole mass spectrometer equipped with an electron impact ionisation mode (70 eV);
- *acquisition system*: single-ion monitoring (SIM) for ethylene oxide quantification and complete spectrum mode (*m/z* = 10-350) for ethylene oxide identification;
- mass spectrometer parameters for the fragmentometric mode (SIM) set as follows: *m/z* = 44 as ethylene oxide quantitation ion; *m/z* = 29 and *m/z* =15 as ethylene oxide qualification ions.

Injection 1 mL of the test sample and reference solutions (c), (d), (e), (f), (g), (h) and (j).

After injection of the test sample, remove the cap from the vial in a fume cupboard, and purge the vial with dry nitrogen for 30 s. Close the vial with the cap and a new septum and repeat the heating and injection to exhaustion. Exhaustion is achieved when the amount of ethylene oxide extracted is less than 10 per cent of the 1st extraction or when no analytically significant increase in the cumulative residue levels is detected.

System suitability:
- *resolution*: minimum 1.5 between the peaks due to ethylene oxide and acetaldehyde in the chromatogram obtained with reference solution (j);
- *signal-to-noise ratio*: minimum 10 for the peak due to ethylene oxide in the chromatogram obtained with reference solution (h).

Verify the absence of peaks interfering with the peak due to ethylene oxide by carrying out the test on an unsterilised sample.

Calculation of content Establish a calibration curve with the mass of ethylene oxide in each reference solution as the abscissa and the corresponding peak areas as the ordinate.

Limit If the label states that ethylene oxide has been used for sterilisation:
- *ethylene oxide*: maximum 10 ppm.

Silicone oil

Calculate the internal surface area of a syringe in square centimetres using the following expression:

$$2\sqrt{V \cdot \pi \cdot h}$$

V = nominal volume of the syringe, in cubic centimetres;
h = height of the graduation, in centimetres.

Take a sufficient number of syringes to give an internal surface area of 100 cm^2 to 200 cm^2. Aspirate into each syringe a volume of *methylene chloride R* equal to half the nominal volume and make up to the nominal volume with air. Rinse the internal surface corresponding to the nominal volume with the solvent by inverting the syringe 10 times in succession with the needle fitting closed by a finger covered by a plastic film inert to methylene chloride. Expel the extracts into a tared dish and repeat the operation. Evaporate the combined extracts to dryness on a water-bath. Dry at 100-105 °C for 1 h. The residue weighs not more than 0.25 mg per square centimetre of internal surface area.

Examine the residue by infrared absorption spectrophotometry (*2.2.24*).

Comparison *silicone oil CRS.*

Reducing substances

To 20.0 mL of solution S add 1 mL of *dilute sulfuric acid R* and 20.0 mL of *0.002 M potassium permanganate*. Boil under a reflux condenser for 3 min and cool immediately. Add 1 g

of *potassium iodide R* and titrate immediately with *0.01 M sodium thiosulfate* using 0.25 mL of *starch solution R* as indicator. Carry out a blank titration using 20.0 mL of *water R*. The difference between the 2 titration volumes is not greater than 3.0 mL.

Transparency
Fill a syringe with *water R* (blank) and fill another with a 10-fold dilution of primary opalescent suspension (*2.2.1*). Use primary opalescent suspension that has been allowed to stand at 20 ± 2 °C for 24 h before use. Compare with the naked eye in diffused light against a dark background. The opalescence of the suspension is detectable when compared with the blank.

Sterility (*2.6.1*)
Syringes stated to be sterile comply with the test for sterility carried out as follows Using aseptic technique, open the package, withdraw the syringe, separate the components and place each in a suitable container containing sufficient culture media to cover the part completely. Use both the recommended media (*2.6.1*).

Syringes stated to be sterile only internally comply with the test for sterility carried out as follows Use 50 mL of inoculation medium for each test syringe. Using aseptic technique, remove the needle protector and submerge the needle in the culture medium. Flush the syringe 5 times by withdrawing the plunger to its fullest extent.

Bacterial endotoxins (*2.6.14*)
Less than 0.5 IU/mL, determined on 10 syringes filled with an extraction volume of 40 mL of water for BET, unless otherwise justified and authorised.

LABELLING
The label states, where applicable, that the syringe has been sterilised using ethylene oxide.

Appendix XX

Materials Used for the Manufacture of Containers

(Ph. Eur. method 3.1)

The materials described in this chapter are used for the manufacture of containers for pharmaceutical use. Their use may also be considered for the manufacture of part or all of objects used for medico-surgical purposes.

Materials and polymers other than those described in the Pharmacopoeia may be used subject to approval in each case by the competent authority responsible for the licensing for sale of the preparation in the container.

Transmissible spongiform encephalopathies (*5.2.8*)
A risk assessment of the product with respect to transmissible spongiform encephalopathies is carried out, and suitable measures are taken to minimise any such risk.

Materials for Containers for Human Blood and Blood Components

(Ph. Eur. method 3.3.1)

This general chapter is published for information.

NOTE: for materials based on plasticised poly(vinyl chloride) for containers for aqueous solutions for intravenous infusion, see text 3.1.14.

Plastic containers for the collection, storage, processing and administration of blood and its components may be manufactured from one or more polymers, if necessary with certain additives.

If all or part of the container consists of a material described in a text of the Pharmacopoeia, the quality of the material is controlled by the methods indicated in that text. (See *3.3.2. Materials based on plasticised poly(vinyl chloride) for containers for human blood and blood components)*

In normal conditions of use the materials and containers made from such materials do not release monomers, or other substances, in amounts likely to be harmful nor do they lead to any abnormal modifications of the blood or blood components.

1. Materials Based on Plasticised Poly(vinyl chloride) for Containers for Human Blood and Blood Components

(Ph. Eur. method 3.3.2)

This general chapter is published for information.

DEFINITION
Materials based on plasticised poly(vinyl chloride) contain not less than 55 per cent of poly(vinyl chloride) and contain various additives, in addition to the high-molecular-mass polymer obtained by polymerisation of vinyl chloride.

Materials based on plasticised poly(vinyl chloride) for containers for human blood and blood components are defined by the nature and the proportions of the substances used in their manufacture.

PRODUCTION
Materials based on plasticised poly(vinyl chloride) are produced by polymerisation methods that guarantee a residual vinyl chloride content of less than 1 ppm.

Vinyl chloride

Head-space gas chromatography (*2.2.28*).

Internal standard solution Using a microsyringe, inject 10 µL of *ether R* into 20.0 mL of *dimethylacetamide R*, immersing the tip of the needle in the solvent. Immediately before use, dilute the solution 1000-fold with *dimethylacetamide R*.

Test solution Place 1.000 g of the material to be examined in a 50 mL vial and add 10.0 mL of the internal standard solution. Close the vial and secure the stopper. Shake, avoiding contact between the stopper and the liquid. Place the vial in a water-bath at 60 ± 1 °C for 2 h.

Vinyl chloride primary solution *Prepare in a fume cupboard.* Place 50.0 mL of *dimethylacetamide R* in a 50 mL vial, stopper the vial, secure the stopper and weigh to the nearest 0.1 mg. Fill a 50 mL polyethylene or polypropylene syringe with gaseous *vinyl chloride R*, allow the gas to remain in contact with the syringe for about 3 min, empty the syringe and fill again with 50 mL of gaseous *vinyl chloride R*. Fit a hypodermic needle to the syringe and reduce the volume of gas in the syringe from 50 mL to 25 mL. Inject the remaining 25 mL of vinyl chloride slowly into the vial shaking gently and avoiding contact between the liquid and the needle. Weigh the vial again; the increase in mass is about 60 mg (1 µL of the solution thus obtained contains about 1.2 µg of vinyl chloride). Allow to stand for 2 h. Keep the primary solution in a refrigerator.

Vinyl chloride standard solution Vinyl chloride primary solution, *dimethylacetamide R* (1:3 *V/V*).

Reference solutions Place 10.0 mL of the internal standard solution in each of six 50 mL vials. Close the vials and secure the stoppers. Inject 1 µL, 2 µL, 3 µL, 5 µL and 10 µL, respectively, of the vinyl chloride standard solution into 5 of the vials. The 6 solutions thus obtained contain, respectively, 0 µg, about 0.3 µg, 0.6 µg, 0.9 µg, 1.5 µg and 3 µg of vinyl chloride. Shake, avoiding contact between the stopper and the liquid. Place the vials in a water-bath at 60 ± 1 °C for 2 h.

Column:
- *material*: stainless steel;
- *size*: *l* = 3 m, Ø = 3 mm;
- *stationary phase*: *silanised diatomaceous earth for gas chromatography R* impregnated with 5 per cent *m/m* of *dimethylstearamide R* and 5 per cent *m/m* of *macrogol 400 R*.

Carrier gas *nitrogen for chromatography R*.

Flow rate 30 mL/min.

Temperature:
- *column*: 45 °C;
- *injection port*: 100 °C;
- *detector*: 150 °C.

Detection Flame ionisation.

Injection 1 mL.

Limit:
- *vinyl chloride*: maximum 1 ppm.

Additives

Depending on the intended use of the polymers, they contain additives to optimise their processing or their chemical, physical and mechanical properties. Unless otherwise justified and authorised, these additives are chosen from the following list, which specifies for each substance the maximum permitted content:
- di(2-ethylhexyl)phthalate (plastic additive 01): maximum 40 per cent;
- zinc octanoate (zinc 2-ethylhexanoate) (plastic additive 02): maximum 1 per cent;
- calcium stearate or zinc stearate: maximum 1 per cent or 1 per cent of a mixture of the two;
- *N,N'*-diacylethylenediamines (plastic additive 03): maximum 1 per cent;
- one of the following epoxidised oils: maximum 10 per cent or 10 per cent of a mixture of the two:
  - epoxidised soya oil (plastic additive 04), of which the oxiran oxygen content is 6 per cent to 8 per cent and the iodine value is not greater than 6;
  - epoxidised linseed oil (plastic additive 05), of which the oxiran oxygen content is not greater than 10 per cent and the iodine value is not greater than 7;
- cyclohexane 1,2-dicarboxylic acid, diisononyl ester (plastic additive 24): maximum 45 per cent;
- butyryl tri-*n*-hexyl citrate (plastic additive 25): maximum 45 per cent;
- tris(2-ethylhexyl) trimellitate (plastic additive 26): maximum 45 per cent;
- bis(2-ethylhexyl) terephthalate (plastic additive 27): maximum 45 per cent.

The supplier of the material must be able to demonstrate that the qualitative and quantitative composition of the type sample is satisfactory for each production batch.

Blood and blood components have different requirements, for example with respect to gas exchange, storage temperature and the mechanical properties of the containers. In addition, the stability and the quality of blood or blood components stored in containers can be influenced by the plasticisers/additives present in the materials used in the composition of the containers. To ensure the stability of blood and blood components during their manufacture and storage, the materials from which the containers are composed must be carefully selected according to the intended use.

CHARACTERS

Almost colourless or pale yellow powder, beads, granules or, after transformation, translucent sheets of varying thickness, with a slight odour. On combustion it gives off dense, black smoke.

IDENTIFICATION

If necessary, cut the samples of the material to be examined into pieces where the maximum size of a side is 1 cm.

To 2.0 g of the material to be examined add 200 mL of *peroxide-free ether R* and heat under a reflux condenser for 8 h. Separate the residue (residue B) and the solution (solution A) by filtration.

Evaporate solution A to dryness under reduced pressure in a water-bath at 30 °C. Dissolve the residue in 10 mL of *toluene R* (solution A1). Dissolve residue B in 60 mL of *ethylene chloride R*, heating on a water-bath under a reflux condenser. Filter. Add the solution obtained dropwise and with vigorous shaking to 600 mL of *heptane R* heated almost to boiling. Separate the coagulum (coagulum B1) and the organic solution by hot filtration. Allow the latter to cool; separate the precipitate that forms (precipitate B2) and filter through a tared sintered-glass filter (40) (*2.1.2*).

A. Infrared absorption spectrophotometry (*2.2.24*).

Preparation Dissolve coagulum B1 in 30 mL of *tetrahydrofuran R* and add, in small volumes with shaking, 40 mL of *anhydrous ethanol R*; separate the precipitate (precipitate B3) by filtration and dry *in vacuo* at a temperature not exceeding 50 °C over *diphosphorus*

pentoxide R; dissolve a few milligrams of precipitate B3 in 1 mL of *tetrahydrofuran R*, place a few drops of the solution obtained on a sodium chloride plate and evaporate to dryness in an oven at 100-105 °C.

Comparison *poly(vinyl chloride) CRS.*

B. Plastic additives 01, 24, 25, 26 and 27 (see Tests).

TESTS

If necessary, cut the samples of the material to be examined into pieces where the maximum size of a side is 1 cm.

Solution S1

Place 5.0 g of the material to be examined in a combustion flask. Add 30 mL of *sulfuric acid R* and heat until a black, syrupy mass is obtained. Cool and add carefully 10 mL of *strong hydrogen peroxide solution R*. Heat gently. Allow to cool and add 1 mL of *strong hydrogen peroxide solution R*; repeat by alternating evaporation and addition of hydrogen peroxide solution until a colourless liquid is obtained. Reduce the volume to about 10 mL. Cool and dilute to 50.0 mL with *water R*.

Solution S2

Place 25 g of the material to be examined in a borosilicate-glass flask. Add 500 mL of *water R* and cover the neck of the flask with a borosilicate-glass beaker. Heat in an autoclave at 121 ± 2 °C for 20 min. Allow to cool, decant the solution and dilute to 500 mL with *water R*.

Appearance of solution S2

Solution S2 is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

Acidity or alkalinity

To 100 mL of solution S2 add 0.15 mL of *BRP indicator solution R*. Not more than 1.5 mL of *0.01 M sodium hydroxide* is required to change the colour of the indicator to blue.

To 100 mL of solution S2 add 0.2 mL of *methyl orange solution R*. Not more than 1.0 mL of *0.01 M hydrochloric acid* is required to initiate the colour change of the indicator from yellow to orange.

Absorbance (*2.2.25*)

Evaporate 100.0 mL of solution S2 to dryness. Dissolve the residue in 5.0 mL of *hexane R*. From 250 nm to 310 nm the absorbance is not greater than 0.25.

Reducing substances

Carry out the test within 4 h of preparation of solution S2 To 20.0 mL of solution S2 add 1 mL of *dilute sulfuric acid R* and 20.0 mL of *0.002 M potassium permanganate*. Boil under a reflux condenser for 3 min and cool immediately. Add 1 g of *potassium iodide R* and titrate immediately with *0.01 M sodium thiosulfate*, using 0.25 mL of *starch solution R* as indicator. Carry out a blank titration using 20 mL of *water R*. The difference between the 2 titration volumes is not more than 2.0 mL.

Primary aromatic amines

Maximum 20 ppm.

To 2.5 mL of solution A1 obtained during the identification, add 6 mL of *water R* and 4 mL of *0.1 M hydrochloric acid*. Shake vigorously and discard the upper layer. To the aqueous layer add 0.4 mL of a freshly prepared 10 g/L solution of *sodium nitrite R*. Mix and allow to stand for 1 min. Add 0.8 mL of a 25 g/L solution of *ammonium sulfamate R*, allow to stand for 1 min and add 2 mL of a 5 g/L solution of *naphthylethylenediamine dihydrochloride R*. After 30 min, any colour in the solution is not more intense than that in a standard prepared at the same time and in the same manner, replacing the aqueous layer with a mixture of 1 mL of a 0.01 g/L solution of *naphthylamine R* in *0.1 M hydrochloric acid*, 5 mL of *water R* and 4 mL of *0.1 M hydrochloric acid*.

Plastic additives 01, 24, 25, 26 and 27

Gas chromatography (*2.2.28*) coupled with mass spectrometry (*2.2.43*).

Internal standard solution S3 1 mg/mL solution of *di-n-octyl phthalate R* in *tetrahydrofuran for chromatography R*.

Internal standard solution S4 5 µg/mL solution of *di-n-octyl phthalate R* in *anhydrous ethanol R*.

Test solution Cut 0.2 g of the material to be examined into pieces about 0.5 cm in length. Dissolve the pieces in 12.5 mL of internal standard solution S3 using a polytetrafluoroethylene magnetic stirring bar. Complete dissolution of the material to be examined is obtained after stirring for about 20-30 min. The poly(vinyl chloride) is precipitated as a white powder by adding dropwise 37.5 mL of *anhydrous ethanol R*. Centrifuge, then dilute 1.0 mL of the supernatant to 50.0 mL with *anhydrous ethanol R*. The final concentration of the internal standard in the test solution is 5 µg/mL.

The stock solutions may be stored at 4 °C for up to 2 weeks.

Stock solution (a) Dissolve 20.0 mg of *plastic additive 01 CRS* in internal standard solution S4 and dilute to 20.0 mL with internal standard solution S4.

Stock solution (b) Dissolve 20.0 mg of *plastic additive 24 CRS* in internal standard solution S4 and dilute to 20.0 mL with internal standard solution S4.

Stock solution (c) Dissolve 20.0 mg of *plastic additive 25 CRS* in internal standard solution S4 and dilute to 20.0 mL with internal standard solution S4.

Stock solution (d) Dissolve 20.0 mg of *plastic additive 26 CRS* in internal standard solution S4 and dilute to 20.0 mL with internal standard solution S4.

Stock solution (e) Dissolve 20.0 mg of *plastic additive 27 CRS* in internal standard solution S4 and dilute to 20.0 mL with internal standard solution S4.

Reference solutions (a1)-(a5) Dilute stock solution (a) with internal standard solution S4 to obtain 5 reference solutions containing 10-40 µg/mL of *plastic additive 01 CRS*.

Reference solutions (b1)-(b5) Dilute stock solution (b) with internal standard solution S4 to obtain 5 reference solutions containing 10-40 µg/mL of *plastic additive 24 CRS*.

Reference solutions (c1)-(c5) Dilute stock solution (c) with internal standard solution S4 to obtain 5 reference solutions containing 10-40 µg/mL of *plastic additive 25 CRS*.

Reference solutions (d1)-(d5) Dilute stock solution (d) with internal standard solution S4 to obtain 5 reference solutions containing 10-40 µg/mL of *plastic additive 26 CRS*.

Reference solutions (e1)-(e5) Dilute stock solution (e) with internal standard solution S4 to obtain 5 reference solutions containing 10-40 µg/mL of *plastic additive 27 CRS*.

Column:

- *material*: fused silica;
- *size*: *l* = 30 m, Ø = 0.25 mm;
- *stationary phase*: *phenyl(5)methyl(95)polysiloxane R* (film thickness 0.25 µm).

Carrier gas *helium for chromatography R.*

Flow rate 1 mL/min.

Split ratio 1:20.

Temperature:

	Time (min)	Temperature (°C)
Column	0 - 3.3	100 → 200
	3.3 - 20	200 → 250
	20 - 22.5	250
	22.5 - 23	250 → 270
	23 - 25	270
	25 - 25.6	270 → 320
	25.6 - 30.6	320
Injection port		300

Detection Mass spectrometer as described below; adjust the detector settings so as to comply with the system suitability criteria:
- quadrupole mass spectrometer equipped with an electron impact ionisation mode (70 eV);
- *ion source temperature*: 230 °C;
- *acquisition system*: performed on full-scan (*m/z* = 40-350) and on single-ion monitoring (SIM) modes;
- *solvent delay*: 2.5 min;
- mass spectrometer parameters for the fragmentometric mode (SIM) set as follows:

Substance	Ion 1 [*m/z*]	Ion 2 [*m/z*]	Ion 3 [*m/z*]
Plastic additive 01	149	167	279
Plastic additive 24	155	127	299
Plastic additive 25	71	213	315
Plastic additive 26	305	193	323
Plastic additive 27	261	149	167
DnOP (internal standard)	149	279	167

Injection 1 µL.

Relative retention With reference to di-*n*-octyl phthalate (retention time = about 22 min): plastic additive 01 = about 0.80; plastic additive 24 = about 0.95-1.09; plastic additive 27 = about 1.02; plastic additive 25 = about 1.14; plastic additive 26 = about 1.34.

The specificity of the detection is checked by monitoring 3 different ions for each substance using a mass spectrometer in SIM mode. Ion ratios are determined from the peak areas after the injection of a standard solution. The ratios in the table below are given for information.

Substance	Ion 1 [*m/z*]	Ion 2 [*m/z*]	Ion 3 [*m/z*]	Ion ratio 2/1 (%)	Ion ratio 3/1 (%)
Plastic additive 01	149	167	279	50	30
Plastic additive 24	155	127	299	30	13
Plastic additive 25	71	213	315	45	20
Plastic additive 26	305	193	323	55	20
Plastic additive 27	261	149	167	130	85
DnOP (internal standard)	149	279	167	/	/

System suitability:
- *resolution*: if plastic additive 27 is tested, minimum 1.5 between the peaks due to the internal standard and plastic additive 27;
- *repeatability*: maximum relative standard deviation of 1.0 per cent for the retention time of the peak due to the plastic additive, determined on 6 injections of a reference solution of each plastic additive tested situated in the middle of the calibration range (e.g. 20 µg/mL);
- maximum relative standard deviation of 3.0 per cent for the ratio of the area of the peak due to the plastic additive to that due to the internal standard, determined on 6 injections of a reference solution of each plastic additive tested situated in the middle of the calibration range (e.g. 20 µg/mL).

From the calibration curve obtained with the reference solutions, calculate the percentage content of plastic additives in the material to be examined.

Limits:
- *plastic additive 01*: maximum 40 per cent;
- *plastic additive 24*: maximum 45 per cent;
- *plastic additive 25*: maximum 45 per cent;
- *plastic additive 26*: maximum 45 per cent;
- *plastic additive 27*: maximum 45 per cent.

Plastic additive 03

Wash precipitate B2 obtained during the identification and contained in the tared sintered-glass filter (40) (*2.1.2*) with *anhydrous ethanol R*. Dry to constant mass over *diphosphorus pentoxide R* and weigh the filter. The residue weighs not more than 20 mg.

Infrared absorption spectrophotometry (*2.2.24*).

Preparation The residue obtained above. When the amount of residue is insufficient to prepare a disc, record the spectrum of the residue placed between 2 plates transparent to infrared radiation or examine by attenuated total reflectance (ATR).

Comparison *plastic additive 03 CRS*.

Plastic additives 04 and 05

Thin-layer chromatography (*2.2.27*).

Reference solutions Prepare 10 mg/mL solutions of *plastic additive 04 CRS* and *plastic additive 05 CRS*, respectively, in *toluene R*.

Plate *TLC silica gel F_{254} plate R*.

Mobile phase *toluene R*.

Application 0.5 mL of solution A1 obtained during the identification, as a band 30 mm by 3 mm, and 5 µL of each reference solution.

Development Over 2/3 of the plate.

Drying In air.

Detection Expose the plate to iodine vapour for 5 min.

Examine the chromatogram and locate the zone corresponding to plastic additives 04 and 05 (R_F = 0). Remove the area of silica gel corresponding to this zone. Similarly remove a corresponding area of silica gel as a blank reference. Separately shake both samples for 15 min with 40 mL of *methanol R*. Filter, rinse with 2 quantities, each of 10 mL, of *methanol R*, add the rinsings to the filtrate and evaporate to dryness. The difference between the masses of both residues is not more than 10 mg.

Barium

Maximum 5 ppm.

Inductively coupled plasma-atomic emission spectrometry (*2.2.57*).

Test solution Ignite 1.0 g of the material to be examined in a silica crucible. Take up the residue with 10 mL of *hydrochloric acid R* and evaporate to dryness on a water-bath. Take up the residue with 20 mL of *0.1 M hydrochloric acid*.

Reference solution A solution containing 0.25 ppm of barium prepared by dilution of *barium standard solution (50 ppm Ba) R* with *0.1 M hydrochloric acid*.

Wavelength Use the emission of barium at 455.40 nm, the spectral background being taken at 455.30 nm.

Verify the absence of barium in the hydrochloric acid used.

Cadmium

Maximum 0.6 ppm.

Atomic absorption spectrometry (*2.2.23, Method I*).

Test solution Evaporate 10 mL of solution S1 to dryness. Take up the residue using 5 mL of a 1 per cent *V/V* solution of *hydrochloric acid R*, filter and dilute the filtrate to 10.0 mL with the same acid solution.

Reference solutions Prepare the reference solutions using *cadmium standard solution (0.1 per cent Cd) R*, diluting with a 1 per cent *V/V* solution of *hydrochloric acid R*.

Source Cadmium hollow-cathode lamp.

Wavelength 228.8 nm.

Atomisation device Air-acetylene flame.

Verify the absence of cadmium in the hydrochloric acid used.

Calcium

Maximum 0.07 per cent.

Inductively coupled plasma-atomic emission spectrometry (*2.2.57*).

Test solution Use the test solution prepared for the determination of barium.

Reference solution A solution containing 50.0 ppm of calcium prepared by dilution of *calcium standard solution (400 ppm Ca) R* with *0.1 M hydrochloric acid*.

Wavelength Use the emission of calcium at 315.89 nm, the spectral background being taken at 315.60 nm.

Verify the absence of calcium in the hydrochloric acid used.

Tin

Maximum 20 ppm.

Inductively coupled plasma-atomic emission spectrometry (*2.2.57*).

Test solution Dilute solution S1 10-fold with *water R* immediately before use.

Reference solution Introduce 2 mL of *tin standard solution (5 ppm Sn) R* into a 50 mL flask containing 5 mL of a 20 per cent *V/V* solution of *sulfuric acid R* and dilute to 50 mL with *water R* immediately before use.

Wavelength Use the emission of tin at 189.99 nm, the spectral background being taken at 190.10 nm.

Verify the absence of tin in the sulfuric acid used.

Zinc

Maximum 0.2 per cent.

Atomic absorption spectrometry (*2.2.23, Method I*).

Test solution Dilute solution S1 100-fold with *0.1 M hydrochloric acid*.

Reference solutions Prepare the reference solutions using *zinc standard solution (100 ppm Zn) R*, diluting with *0.1 M hydrochloric acid*.

Source Zinc hollow-cathode lamp.

Wavelength 213.9 nm.

Atomisation device Air-acetylene flame.

Verify the absence of zinc in the hydrochloric acid used.

Heavy metals (*2.4.8*)

Maximum 50 ppm.

To 10 mL of solution S1 add 0.5 mL of *phenolphthalein solution R* and then *strong sodium hydroxide solution R* until a pale pink colour is obtained. Dilute to 25 mL with *water R*. 12 mL of the solution complies with test A. Prepare the reference solution using *lead standard solution (2 ppm Pb) R*.

Water extractable substances

Maximum 0.3 per cent.

Evaporate 50 mL of solution S2 to dryness on a water-bath and dry in an oven at 100-105 °C to constant mass. Carry out a blank test with 50.0 mL of *water R*. The residue weighs not more than 7.5 mg taking into account the blank test.

ASSAY

Carry out the oxygen-flask method (*2.5.10*) using 50.0 mg of the material to be examined. Absorb the combustion products in 20 mL of *1 M sodium hydroxide*. To the solution obtained add 2.5 mL of *nitric acid R*. Titrate with *0.1 M silver nitrate*, determining the end-point potentiometrically (*2.2.20*). Carry out a blank titration.

1 mL of *0.1 M silver nitrate* is equivalent to 6.25 mg of poly(vinyl chloride).

Additional tests for sterile plastic containers for human blood and blood components are described in chapter 3.3. Containers for human blood and blood components, and materials used in their manufacture; transfusion sets and materials used in their manufacture; syringes and relevant subsections.

An additional test for the absorbance of an anticoagulant solution is described in general chapter 3.3.6. Sterile containers of plasticised poly(vinyl chloride) for human blood containing anticoagulant solution.

2. Materials Based on Plasticised Poly(vinyl chloride) for Tubing Used In Sets for the Transfusion of Blood and Blood Components

(Ph. Eur. method 3.3.3)

This general chapter is published for information.

DEFINITION

Materials based on plasticised poly(vinyl chloride) contain not less than 55 per cent of poly(vinyl chloride) and contain various additives, in addition to the high-molecular-mass polymer obtained by polymerisation of vinyl chloride.

Materials based on plasticised poly(vinyl chloride) for tubing used in sets for the transfusion of blood and blood components are defined by the nature and the proportions of the substances used in their manufacture.

Connecting ports are also to be considered as tubing.

PRODUCTION

Materials based on plasticised poly(vinyl chloride) are produced by polymerisation methods that guarantee a residual vinyl chloride content of less than 1 ppm.

Vinyl chloride

Head-space gas chromatography (*2.2.28*).

Internal standard solution Using a microsyringe, inject 10 µL of *ether R* into 20.0 mL of *dimethylacetamide R*, immersing the tip of the needle in the solvent. Immediately before use, dilute the solution 1000-fold with *dimethylacetamide R*.

Test solution Place 1.000 g of the material to be examined in a 50 mL vial and add 10.0 mL of the internal standard solution. Close the vial and secure the stopper. Shake, avoiding contact between the stopper and the liquid. Place the vial in a water-bath at 60 ± 1 °C for 2 h.

Vinyl chloride primary solution *Prepare in a fume cupboard.* Place 50.0 mL of *dimethylacetamide R* in a 50 mL vial, stopper the vial, secure the stopper and weigh to the nearest 0.1 mg. Fill a 50 mL polyethylene or polypropylene syringe with gaseous *vinyl chloride R*, allow the gas to remain

in contact with the syringe for about 3 min, empty the syringe and fill again with 50 mL of gaseous *vinyl chloride R*. Fit a hypodermic needle to the syringe and reduce the volume of gas in the syringe from 50 mL to 25 mL. Inject the remaining 25 mL of vinyl chloride slowly into the vial shaking gently and avoiding contact between the liquid and the needle. Weigh the vial again; the increase in mass is about 60 mg (1 µL of the solution thus obtained contains about 1.2 µg of vinyl chloride). Allow to stand for 2 h. Keep the primary solution in a refrigerator.

Vinyl chloride standard solution Vinyl chloride primary solution, *dimethylacetamide R* (1:3 *V/V*).

Reference solutions Place 10.0 mL of the internal standard solution in each of six 50 mL vials. Close the vials and secure the stoppers. Inject 1 µL, 2 µL, 3 µL, 5 µL and 10 µL, respectively, of the vinyl chloride standard solution into 5 of the vials. The 6 solutions thus obtained contain respectively, 0 µg, about 0.3 µg, 0.6 µg, 0.9 µg, 1.5 µg and 3 µg of vinyl chloride. Shake, avoiding contact between the stopper and the liquid. Place the vials in a water-bath at 60 ± 1 °C for 2 h.

Column:
- *material*: stainless steel;
- *size*: *l* = 3 m, Ø = 3 mm;
- *stationary phase*: *silanised diatomaceous earth for gas chromatography R* impregnated with 5 per cent *m/m* of *dimethylstearamide R* and 5 per cent *m/m* of *macrogol 400 R*.

Carrier gas *nitrogen for chromatography R*.

Flow rate 30 mL/min.

Temperature:
- *column*: 45 °C;
- *injection port*: 100 °C;
- *detector*: 150 °C.

Detection Flame ionisation.

Injection 1 mL.

Limit:
- *vinyl chloride*: maximum 1 ppm.

Additives

Depending on the intended use of the polymers, they contain additives to optimise their processing or their chemical, physical and mechanical properties. Unless otherwise justified and authorised, these additives are chosen from the following list, which specifies for each substance the maximum permitted content:
- di(2-ethylhexyl)phthalate (plastic additive 01): maximum 40 per cent;
- cyclohexane 1,2-dicarboxylic acid, diisononyl ester (plastic additive 24): maximum 45 per cent;
- butyryl tri-*n*-hexyl citrate (plastic additive 25): maximum 45 per cent;
- tris(2-ethylhexyl) trimellitate (plastic additive 26): maximum 45 per cent;
- bis(2-ethylhexyl) terephthalate (plastic additive 27): maximum 45 per cent.

The supplier of the material must be able to demonstrate that the qualitative and quantitative composition of the type sample is satisfactory for each production batch.

Blood and blood components have different requirements, for example with respect to gas exchange, storage temperature and the mechanical properties of the tubing. In addition, the stability and the quality of blood or blood components during transfusion can be influenced by the plasticisers/additives present in the materials used in the composition of the tubing. To ensure the stability of blood and blood components during transfusion, the materials from which the tubing is composed must be carefully selected according to the intended use.

CHARACTERS

Almost colourless or pale-yellow material in the form of powder, beads, granules or, after transformation, tubes with a slight odour. On combustion it gives off dense, black smoke.

IDENTIFICATION

If necessary, cut the samples of the material to be examined into pieces where the maximum size of a side is 1 cm.

A. Infrared absorption spectrophotometry (*2.2.24*).

Preparation To 0.5 g of the material to be examined add 30 mL of *tetrahydrofuran R*. Heat with stirring on a water-bath in a fume cupboard for 10 min; the material dissolves completely. Add *methanol R* dropwise with stirring; a granular precipitate is formed. Filter the precipitate and dry at 60 °C. Examine the precipitate by infrared absorption spectrophotometry (*2.2.24*). Dissolve 50 mg in 2 mL of *tetrahydrofuran R* and pour on a glass slide. Dry in an oven at 80 °C, remove the film and fix on a suitable mount. Examine by infrared absorption spectrophotometry (*2.2.24*).

Comparison *poly(vinyl chloride) CRS*.

B. Plastic additives 01, 24, 25, 26 and 27 (see Tests).

TESTS

If necessary, cut the samples of the material to be examined into pieces where the maximum size of a side is 1 cm.

Solution S1

Place 5.0 g of the material to be examined in a combustion flask. Add 30 mL of *sulfuric acid R* and heat until a black, syrupy mass is obtained. Cool and add carefully 10 mL of *strong hydrogen peroxide solution R*. Heat gently. Allow to cool and add 1 mL of *strong hydrogen peroxide solution R*; repeat by alternating evaporation and addition of hydrogen peroxide solution until a colourless liquid is obtained. Reduce the volume to about 10 mL. Cool and dilute to 50.0 mL with *water R*.

Solution S2

Place 25 g of the material to be examined in a borosilicate-glass flask. Add 500 mL of *water R* and cover the neck of the flask with a borosilicate-glass beaker. Heat in an autoclave at 121 ± 2 °C for 20 min. Allow to cool. Decant the solution and dilute to 500 mL with *water R*.

Appearance of solution S2

Solution S2 is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

Plastic additives 01, 24, 25, 26 and 27

Gas chromatography (*2.2.28*) coupled with mass spectrometry (*2.2.43*).

Internal standard solution S3 1 mg/mL solution of *di-n-octyl phthalate R* in *tetrahydrofuran for chromatography R*.

Internal standard solution S4 5 µg/mL solution of *di-n-octyl phthalate R* in *anhydrous ethanol R*.

Test solution Cut 0.2 g of the material to be examined into pieces about 0.5 cm in length. Dissolve the pieces in 12.5 mL of internal standard solution S3 using a polytetrafluoroethylene magnetic stirring bar. Complete dissolution of the material to be examined is obtained after stirring for about 20-30 min. The poly(vinyl chloride) is precipitated as a white powder by adding dropwise 37.5 mL of *anhydrous ethanol R*. Centrifuge, then dilute 1.0 mL of the supernatant to 50.0 mL with *anhydrous ethanol R*. The final

concentration of the internal standard in the test solution is 5 µg/mL.

The stock solutions may be stored at 4 °C for up to 2 weeks.

Stock solution (a) Dissolve 20.0 mg of *plastic additive 01 CRS* in internal standard solution S4 and dilute to 20.0 mL with internal standard solution S4.

Stock solution (b) Dissolve 20.0 mg of *plastic additive 24 CRS* in internal standard solution S4 and dilute to 20.0 mL with internal standard solution S4.

Stock solution (c) Dissolve 20.0 mg of *plastic additive 25 CRS* in internal standard solution S4 and dilute to 20.0 mL with internal standard solution S4.

Stock solution (d) Dissolve 20.0 mg of *plastic additive 26 CRS* in internal standard solution S4 and dilute to 20.0 mL with internal standard solution S4.

Stock solution (e) Dissolve 20.0 mg of *plastic additive 27 CRS* in internal standard solution S4 and dilute to 20.0 mL with internal standard solution S4.

Reference solutions (a1)-(a5) Dilute stock solution (a) with internal standard solution S4 to obtain 5 reference solutions containing 10-40 µg/mL of *plastic additive 01 CRS*.

Reference solutions (b1)-(b5) Dilute stock solution (b) with internal standard solution S4 to obtain 5 reference solutions containing 10-40 µg/mL of *plastic additive 24 CRS*.

Reference solutions (c1)-(c5) Dilute stock solution (c) with internal standard solution S4 to obtain 5 reference solutions containing 10-40 µg/mL of *plastic additive 25 CRS*.

Reference solutions (d1)-(d5) Dilute stock solution (d) with internal standard solution S4 to obtain 5 reference solutions containing 10-40 µg/mL of *plastic additive 26 CRS*.

Reference solutions (e1)-(e5) Dilute stock solution (e) with internal standard solution S4 to obtain 5 reference solutions containing 10-40 µg/mL of *plastic additive 27 CRS*.

Column:
- *material*: fused silica;
- *size*: *l* = 30 m, Ø = 0.25 mm;
- *stationary phase*: *phenyl(5)methyl(95)polysiloxane R* (film thickness 0.25 µm).

Carrier gas *helium for chromatography R*.

Flow rate 1 mL/min.

Split ratio 1:20.

Temperature:

	Time (min)	Temperature (°C)
Column	0 - 3.3	100 → 200
	3.3 - 20	200 → 250
	20 - 22.5	250
	22.5 - 23	250 → 270
	23 - 25	270
	25 - 25.6	270 → 320
	25.6 - 30.6	320
Injection port		300

Detection Mass spectrometer as described below; adjust the detector settings so as to comply with the system suitability criteria:
- quadrupole mass spectrometer equipped with an electron impact ionisation mode (70 eV);
- *ion source temperature*: 230 °C;
- *acquisition system*: performed on full-scan (*m/z* = 40-350) and on single-ion monitoring (SIM) modes;
- *solvent delay*: 2.5 min;
- mass spectrometer parameters for the fragmentometric mode (SIM) set as follows:

Substance	Ion 1 [*m/z*]	Ion 2 [*m/z*]	Ion 3 [*m/z*]
Plastic additive 01	149	167	279
Plastic additive 24	155	127	299
Plastic additive 25	71	213	315
Plastic additive 26	305	193	323
Plastic additive 27	261	149	167
DnOP (internal standard)	149	279	167

Injection 1 µL.

Relative retention With reference to di-*n*-octyl phthalate (retention time = about 22 min): plastic additive 01 = about 0.80; plastic additive 24 = about 0.95-1,09; plastic additive 27 = about 1.02; plastic additive 25 = about 1.14; plastic additive 26 = about 1.34.

The specificity of the detection is checked by monitoring 3 different ions for each substance using a mass spectrometer in SIM mode. Ion ratios are determined from the peak areas after the injection of a standard solution. The ratios in the table below are given for information.

Substance	Ion 1 [*m/z*]	Ion 2 [*m/z*]	Ion 3 [*m/z*]	Ion ratio 2/1 (%)	Ion ratio 3/1 (%)
Plastic additive 01	149	167	279	50	30
Plastic additive 24	155	127	299	30	13
Plastic additive 25	71	213	315	45	20
Plastic additive 26	305	193	323	55	20
Plastic additive 27	261	149	167	130	85
DnOP (internal standard)	149	279	167	/	/

System suitability:
- *resolution*: if plastic additive 27 is tested, minimum 1.5 between the peaks due to the internal standard and plastic additive 27;
- *repeatability*: maximum relative standard deviation of 1.0 per cent for the retention time of the peak due to the plastic additive, determined on 6 injections of a reference solution of each plastic additive tested situated in the middle of the calibration range (e.g. 20 µg/mL); maximum relative standard deviation of 3.0 per cent for the ratio of the area of the peak due to the plastic additive to that due to the internal standard, determined on 6 injections of a reference solution of each plastic additive tested situated in the middle of the calibration range (e.g. 20 µg/mL).

From the calibration curve obtained with the reference solutions, calculate the percentage content of plastic additives in the material to be examined.

Limits:
- *plastic additive 01*: maximum 40 per cent;
- *plastic additive 24*: maximum 45 per cent;
- *plastic additive 25*: maximum 45 per cent;
- *plastic additive 26*: maximum 45 per cent;
- *plastic additive 27*: maximum 45 per cent.

Barium

Maximum 5 ppm.

Inductively coupled plasma-atomic emission spectrometry (*2.2.57*).

Test solution Ignite 1.0 g of the material to be examined in a silica crucible. Take up the residue with 10 mL of

hydrochloric acid R and evaporate to dryness on a water-bath. Take up the residue with 20 mL of *0.1 M hydrochloric acid.*

Reference solution A solution containing 0.25 ppm of barium prepared by dilution of *barium standard solution (50 ppm Ba) R* with *0.1 M hydrochloric acid.*

Wavelength Use the emission of barium at 455.40 nm, the spectral background being taken at 455.30 nm.

Verify the absence of barium in the hydrochloric acid used.

Cadmium

Maximum 0.6 ppm.

Atomic absorption spectrometry (*2.2.23, Method I*).

Test solution Evaporate 10.0 mL of solution S1 to dryness. Take up the residue using 5 mL of a 1 per cent *V/V* solution of *hydrochloric acid R*, filter and dilute the filtrate to 10.0 mL with the same acid.

Reference solutions Prepare the reference solutions using *cadmium standard solution (0.1 per cent Cd) R*, diluting with a 1 per cent *V/V* solution of *hydrochloric acid R.*

Source Cadmium hollow-cathode lamp.

Wavelength 228.8 nm.

Atomisation device Air-acetylene flame.

Verify the absence of cadmium in the hydrochloric acid used.

Tin

Maximum 20 ppm.

Inductively coupled plasma-atomic emission spectrometry (*2.2.57*).

Test solution Dilute solution S1 10-fold with *water R* immediately before use.

Reference solution Introduce 2 mL of *tin standard solution (5 ppm Sn) R* into a 50 mL flask containing 5 mL of a 20 per cent *V/V* solution of *sulfuric acid R* and dilute to 50 mL with *water R* immediately before use.

Wavelength Use the emission of tin at 189.99 nm, the spectral background being taken at 190.10 nm.

Verify the absence of tin in the sulfuric acid used.

Heavy metals (*2.4.8*)

Maximum 50 ppm.

To 10 mL of solution S1 add 0.5 mL of *phenolphthalein solution R* and then *strong sodium hydroxide solution R* until a pale pink colour is obtained. Dilute to 25 mL with *water R.* 12 mL of the solution complies with test A. Prepare the reference solution using *lead standard solution (2 ppm Pb) R.*

ASSAY

To 0.500 g of the material to be examined add 30 mL of *tetrahydrofuran R* and heat with stirring on a water-bath in a fume cupboard for 10 min. The material dissolves completely. Add 60 mL of *methanol R* dropwise with stirring. A granular precipitate of poly(vinyl chloride) is formed. Allow to stand for a few minutes. Continue addition of *methanol R* until no further precipitation is observed. Transfer to a sintered-glass filter (40) (*2.1.2*), using 3 small quantities of *methanol R* to aid transfer and to wash the precipitate. Dry the filter and the precipitate to constant mass at 60 °C and weigh.

Additional tests for sterilised sets are described in general chapter 3.3.7. Sets for the transfusion of blood and blood components.

3. Materials Based on Non-plasticised Poly(Vinyl Chloride) for Containers for Non-injectable, Aqueous Solutions

(Ph. Eur. method 3.1.10)

DEFINITION

Materials based on non-plasticised poly(vinyl chloride) that comply with the following specifications are suitable for the manufacture of containers for non-injectable aqueous solutions. They may also be used for solid forms for oral administration and in some cases, subject to special studies on the compatibility of the container with its contents, these materials may be suitable for the preparation of containers for suppositories. They consist of 1 or more poly(vinyl chloride/vinyl acetate) or of a mixture of poly(vinyl chloride) and poly(vinyl acetate) or of poly(vinyl chloride).

The chlorine content expressed as poly(vinyl chloride) is not less than 80 per cent.

They may contain not more than 15 per cent of copolymers based on acrylic and/or methacrylic acids and/or their esters, and/or on styrene and/or butadiene.

PRODUCTION

Materials based on non-plasticised poly(vinyl chloride) are produced by polymerisation methods that guarantee a residual vinyl chloride content of less than 1 ppm.
The manufacturing process is validated to demonstrate that the product complies with the following test.

Vinyl chloride

Head-space gas chromatography (*2.2.28*).

Internal standard solution Using a microsyringe, inject 10 µL of *ether R* into 20.0 mL of *dimethylacetamide R*, immersing the tip of the needle in the solvent. Immediately before use, dilute the solution 1000-fold with *dimethylacetamide R.*

Test solution Place 1.000 g of the material to be examined in a 50 mL vial and add 10.0 mL of the internal standard solution. Close the vial and secure the stopper. Shake, avoiding contact between the stopper and the liquid. Place the vial in a water-bath at 60 ± 1 °C for 2 h.

Vinyl chloride primary solution *Prepare in a fume cupboard.* Place 50.0 mL of *dimethylacetamide R* in a 50 mL vial, stopper the vial, secure the stopper and weigh to the nearest 0.1 mg. Fill a 50 mL polyethylene or polypropylene syringe with gaseous *vinyl chloride R*, allow the gas to remain in contact with the syringe for about 3 min, empty the syringe and fill again with 50 mL of gaseous *vinyl chloride R.* Fit a hypodermic needle to the syringe and reduce the volume of gas in the syringe from 50 mL to 25 mL. Inject these 25 mL of vinyl chloride slowly into the vial, shaking gently and avoiding contact between the liquid and the needle. Weigh the vial again; the increase in mass is about 60 mg (1 µL of the solution thus obtained contains about 1.2 µg of vinyl chloride). Allow to stand for 2 h. Keep the primary solution in a refrigerator.

Vinyl chloride standard solution Vinyl chloride primary solution, *dimethylacetamide R* (1:3 *V/V*).

Reference solutions Place 10.0 mL of the internal standard solution in each of six 50 mL vials. Close the vials and secure the stoppers. Inject 1 µL, 2 µL, 3 µL, 5 µL and 10 µL, respectively, of the vinyl chloride standard solution into 5 of the vials. The 6 solutions thus obtained contain respectively, 0 µg, about 0.3 µg, 0.6 µg, 0.9 µg, 1.5 µg and 3 µg of vinyl chloride. Shake, avoiding contact between the stopper and the liquid. Place the vials in a water-bath at 60 ± 1 °C for 2 h.

Column:
— *material*: stainless steel;
— *size*: *l* = 3 m, Ø = 3 mm;
— *stationary phase*: *silanised diatomaceous earth for gas chromatography R* impregnated with 5 per cent *m/m* of *dimethylstearamide R* and 5 per cent *m/m* of *macrogol 400 R*.

Carrier gas *nitrogen for chromatography R*.

Flow rate 30 mL/min.

Temperature:
— *column*: 45 °C;
— *injection port*: 100 °C;
— *detector*: 150 °C.

Detection Flame ionisation.

Injection 1 mL of the head space.

Limit:
— *vinyl chloride*: maximum 1 ppm.

Additives

Depending on the intended use of the polymers, they may contain additives to optimise their processing or their chemical, physical and mechanical properties. These additives are chosen from the following list, which specifies for each substance the maximum allowable content:
— epoxidised soya oil of which the oxiran oxygen content is 6 per cent to 8 per cent and the iodine value is not greater than 6: maximum 8 per cent;
— calcium salt or zinc salts of aliphatic fatty acids with more than 7 carbon atoms: maximum 1.5 per cent or maximum 1.5 per cent of their mixture;
— liquid paraffin: maximum 1.5 per cent;
— waxes: maximum 1.5 per cent;
— hydrogenated oils or esters of aliphatic fatty acids: maximum 2 per cent;
— macrogol esters: maximum 1.5 per cent;
— sorbitol: maximum 1.5 per cent;
— 2,4-dinonylphenyl phosphite, or di(4-nonylphenyl) phosphite or tris(nonylphenyl) phosphite: maximum 1 per cent.

They may contain one of the following groups of stabilisers (where isooctyl is, for example, 2-ethylhexyl):
— tin as di(isooctyl) 2,2′-[(dioctylstannylene)bis (thio)]diacetate containing about 27 per cent of tri (isooctyl) 2,2′,2″-[(monooctylstannylidyne)tris(thio)] triacetate: maximum 0.25 per cent;
— tin as a mixture containing not more than 76 per cent of di(isooctyl) 2,2′-[(dimethylstannylene)bis(thio)]diacetate and not more than 85 per cent of tri(isooctyl) 2,2′,2″-[(monomethylstannylidyne)tris(thio)]triacetate: maximum 0.25 per cent;
— 1-phenyleicosane-1,3-dione (benzoylstearoylmethane) or 2-(4-dodecylphenyl)indole or didodecyl 1,4-dihydropyridine-2,6-dimethyl-3,5-dicarboxylate: maximum 1 per cent or 1 per cent of a mixture of 2 of these.

Colouring materials may be added, provided that the safety of the material is demonstrated to the satisfaction of the competent authority. The material may be opacified with titanium dioxide.

The supplier of the material must be able to demonstrate that the qualitative and quantitative composition of the type sample is satisfactory for each production batch.

CHARACTERS

Appearance

Powder, beads, granules, sheets of varying thicknesses or samples taken from finished objects.

Solubility

Practically insoluble in water, soluble in tetrahydrofuran, slightly soluble in methylene chloride, insoluble in anhydrous ethanol.

They burn with an orange-yellow flame edged with green, giving off thick black smoke.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

Preparation Dissolve residue A (see Tests: solution S2) in 5 mL of *tetrahydrofuran R*. Apply a few drops of the solution to a sodium chloride plate and evaporate to dryness in an oven at 100-105 °C.

Absorption maxima (tolerance ± 5 cm^{-1}): at 2910 cm^{-1}, 1425 cm^{-1}, 1330 cm^{-1}, 1252 cm^{-1}, 958 cm^{-1} and 690 cm^{-1}.

The spectrum obtained is identical to that of the material selected for the type sample.

TESTS

If necessary, cut the samples of the material to be examined into pieces with a maximum dimension on a side of not greater than 1 cm.

Solution S1

Place 25 g of the material to be examined in a borosilicate-glass flask. Add 500 mL of *water R* and cover the neck of the flask with a borosilicate-glass beaker. Heat in an autoclave for 121 ± 2 °C for 20 min. Allow to cool, decant the solution and make up to 500 mL with *water R*.

Solution S2

Dissolve 5.0 g of the material to be examined in 80 mL of *tetrahydrofuran R* and dilute to 100 mL with the same solvent. Filter if necessary (the solution may remain opalescent). To 20 mL of the solution add, dropwise and with gentle shaking, 70 mL of *ethanol (96 per cent) R*. Cool in ice for 1 h. Filter or centrifuge (residue A). Wash residue A with *ethanol (96 per cent) R*, add the washings to the filtrate or the centrifugation liquid and dilute to 100 mL with *ethanol (96 per cent) R*.

Solution S3

Place 5 g of the material to be examined in a borosilicate-glass flask with a ground-glass neck. Add 100 mL of *0.1 M hydrochloric acid* and boil under a reflux condenser for 1 h. Allow to cool and allow the solids to settle.

Appearance of solution S1

Solution S1 is not more opalescent than reference suspension II (*2.2.1*) and is colourless (*2.2.2, Method II*).

Absorbance of solution S1 (*2.2.25*)

Evaporate 100 mL of solution S1 to dryness. Dissolve the residue in 5 mL of *hexane R*. Filter if necessary through a filter previously rinsed with *hexane R*. At wavelengths from 250 nm to 310 nm, the absorbance of the filtrate is not greater than 0.25.

Absorbance of solution S2 (*2.2.25*)

Maximum 0.2 for tin-stabilised materials or 0.4 for other materials determined between wavelengths of 250 nm and 330 nm on solution S2.

Extractable barium

Maximum 2 ppm.

Inductively coupled plasma-atomic emission spectrometry (*2.2.57*).

Test solution Solution S3.

Reference solution A solution containing 0.1 ppm of barium prepared by dilution of *barium standard solution (50 ppm Ba) R* with *0.1 M hydrochloric acid.*

Wavelength Use the emission of barium at 455.40 nm, the spectral background being taken at 455.30 nm.

Verify the absence of barium in the hydrochloric acid used.

Examined at 455.40 nm, the emission of the test solution is not greater than that of the reference solution.

Extractable cadmium
Maximum 0.6 ppm.

Atomic absorption spectrometry (*2.2.23, Method I*).

Test solution Solution S3.

Reference solution A solution containing 0.03 ppm of cadmium prepared by diluting *cadmium standard solution (0.1 per cent Cd) R* with *0.1 M hydrochloric acid.*

Verify the absence of cadmium in the hydrochloric acid used.

Examined at 228.8 nm, the absorbance of the test solution is not greater than that of the reference solution.

Tin-stabilised materials
Maximum 0.25 per cent of Sn.

Tin stock solution Dilute 81 mg of *plastic additive 23 GRS* to 100.0 mL with *tetrahydrofuran R.*

Tin standard solution Dilute 20 mL of the tin stock solution to 100.0 mL with *ethanol (96 per cent) R.*

To 0.10 mL of solution S2 in a test tube add 0.05 mL of *1 M hydrochloric acid,* 0.5 mL of *potassium iodide solution R* and 5 mL of *ethanol (96 per cent) R.* Mix thoroughly and wait for 5 min. Add 9 mL of *water R* and 0.1 mL of a 5 g/L solution of *sodium sulfite heptahydrate R* and mix thoroughly. Add 1.5 mL of *dithizone solution R* freshly diluted 100-fold with *methylene chloride R,* shake for 15 s and allow to stand for 2 min. At the same time prepare a reference solution in the same manner using 0.1 mL of the tin standard solution.

Any violet colour in the lower layer obtained with solution S2 is not more intense than that obtained with the reference solution. The greenish-blue colour of dithizone solution turns pink in the presence of tin.

Non-tin stabilised materials
Maximum 25 ppm of Sn.

To 5 mL of solution S2 in a test tube add 0.05 mL of *1 M hydrochloric acid* and 0.5 mL of *potassium iodide solution R.* Mix thoroughly and wait for 5 min. Add 9 mL of *water R* and 0.1 mL of a 5 g/L solution of *sodium sulfite heptahydrate R* and mix thoroughly. If the solution obtained is not colourless, add the sodium sulfite solution in 0.05 mL fractions. Add 1.5 mL of *dithizone solution R* freshly diluted 100-fold with *methylene chloride R,* shake for 15 s and allow to stand for 2 min. At the same time prepare a reference solution in the same manner using 0.05 mL of the tin standard solution (see test above).

Any violet colour in the lower layer obtained with solution S2 is not more intense than that obtained with the reference solution.

Extractable heavy metals (*2.4.8*)
Maximum 20 ppm.

12 mL of solution S3 complies with test A. Prepare the reference solution using 10 mL of *lead standard solution (1 ppm Pb) R.*

Extractable zinc
Maximum 100 ppm.

Atomic absorption spectrometry (*2.2.23, Method I*).

Test solution Solution S3 diluted 10-fold with *water R.*

Reference solution A solution containing 0.50 ppm of zinc prepared by dilution of *zinc standard solution (5 mg/mL Zn) R* with *0.01 M hydrochloric acid.*

Verify the absence of zinc in the hydrochloric acid used.

Examined at 214.0 nm, the absorbance of the test solution is not greater than that of the reference solution.

Sulfated ash (*2.4.14*)
Maximum 1.0 per cent, determined on 1.0 g; maximum 4.0 per cent when the materials are opacified with titanium dioxide.

ASSAY
Carry out the oxygen-flask method (*2.5.10*) using 50.0 mg of the material to be examined. Absorb the combustion products in 20 mL of *1 M sodium hydroxide.* To the solution obtained add 2.5 mL of *nitric acid R.* Titrate with *0.1 M silver nitrate,* determining the end-point potentiometrically (*2.2.20*). Carry out a blank titration.

1 mL of *0.1 M silver nitrate* is equivalent to 6.25 mg of poly(vinyl chloride).

4. Materials based on Non-Plasticised Poly(Vinyl Chloride) for Containers for Solid Dosage Forms for Oral Administration

(Ph. Eur. method 3.1.11)

DEFINITION
Materials based on non-plasticised poly(vinyl chloride) for containers for solid dosage forms for oral administration are suitable for the manufacture of sheets or containers, and consist of 1 or more poly(vinyl chloride/vinyl acetate) or of a mixture of poly(vinyl chloride) and poly(vinyl acetate) or of poly(vinyl chloride).

The chlorine content expressed as poly(vinyl chloride) is not less than 80 per cent.

They may contain not more than 15 per cent of copolymers based on acrylic and/or methacrylic acids and/or their esters, and/or on styrene and/or butadiene.

PRODUCTION
Materials based on non-plasticised poly(vinyl chloride) are produced by polymerisation methods that guarantee a residual vinyl chloride content of less than 1 ppm.
The manufacturing process is validated to demonstrate that the product complies with the following test for vinyl chloride.

Vinyl chloride
Head-space gas chromatography (*2.2.28*).

Internal standard solution Using a microsyringe, inject 10 µL of *ether R* into 20.0 mL of *dimethylacetamide R,* immersing the tip of the needle in the solvent. Immediately before use, dilute the solution 1000-fold with *dimethylacetamide R.*

Test solution Place 1.000 g of the material to be examined in a 50 mL vial and add 10.0 mL of the internal standard solution. Close the vial and secure the stopper. Shake, avoiding contact between the stopper and the liquid. Place the vial in a water-bath at 60 ± 1 °C for 2 h.

Vinyl chloride primary solution *Prepare in a fume cupboard.* Place 50.0 mL of *dimethylacetamide R* in a 50 mL vial, stopper the vial, secure the stopper and weigh to the nearest 0.1 mg. Fill a 50 mL polyethylene or polypropylene syringe with gaseous *vinyl chloride R,* allow the gas to remain in contact with the syringe for about 3 min, empty the syringe and fill again with 50 mL of gaseous *vinyl chloride R.*

Fit a hypodermic needle to the syringe and reduce the volume of gas in the syringe from 50 mL to 25 mL. Inject the 25 mL of vinyl chloride slowly into the vial, shaking gently and avoiding contact between the liquid and the needle. Weigh the vial again; the increase in mass is about 60 mg (1 µL of the solution thus obtained contains about 1.2 µg of vinyl chloride). Allow to stand for 2 h. Keep the primary solution in a refrigerator.

Vinyl chloride standard solution Vinyl chloride primary solution, *dimethylacetamide R* (1:3 *V/V*).

Reference solutions Place 10.0 mL of the internal standard solution in each of six 50 mL vials. Close the vials and secure the stoppers. Inject 1 µL, 2 µL, 3 µL, 5 µL and 10 µL, respectively, of the vinyl chloride standard solution into 5 of the vials. The 6 solutions thus obtained contain respectively, 0 µg, about 0.3 µg, 0.6 µg, 0.9 µg, 1.5 µg and 3 µg of vinyl chloride. Shake, avoiding contact between the stopper and the liquid. Place the vials in a water-bath at 60 ± 1 °C for 2 h.

Column:
- *material*: stainless steel;
- *size*: *l* = 3 m, Ø = 3 mm;
- *stationary phase*: *silanised diatomaceous earth for gas chromatography R* impregnated with 5 per cent *m/m* of *dimethylstearamide R* and 5 per cent *m/m* of *macrogol 400 R*.

Carrier gas *nitrogen for chromatography R*.

Flow rate 30 mL/min.

Temperature:
- *column*: 45 °C;
- *injection port*: 100 °C;
- *detector*: 150 °C.

Detection Flame ionisation.

Injection 1 mL of the head space.

Limit:
- *vinyl chloride*: maximum 1 ppm.

Additives

Depending on the intended use of the polymers, they may contain additives to optimise their processing or their chemical, physical and mechanical properties. These additives are chosen from the following list, which specifies for each substance the maximum allowable content:
- epoxidised soya oil of which the oxiran oxygen content is 6 per cent to 8 per cent and the iodine value is not greater than 6 for tin-stabilised materials: maximum 2 per cent;
- epoxidised soya oil of which the oxiran oxygen content is 6 per cent to 8 per cent and the iodine value is not greater than 6 for non-tin-stabilised materials: maximum 3 per cent;
- calcium, magnesium or zinc salts of aliphatic fatty acids with more than 7 carbon atoms: maximum 1.5 per cent or maximum 1.5 per cent of their mixture;
- waxes: maximum 4 per cent;
- liquid paraffin: maximum 1.5 per cent;
- hydrogenated oils or esters of aliphatic fatty acids: maximum 2 per cent;
- the percentage sum of the 3 lubricants above: maximum 4 per cent;
- macrogol esters: maximum 1.5 per cent;
- sorbitol: maximum 1.5 per cent;
- 2,4-dinonylphenyl phosphite, or di(4-nonylphenyl) phosphite or tris(nonylphenyl) phosphite: maximum 1 per cent;
- calcium carbonate: maximum 1 per cent;
- silica: maximum 1 per cent.

They may contain one of the following groups of stabilisers (where isooctyl is, for example, 2-ethylhexyl):
- tin as di(isooctyl) 2,2′-[(dioctylstannylene)bis (thio)]diacetate containing about 27 per cent of tri (isooctyl) 2,2′2″-[(monooctylstannylidyne)tris(thio)] triacetate: maximum 0.25 per cent;
- tin as tri(isooctyl) 2,2′2″-[(monooctylstannylidyne)tris (thio)]triacetate: maximum 0.25 per cent;
- tin as a mixture containing not more than 76 per cent of di(isooctyl) 2,2′-[(dimethylstannylene)bis(thio)]diacetate and not more than 85 per cent of tri(isooctyl) 2,2′,2″-[(monomethylstannylidyne)tris(thio)]triacetate: maximum 0.25 per cent;
- 1-phenyleicosane-1,3-dione (benzoylstearoylmethane): maximum 1 per cent.

Colouring materials may be added, provided that the safety of the material is demonstrated to the satisfaction of the competent authority. The material may be opacified with titanium dioxide.

The supplier of the material must be able to demonstrate that the qualitative and quantitative composition of the type sample is satisfactory for each production batch.

CHARACTERS

Appearance

Powder, beads, granules, sheets of varying thicknesses or samples taken from finished objects.

Solubility

Practically insoluble in water, soluble in tetrahydrofuran, slightly soluble in methylene chloride, practically insoluble in anhydrous ethanol.

They burn with an orange-yellow flame edged with green, giving off thick black smoke.

IDENTIFICATION

Infrared absorption spectrophotometry (*2.2.24*).

Preparation Dissolve residue A (see Tests: solution S2) in 5 mL of *tetrahydrofuran R*. Apply a few drops of the solution to a sodium chloride plate and evaporate to dryness in an oven at 100-105 °C.

Absorption maxima (tolerance ± 5 cm^{-1}): at 2910 cm^{-1}, 1425 cm^{-1}, 1330 cm^{-1}, 1252 cm^{-1}, 958 cm^{-1} and 690 cm^{-1}.

The spectrum obtained is identical to that of the material selected for the type sample.

TESTS

If necessary, cut the samples of the material to be examined into pieces with a maximum dimension on a side of not greater than 1 cm.

Solution S1

Place 25 g of the material to be examined in a borosilicate-glass flask. Add 500 mL of *water R* and cover the neck of the flask with a borosilicate glass beaker. Heat in an autoclave for 121 ± 2 °C for 20 min. Allow to cool, decant the solution and make up to 500 mL with *water R*.

Solution S2

Dissolve 5.0 g of the material to be examined in 80 mL of *tetrahydrofuran R* and dilute to 100 mL with the same solvent. Filter if necessary (the solution may remain opalescent). To 20 mL of the solution add, dropwise and with gentle shaking, 70 mL of *ethanol (96 per cent) R*. Cool in ice for 1 h. Filter or centrifuge (residue A). Wash residue A with *ethanol (96 per cent) R*, add the washings to the filtrate

or the centrifugation liquid and dilute to 100 mL with *ethanol (96 per cent) R*.

Solution S3
Place 5 g of the material to be examined in a borosilicate-glass flask with a ground-glass neck. Add 100 mL of *0.1 M hydrochloric acid* and boil under a reflux condenser for 1 h. Allow to cool and allow the solids to settle.

Appearance of solution S1
Solution S1 is not more opalescent than reference suspension II (*2.2.1*) and is colourless (*2.2.2, Method II*).

Absorbance of solution S1 (*2.2.25*)
Evaporate 100 mL of solution S1 to dryness. Dissolve the residue in 5 mL of *hexane R*. Filter if necessary through a filter previously rinsed with *hexane R*. At wavelengths from 250 nm to 310 nm, the absorbance of the filtrate is not greater than 0.3.

Absorbance of solution S2 (*2.2.25*)
Maximum 1.0, determined between wavelengths of 250 nm and 330 nm on solution S2 for material that does not contain 1-phenyleicosane-1,3-dione; maximum 0.4, determined between wavelengths of 250 nm and 330 nm on a 10-fold dilution of solution S2 in *ethanol (96 per cent) R* for material that contains 1-phenyleicosane-1,3-dione.

Tin-stabilised materials
Maximum 0.25 per cent of Sn.

Tin stock solution Dilute 81 mg of *plastic additive 23 CRS* to 100.0 mL with *tetrahydrofuran R*.

Tin standard solution Dilute 20 mL of the tin stock solution to 100.0 mL with *ethanol (96 per cent) R*.

To 0.10 mL of solution S2 in a test-tube add 0.05 mL of *1 M hydrochloric acid*, 0.5 mL of *potassium iodide solution R* and 5 mL of *ethanol (96 per cent) R*. Mix thoroughly and wait for 5 min. Add 9 mL of *water R* and 0.1 mL of a 5 g/L solution of *sodium sulfite heptahydrate R* and mix thoroughly. Add 1.5 mL of *dithizone solution R* freshly diluted 100-fold with *methylene chloride R*, shake for 15 s and allow to stand for 2 min. At the same time prepare a reference solution in the same manner using 0.1 mL of the tin standard solution.

Any violet colour in the lower layer obtained with solution S2 is not more intense than that obtained with the reference solution. The greenish-blue colour of dithizone solution turns pink in the presence of tin.

Non-tin-stabilised materials
Maximum 25 ppm of Sn.

To 5 mL of solution S2 in a test-tube add 0.05 mL of *1 M hydrochloric acid* and 0.5 mL of *potassium iodide solution R*. Mix thoroughly and wait for 5 min. Add 9 mL of *water R* and 0.1 mL of a 5 g/L solution of *sodium sulfite heptahydrate R* and mix thoroughly. If the solution obtained is not colourless, add the sodium sulfite solution in 0.05 mL fractions. Add 1.5 mL of *dithizone solution R* freshly diluted 100-fold with *methylene chloride R*, shake for 15 s and allow to stand for 2 min. At the same time prepare a reference solution in the same manner using 0.05 mL of the tin standard solution (see test above).

Any violet colour in the lower layer obtained with solution S2 is not more intense than that obtained with the reference solution.

Extractable heavy metals (*2.4.8*)
Maximum 20 ppm.

12 mL of solution S3 complies with test A. Prepare the reference solution using 10 mL of *lead standard solution (1 ppm Pb) R*.

Extractable zinc
Maximum 100 ppm.

Atomic absorption spectrometry (*2.2.23, Method I*).

Test solution Solution S3 diluted 10-fold with *water R*.

Reference solution A solution containing 0.50 ppm of zinc prepared by dilution of *zinc standard solution (5 mg/mL Zn) R* with *0.01 M hydrochloric acid*.

Verify the absence of zinc in the hydrochloric acid used.

Examined at 214.0 nm, the absorbance of the test solution is not greater than that of the reference solution.

Sulfated ash (*2.4.14*)
Maximum 1.0 per cent, determined on 1.0 g; maximum 4.0 per cent when the materials are opacified with titanium dioxide.

ASSAY
Carry out the oxygen-flask method (*2.5.10*) using 50.0 mg of the material to be examined. Absorb the combustion products in 20 mL of *1 M sodium hydroxide*. To the solution obtained add 2.5 mL of *nitric acid R*. Titrate with *0.1 M silver nitrate*, determining the end-point potentiometrically (*2.2.20*). Carry out a blank titration.

1 mL of *0.1 M silver nitrate* is equivalent to 6.25 mg of poly(vinyl chloride).

5. Materials Based on Plasticised Poly(Vinyl Chloride) for Containers for Aqueous Solutions for Intravenous Infusion

(Ph. Eur. method 3.1.14)

DEFINITION
Materials based on plasticised poly(vinyl chloride) contain not less than 55 per cent of poly(vinyl chloride) and contain various additives, in addition to the high-molecular-mass polymer obtained by polymerisation of vinyl chloride.

Materials based on plasticised poly(vinyl chloride) for containers for aqueous solutions for intravenous infusion are defined by the nature and the proportions of the substances used in their manufacture.

PRODUCTION
Materials based on plasticised poly(vinyl chloride) are produced by polymerisation methods that guarantee a residual vinyl chloride content of less than 1 ppm.

Vinyl chloride
Head-space gas chromatography (*2.2.28*).

Internal standard solution Using a microsyringe, inject 10 µL of *ether R* into 20.0 mL of *dimethylacetamide R*, immersing the tip of the needle in the solvent. Immediately before use, dilute the solution 1000-fold with *dimethylacetamide R*.

Test solution Place 1.000 g of the material to be examined in a 50 mL vial and add 10.0 mL of the internal standard solution. Close the vial and secure the stopper. Shake, avoiding contact between the stopper and the liquid. Place the vial in a water-bath at 60 ± 1 °C for 2 h.

Vinyl chloride primary solution *Prepare in a fume cupboard.* Place 50.0 mL of *dimethylacetamide R* in a 50 mL vial, stopper the vial, secure the stopper and weigh to the nearest 0.1 mg. Fill a 50 mL polyethylene or polypropylene syringe with gaseous *vinyl chloride R*, allow the gas to remain in contact with the syringe for about 3 min, empty the syringe and fill again with 50 mL of gaseous *vinyl chloride R*. Fit a hypodermic needle to the syringe and reduce the volume of gas in the syringe from 50 mL to 25 mL. Inject the remaining 25 mL of vinyl chloride slowly into the vial

shaking gently and avoiding contact between the liquid and the needle. Weigh the vial again; the increase in mass is about 60 mg (1 µL of the solution thus obtained contains about 1.2 µg of vinyl chloride). Allow to stand for 2 h. Keep the primary solution in a refrigerator.

Vinyl chloride standard solution Vinyl chloride primary solution, *dimethylacetamide R* (1:3 *V/V*).

Reference solutions Place 10.0 mL of the internal standard solution in each of six 50 mL vials. Close the vials and secure the stoppers. Inject 1 µL, 2 µL, 3 µL, 5 µL and 10 µL, respectively, of the vinyl chloride standard solution into 5 of the vials. The 6 solutions thus obtained contain, respectively, 0 µg, about 0.3 µg, 0.6 µg, 0.9 µg, 1.5 µg and 3 µg of vinyl chloride. Shake, avoiding contact between the stopper and the liquid. Place the vials in a water-bath at 60 ± 1 °C for 2 h.

Column:
- *material*: stainless steel;
- *size*: *l* = 3 m, Ø = 3 mm;
- *stationary phase*: *silanised diatomaceous earth for gas chromatography R* impregnated with 5 per cent *m/m* of *dimethylstearamide R* and 5 per cent *m/m* of *macrogol 400 R*.

Carrier gas *nitrogen for chromatography R*.

Flow rate 30 mL/min.

Temperature:
- *column*: 45 °C;
- *injection port*: 100 °C;
- *detector*: 150 °C.

Detection Flame ionisation.

Injection 1 mL of the head-space.

Limit:
- *vinyl chloride*: maximum 1 ppm.

Additives

Depending on the intended use of the polymers, they contain additives to optimise their processing or their chemical, physical and mechanical properties. Unless otherwise justified and authorised, these additives are chosen from the following list, which specifies for each substance the maximum permitted content:
- di(2-ethylhexyl) phthalate (plastic additive 01): maximum 40 per cent;
- zinc octanoate (zinc 2-ethylhexanoate) (plastic additive 02): maximum 1 per cent;
- calcium stearate or zinc stearate: maximum 1 per cent or 1 per cent of a mixture of the two;
- *N,N'*-diacylethylenediamines (plastic additive 03): maximum 1 per cent;
- maximum 10 per cent of one of the following epoxidised oils or 10 per cent of a mixture of the two:
  - epoxidised soya oil (plastic additive 04) of which the oxiran oxygen content is 6 per cent to 8 per cent and the iodine value is not greater than 6;
  - epoxidised linseed oil (plastic additive 05) of which the oxiran oxygen content is not greater than 10 per cent and the iodine value is not greater than 7.

When colouring materials are added, ultramarine blue (plastic additive 06) is used. Other colouring materials may be added, provided that the safety of the material is demonstrated to the satisfaction of the competent authority.

The supplier of the material must be able to demonstrate that the qualitative and quantitative composition of the type sample is satisfactory for each production batch.

CHARACTERS

Colourless, slightly blue or pale yellow powder, beads, granules or, after transformation, translucent sheets of varying thickness, with a slight odour. On combustion it gives off dense, black smoke.

IDENTIFICATION

If necessary, cut the samples of the material to be examined into pieces where the maximum size of a side is 1 cm.

To 2.0 g of the material to be examined add 200 mL of *peroxide-free ether R* and heat under a reflux condenser for 8 h. Separate the residue (residue B) and the solution (solution A) by filtration.

Evaporate solution A to dryness under reduced pressure in a water-bath at 30 °C. Dissolve residue in 10 mL of *toluene R* (solution A1). Dissolve residue B in 60 mL of *ethylene chloride R*, heating on a water-bath under a reflux condenser. Filter. Add the obtained solution dropwise and with vigorous shaking to 600 mL of *heptane R* heated almost to boiling. Separate by hot filtration the coagulum (coagulum B1) and the organic solution. Allow the latter to cool; separate the precipitate that forms (precipitate B2) and filter through a tared sintered-glass filter (40) (*2.1.2*).

A. Infrared absorption spectrophotometry (*2.2.24*).

Preparation Dissolve coagulum B1 in 30 mL of *tetrahydrofuran R* and add, in small volumes with shaking, 40 mL of *anhydrous ethanol R*; separate the precipitate (precipitate B3) by filtration and dry *in vacuo* at a temperature not exceeding 50 °C over *diphosphorus pentoxide R*; dissolve a few milligrams of precipitate B3 in 1 mL of *tetrahydrofuran R*, place a few drops of the solution obtained on a sodium chloride plate and evaporate to dryness in an oven at 100-105 °C.

Comparison *poly(vinyl chloride) CRS*.

B. Plastic additive 01 (see Tests).

TESTS

If necessary, cut the samples of the material to be examined into pieces where the maximum size of a side is 1 cm.

Solution S1

Place 5.0 g of the material to be examined in a combustion flask. Add 30 mL of *sulfuric acid R* and heat until a black, syrupy mass is obtained. Cool and add carefully 10 mL of *strong hydrogen peroxide solution R*. Heat gently. Allow to cool and add 1 mL of *strong hydrogen peroxide solution R*; repeat by alternating evaporation and addition of strong hydrogen peroxide solution until a colourless liquid is obtained. Reduce the volume to about 10 mL. Cool and dilute to 50.0 mL with *water R*.

Solution S2

Place 25 g of the material to be examined in a borosilicate-glass flask. Add 500 mL of *water R* and cover the neck of the flask with a borosilicate-glass beaker. Heat in an autoclave at 121 ± 2 °C for 20 min. Allow to cool, decant the solution and make up to 500 mL with *water R*.

Appearance of solution S2

Solution S2 is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

Acidity or alkalinity

To 100 mL of solution S2 add 0.15 mL of *BRP indicator solution R*. Not more than 1.5 mL of *0.01 M sodium hydroxide* is required to change the colour of the indicator to blue.

To 100 mL of solution S2 add 0.2 mL of *methyl orange solution R*. Not more than 1.0 mL of *0.01 M hydrochloric acid* is required to initiate the colour change of the indicator from yellow to orange.

Absorbance (*2.2.25*)
Evaporate 100.0 mL of solution S2 to dryness. Dissolve the residue in 5.0 mL of *hexane R*. From 250 nm to 310 nm the absorbance is not greater than 0.25.

Reducing substances
Carry out the test within 4 h of preparation of solution S2 To 20.0 mL of solution S2 add 1 mL of *dilute sulfuric acid R* and 20.0 mL of *0.002 M potassium permanganate*. Boil under a reflux condenser for 3 min and cool immediately. Add 1 g of *potassium iodide R* and titrate immediately with *0.01 M sodium thiosulfate*, using 0.25 mL of *starch solution R* as indicator. Carry out a blank titration using 20 mL of *water R*. The difference between the 2 titration volumes is not greater than 2.0 mL.

Primary aromatic amines
Maximum 20 ppm.

To 2.5 mL of solution A1 obtained during the identification, add 6 mL of *water R* and 4 mL of *0.1 M hydrochloric acid*. Shake vigorously and discard the upper layer. To the aqueous layer add 0.4 mL of a freshly prepared 10 g/L solution of *sodium nitrite R*. Mix and allow to stand for 1 min. Add 0.8 mL of a 25 g/L solution of *ammonium sulfamate R*, allow to stand for 1 min and add 2 mL of a 5 g/L solution of *naphthylethylenediamine dihydrochloride R*. After 30 min, any colour in the solution is not more intense than that in a standard prepared at the same time and in the same manner, replacing the aqueous layer with a mixture of 1 mL of a 0.01 g/L solution of *naphthylamine R* in *0.1 M hydrochloric acid*, 5 mL of *water R* and 4 mL of *0.1 M hydrochloric acid*.

Plastic additive 01
Gas chromatography (*2.2.28*) coupled with mass spectrometry (*2.2.43*).

Internal standard solution S3 1 mg/mL solution of *di-n-octyl phthalate R* in *tetrahydrofuran for chromatography R*.

Internal standard solution S4 5 µg/mL solution of *di-n-octyl phthalate R* in *anhydrous ethanol R*.

Test solution Cut 0.2 g of the material to be examined into pieces about 0.5 cm in length. Dissolve the pieces in 12.5 mL of internal standard solution S3 using a polytetrafluoroethylene magnetic stirring bar. Complete dissolution of the material to be examined is obtained after stirring for about 20-30 min. The poly(vinyl chloride) is precipitated as a white powder by adding dropwise 37.5 mL of *anhydrous ethanol R*. Centrifuge, then dilute 1.0 mL of the supernatant to 50.0 mL with *anhydrous ethanol R*. The final concentration of the internal standard in the test solution is 5 µg/mL.

The stock solution may be stored at 4 °C for up to 2 weeks.

Stock solution Dissolve 20.0 mg of *plastic additive 01 CRS* in internal standard solution S4 and dilute to 20.0 mL with the internal standard solution S4.

Reference solutions (a1)-(a5) Dilute the stock solution with internal standard solution S4 to obtain 5 reference solutions containing 10-40 µg/mL of *plastic additive 01 CRS*.

Column:
— *material*: fused silica;
— *size*: *l* = 30 m, Ø = 0.25 mm;
— *stationary phase*: *phenyl(5)methyl(95)polysiloxane R* (film thickness 0.25 µm).

Carrier gas *helium for chromatography R*.

Flow rate 1 mL/min.

Split ratio 1:20.

Temperature:

	Time (min)	Temperature (°C)
Column	0	100
	0 - 3.3	100 → 200
	3.3 - 20	200 → 250
	20 - 22.5	250
	22.5 - 23	250 → 270
	23 - 25	270
	25 - 25.6	270 → 320
	25.6 - 30.6	320
Injection port		300

Detection Mass spectrometer as described below; adjust the detector settings so as to comply with the system suitability criteria:
— quadrupole mass spectrometer equipped with an electron impact ionisation mode (70 eV);
— *ion source temperature*: 230 °C;
— *acquisition system*: performed on full-scan (*m/z* = 40-350) and on single-ion monitoring (SIM) modes;
— *solvent delay*: 2.5 min;
— mass spectrometer parameters for the fragmentometric mode (SIM) set as follows:

Substance	Ion 1 [*m/z*]	Ion 2 [*m/z*]	Ion 3 [*m/z*]
Plastic additive 01	149	167	279
DnOP (internal standard)	149	279	167

Injection 1 µL.

Relative retention With reference to di-*n*-octyl phthalate (retention time = about 22 min): plastic additive 01 = about 0.80.

The specificity of the detection is checked by monitoring 3 different ions for each substance using a mass spectrometer in SIM mode. Ion ratios are determined from the peak areas after the injection of a standard solution. The ratios in the table below are given for information.

Substance	Ion 1 [*m/z*]	Ion 2 [*m/z*]	Ion 3 [*m/z*]	Ion ratio 2/1 (%)	Ion ratio 3/1 (%)
Plastic additive 01	149	167	279	50	30
DnOP (internal standard)	149	279	167	/	/

System suitability:
— *repeatability*: maximum relative standard deviation of 1.0 per cent for the retention time of the peak due to plastic additive 01, determined on 6 injections of a reference solution situated in the middle of the calibration range (e.g. 20 µg/mL); maximum relative standard deviation of 3.0 per cent for the ratio of the area of the peak due to plastic additive 01 to that due to the internal standard, determined on 6 injections of a reference solution situated in the middle of the calibration range (e.g. 20 µg/mL).

From the calibration curve obtained with the reference solutions, calculate the percentage content of plastic additive 01 in the material to be examined.

Limit:
— *plastic additive 01*: maximum 40 per cent.

Plastic additive 03

Wash precipitate B2 obtained during the identification and contained in the tared sintered-glass filter (40) (*2.1.2*) with *anhydrous ethanol R*. Dry to constant mass over *diphosphorus pentoxide R* and weigh the filter. The residue weighs not more than 20 mg.

Infrared absorption spectrophotometry (*2.2.24*).

Preparation The residue obtained above. When the amount of residue is insufficient to prepare a disc, record the spectrum of the residue placed between 2 plates transparent to infrared radiation or examine by attenuated total reflectance (ATR).

Comparison *plastic additive 03 CRS.*

Plastic additives 04 and 05

Thin-layer chromatography (*2.2.27*).

Reference solutions Prepare 10 mg/mL solutions of *plastic additive 04 CRS* and *plastic additive 05 CRS*, respectively, in *toluene R*.

Plate *TLC silica gel F_{254} plate R.*

Mobile phase *toluene R.*

Application 0.5 mL of solution A1 obtained during the identification, as a band 30 mm by 3 mm, and 5 µL of each reference solution.

Development Over 2/3 of the plate.

Drying In air.

Detection Expose the plate to iodine vapour for 5 min.

Examine the chromatogram and locate the zone corresponding to plastic additives 04 and 05 ($R_F = 0$). Remove the area of silica gel corresponding to this zone. Similarly remove a corresponding area of silica gel as a blank reference. Separately shake both samples for 15 min with 40 mL of *methanol R*. Filter, rinse with 2 quantities, each of 10 mL, of *methanol R*, add the rinsings to the filtrate and evaporate to dryness. The difference between the masses of both residues is not more than 10 mg.

Barium

Maximum 5 ppm.

Inductively coupled plasma-atomic emission spectrometry (*2.2.57*).

Test solution Ignite 1.0 g of the material to be examined in a silica crucible. Take up the residue with 10 mL of *hydrochloric acid R* and evaporate to dryness on a water-bath. Take up the residue with 20 mL of *0.1 M hydrochloric acid.*

Reference solution A solution containing 0.25 ppm of barium prepared by dilution of *barium standard solution (50 ppm Ba) R* with *0.1 M hydrochloric acid.*

Wavelength Use the emission of barium at 455.40 nm, the spectral background being taken at 455.30 nm.

Verify the absence of barium in the hydrochloric acid used.

Cadmium

Maximum 0.6 ppm.

Atomic absorption spectrometry (*2.2.23, Method I*).

Test solution Evaporate 10 mL of solution S1 to dryness. Take up the residue using 5 mL of a 1 per cent *V/V* solution of *hydrochloric acid R*, filter and dilute the filtrate to 10.0 mL with the same acid solution.

Reference solutions Prepare the reference solutions using *cadmium standard solution (0.1 per cent Cd) R*, diluting with a 1 per cent *V/V* solution of *hydrochloric acid R*.

Source Cadmium hollow-cathode lamp.

Wavelength 228.8 nm.

Atomisation device Air-acetylene flame.

Verify the absence of cadmium in the hydrochloric acid used.

Calcium

Maximum 0.07 per cent.

Inductively coupled plasma-atomic emission spectrometry (*2.2.57*).

Test solution Use the test solution prepared for the determination of barium.

Reference solution A solution containing 50.0 ppm of calcium prepared by dilution of *calcium standard solution (400 ppm Ca) R* with *0.1 M hydrochloric acid.*

Wavelength Use the emission of calcium at 315.89 nm, the spectral background being taken at 315.60 nm.

Verify the absence of calcium in the hydrochloric acid used.

Tin

Maximum 20 ppm.

Inductively coupled plasma-atomic emission spectrometry (*2.2.57*).

Test solution Dilute solution S1 10-fold with *water R* immediately before use.

Reference solution Introduce 2 mL of *tin standard solution (5 ppm Sn) R* into a 50 mL flask containing 5 mL of a 20 per cent *V/V* solution of *sulfuric acid R* and dilute to 50 mL with *water R* immediately before use.

Wavelength Use the emission of tin at 189.99 nm, the spectral background being taken at 190.10 nm.

Verify the absence of tin in the hydrochloric acid used.

Zinc

Maximum 0.2 per cent.

Atomic absorption spectrometry (*2.2.23, Method I*).

Test solution Dilute solution S1 100-fold with *0.1 M hydrochloric acid.*

Reference solutions Prepare the reference solutions using *zinc standard solution (100 ppm Zn) R*, diluting with *0.1 M hydrochloric acid.*

Source Zinc hollow-cathode lamp.

Wavelength 213.9 nm.

Atomisation device Air-acetylene flame.

Verify the absence of zinc in the hydrochloric acid used.

Heavy metals (*2.4.8*)

Maximum 50 ppm.

To 10 mL of solution S1 add 0.5 mL of *phenolphthalein solution R* and then *strong sodium hydroxide solution R* until a pale pink colour is obtained. Dilute to 25 mL with *water R*. 12 mL of the solution complies with test A. Prepare the reference solution using *lead standard solution (2 ppm Pb) R.*

Water extractable substances

Maximum 0.3 per cent.

Evaporate 50.0 mL of solution S2 to dryness on a water-bath and dry in an oven at 100-105 °C to constant mass. Carry out a blank test with 50.0 mL of *water R*. The residue weighs not more than 7.5 mg taking into account the blank test.

ASSAY

Carry out the oxygen-flask method (*2.5.10*) using 50.0 mg of the material to be examined. Absorb the combustion products in 20 mL of *1 M sodium hydroxide*. To the solution obtained add 2.5 mL of *nitric acid R*. Titrate with *0.1 M silver nitrate*, determining the end-point potentiometrically (*2.2.20*). Carry out a blank titration.

1 mL of *0.1 M silver nitrate* is equivalent to 6.25 mg of poly(vinyl chloride).

B. Polyolefins

(Ph. Eur. method 3.1.3)

DEFINITION

Polyolefins are obtained by polymerisation of ethylene or propylene or by copolymerisation of these substances with not more than 25 per cent of higher homologues (C_4 to C_{10}) or of carboxylic acids or of esters. Certain materials may be mixtures of polyolefins.

PRODUCTION

Depending on the intended use of the polymers, they may contain additives to optimise their processing or their chemical, physical and mechanical properties. Unless otherwise justified and authorised, these additives are chosen from the following list, which specifies for each substance the maximum permitted content.

Polyolefins may contain at most 3 antioxidants, 1 or several lubricants or antiblocking agents as well as titanium dioxide as an opacifying agent when the material must provide protection from light.

- butylhydroxytoluene (plastic additive 07): maximum 0.125 per cent;
- pentaerythrityl tetrakis[3-(3,5-di-*tert*-butyl-4-hydroxyphenyl)propionate] (plastic additive 09): maximum 0.3 per cent;
- 1,3,5-tris(3,5-di-*tert*-butyl-4-hydroxybenzyl)-*S*-triazine-2,4,6(1*H*,3*H*,5*H*)-trione (plastic additive 13): maximum 0.3 per cent;
- octadecyl 3-(3,5-di-*tert*-butyl-4-hydroxyphenyl)propionate (plastic additive 11): maximum 0.3 per cent;
- ethylene bis[3,3-bis[3-(1,1-dimethylethyl)-4-hydroxyphenyl]butanoate] (plastic additive 08): maximum 0.3 per cent;
- dioctadecyl disulfide (plastic additive 15): maximum 0.3 per cent;
- 4,4′,4″-(2,4,6-trimethylbenzene-1,3,5-triyltrismethylene) tris[2,6-bis(1,1-dimethylethyl)phenol] (plastic additive 10): maximum 0.3 per cent;
- 2,2′-bis(octadecyloxy)-5,5′-spirobi[1,3,2-dioxaphosphinane] (plastic additive 14): maximum 0.3 per cent;
- didodecyl 3,3′-thiodipropionate (plastic additive 16): maximum 0.3 per cent;
- dioctadecyl 3,3′-thiodipropionate (plastic additive 17): maximum 0.3 per cent;
- tris[2,4-bis(1,1-dimethylethyl)phenyl] phosphite (plastic additive 12): maximum 0.3 per cent;
- plastic additive 18: maximum 0.1 per cent;
- copolymer of dimethyl succinate and (4-hydroxy-2,2,6,6-tetramethylpiperidin-1-yl)ethanol (plastic additive 22): maximum 0.3 per cent.

The total of antioxidant additives listed above does not exceed 0.3 per cent.

- hydrotalcite: maximum 0.5 per cent;
- alkanamides: maximum 0.5 per cent;
- alkenamides: maximum 0.5 per cent;
- sodium silico-aluminate: maximum 0.5 per cent;
- silica (natural or synthetic, coated or uncoated): maximum 0.5 per cent;
- sodium benzoate: maximum 0.5 per cent;
- fatty acid esters or salts: maximum 0.5 per cent;
- trisodium phosphate: maximum 0.5 per cent;
- liquid paraffin: maximum 0.5 per cent;
- zinc oxide: maximum 0.5 per cent;
- talc: maximum 0.5 per cent;
- magnesium oxide: maximum 0.2 per cent;
- calcium stearate or zinc stearate or a mixture of both: maximum 0.5 per cent;
- titanium dioxide: maximum 4 per cent.

The supplier of the material must be able to demonstrate that the qualitative and quantitative composition of the type sample (see chapter *3.2.2. Plastic containers and closures for pharmaceutical use* for the definition of 'type sample') is satisfactory for each production batch.

CHARACTERS

Appearance

Powder, beads, granules or, after transformation, sheets of varying thickness or containers.

Solubility

Practically insoluble in water, soluble in hot aromatic hydrocarbons, practically insoluble in anhydrous ethanol, in hexane and in methanol.

They soften at temperatures between 65 °C and 165 °C. They burn with a blue flame.

IDENTIFICATION

If necessary, cut the samples of the material to be examined into pieces of maximum dimension on a side of not greater than 1 cm.

A. Infrared absorption spectrophotometry (*2.2.24*).

Preparation To 0.25 g add 10 mL of *toluene R* and boil under a reflux condenser for about 15 min. Place a few drops of the solution on a sodium chloride slide or on a disc of *potassium bromide R* and evaporate the solvent in an oven at 80 °C.

Alternatively, the spectrum may be recorded directly on a cut piece of suitable size (sheets), granules or hot pressed films by attenuated total reflection (ATR).

The spectrum obtained is identical to that obtained with the material selected for the type sample.

B. It complies with the supplementary tests corresponding to the additives present (see Tests).

C. *(To be performed only on opacified material.)* In a platinum crucible, mix about 20 mg with 1 g of *potassium hydrogen sulfate R* and heat until completely melted. Allow to cool and add 20 mL of *dilute sulfuric acid R*. Heat gently. Filter the resulting solution. To the filtrate add 1 mL of *phosphoric acid R* and 1 mL of *strong hydrogen peroxide solution R*. If the substance is opacified with titanium dioxide, an orange-yellow colour develops.

TESTS

If necessary, cut the samples of the material to be examined into pieces of maximum dimension on a side of not greater than 1 cm.

Solution S1

Place 25 g in a borosilicate-glass flask with a ground-glass neck. Add 500 mL of *water R* and boil under a reflux condenser for 5 h. Allow to cool and decant. Reserve a portion of the solution for the test for appearance of solution and filter the rest through a sintered-glass filter (16) (*2.1.2*). Use within 4 h of preparation.

Solution S2

Place 2.0 g in a conical borosilicate-glass flask with a ground-glass neck. Add 80 mL of *toluene R* and boil under a reflux condenser, for 90 min with constant stirring. Allow to cool to 60 °C and add, with constant stirring, 120 mL of *methanol R*. Filter the solution through a sintered-glass filter (16) (*2.1.2*). Rinse the flask and the filter with 25 mL of a mixture of 40 mL of *toluene R* and 60 mL of *methanol R*, add the rinsings to the filtrate and dilute to 250 mL with the same mixture of solvents. Prepare a blank solution.

Solution S3
Place 100 g in a conical borosilicate-glass flask with a ground-glass neck. Add 250 mL of *0.1 M hydrochloric acid* and boil under a reflux condenser for 1 h with constant stirring. Allow to cool and decant the solution.

Appearance of solution
Solution S1 is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

Acidity or alkalinity
To 100 mL of solution S1 add 0.15 mL of *BRP indicator solution R*. Not more than 1.5 mL of *0.01 M sodium hydroxide* is required to change the colour of the indicator to blue.
To 100 mL of solution S1 add 0.2 mL of *methyl orange solution R*. Not more than 1.0 mL of *0.01 M hydrochloric acid* is required to initiate the colour change of the indicator from yellow to orange.

Absorbance (*2.2.25*)
Maximum 0.2, determined between wavelengths of 220 nm and 340 nm on solution S1.

Reducing substances
To 20 mL of solution S1 add 1 mL of *dilute sulfuric acid R* and 20 mL of *0.002 M potassium permanganate*. Boil under a reflux condenser for 3 min and cool immediately. Add 1 g of *potassium iodide R* and titrate immediately with *0.01 M sodium thiosulfate*, using 0.25 mL of *starch solution R* as indicator. Carry out a blank titration. The difference between the titration volumes is not more than 3.0 mL.

Extractable aluminium
Maximum 1 ppm.

Inductively coupled plasma-atomic emission spectrometry (*2.2.57*).

Test solution Use solution S3.

Reference solutions Prepare the reference solutions using *aluminium standard solution (200 ppm Al) R*, diluting with *0.1 M hydrochloric acid*.

Wavelength Use the emission of aluminium at 396.15 nm, the spectral background being taken as 396.25 nm.

Verify the absence of aluminium in the hydrochloric acid used.

Extractable titanium *(not for materials opacified with titanium dioxide)*
Maximum 1 ppm.

Inductively coupled plasma-atomic emission spectrometry (*2.2.57*).

Test solution Use solution S3.

Reference solutions Prepare the reference solutions using *titanium standard solution (100 ppm Ti) R*, diluting with *0.1 M hydrochloric acid*.

Wavelength Use the emission of titanium at 336.12 nm, the spectral background being taken as 336.16 nm.

Verify the absence of titanium in the hydrochloric acid used.

Extractable zinc
Maximum 1 ppm.

Atomic absorption spectrometry (*2.2.23, Method I*).

Test solution Use solution S3.

Reference solutions Prepare the reference solutions using *zinc standard solution (10 ppm Zn) R*, diluting with *0.1 M hydrochloric acid*.

Source Zinc hollow-cathode lamp.

Wavelength 213.9 nm.

Atomisation device Air-acetylene flame.

Verify the absence of zinc in the hydrochloric acid used.

Extractable heavy metals (*2.4.8*)
Maximum 2.5 ppm.

Evaporate 50 mL of solution S3 to about 5 mL on a water-bath and dilute to 20.0 mL with *water R*. 12 mL of the solution complies with test A. Prepare the reference solution using 2.5 mL of *lead standard solution (10 ppm Pb) R*.

Sulfated ash (*2.4.14*)
Maximum 1.0 per cent, determined on 5.0 g. This limit does not apply to material that has been opacified with titanium dioxide.

SUPPLEMENTARY TESTS
These tests are to be carried out, in whole or in part, only if required by the stated composition or the use of the material.

Phenolic antioxidants
Liquid chromatography (*2.2.29*).

Solvent mixture *acetonitrile R, tetrahydrofuran R* (50:50 *V/V*).

Test solution S21 Evaporate 50 mL of solution S2 to dryness *in vacuo* at 45 °C. Dissolve the residue in 5.0 mL of the solvent mixture. Prepare a blank solution from the blank solution corresponding to solution S2.

Test solution S22 Evaporate 50 mL of solution S2 to dryness *in vacuo* at 45 °C. Dissolve the residue in 5.0 mL of a mixture of equal volumes of *acetonitrile R* and a 10 g/L solution of *tert-butylhydroperoxide R* in *tetrahydrofuran R*. Close the flask and allow to stand for 1 h. Prepare a blank solution using the blank of solution S2.

Of the following reference solutions, prepare only those that are necessary for the analysis of the phenolic antioxidants stated in the composition of the substance to be examined.

Reference solution (a) Dissolve 25.0 mg of *butylhydroxytoluene CRS* (plastic additive 07) and 60.0 mg of *plastic additive 08 CRS* in 10.0 mL of the solvent mixture. Dilute 2.0 mL of this solution to 50.0 mL with the solvent mixture.

Reference solution (b) Dissolve 60.0 mg of *plastic additive 09 CRS* and 60.0 mg of *plastic additive 10 CRS* in 10.0 mL of the solvent mixture. Dilute 2.0 mL of this solution to 50.0 mL with the solvent mixture.

Reference solution (c) *Prepare immediately before use.* Dissolve 60.0 mg of *plastic additive 11 CRS* and 60.0 mg of *plastic additive 12 CRS* in 10.0 mL of the solvent mixture. Dilute 2.0 mL of this solution to 50.0 mL with the solvent mixture.

Reference solution (d) Dissolve 25.0 mg of *butylhydroxytoluene CRS* (plastic additive 07) in 10.0 mL of the solvent mixture. Dilute 2.0 mL of this solution to 50.0 mL with the solvent mixture.

Reference solution (e) Dissolve 60.0 mg of *plastic additive 08 CRS* in 10.0 mL of the solvent mixture. Dilute 2.0 mL of this solution to 50.0 mL with the solvent mixture.

Reference solution (f) Dissolve 60.0 mg of *plastic additive 13 CRS* in 10.0 mL of the solvent mixture. Dilute 2.0 mL of this solution to 50.0 mL with the solvent mixture.

Reference solution (g) Dissolve 60.0 mg of *plastic additive 09 CRS* in 10.0 mL of the solvent mixture. Dilute 2.0 mL of this solution to 50.0 mL with the solvent mixture.

Reference solution (h) Dissolve 60.0 mg of *plastic additive 10 CRS* in 10.0 mL of the solvent mixture. Dilute 2.0 mL of this solution to 50.0 mL with the solvent mixture.

Reference solution (i) Dissolve 60.0 mg of *plastic additive 11 CRS* in 10.0 mL of the solvent mixture. Dilute 2.0 mL of this solution to 50.0 mL with the solvent mixture.

Reference solution (j) *Prepare immediately before use.* Dissolve 60.0 mg of *plastic additive 12 CRS* in 10.0 mL of the solvent mixture. Dilute 2.0 mL of this solution to 50.0 mL with the solvent mixture.

Reference solution (k) Dissolve 20.0 mg of *plastic additive 18 CRS* in 10.0 mL of a mixture of equal volumes of *acetonitrile R* and a 10 g/L solution of *tert-butylhydroperoxide R* in *tetrahydrofuran R*. Allow to stand in a closed container for 1 h. Dilute 2.0 mL of this solution to 50.0 mL with the solvent mixture.

A. If the substance to be examined contains plastic additive 07 and/or plastic additive 08, proceed as follows.

Column:
- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

Mobile phase *water for chromatography R, acetonitrile R* (30:70 *V/V*).

Flow rate 2 mL/min.

Detection Spectrophotometer at 280 nm.

Injection 20 µL of test solution S21, the corresponding blank solution, reference solution (a), and either the reference solution (d) or (e) or reference solutions (d) and (e).

Run time 30 min.

System suitability:
- *resolution*: minimum 5.0 between the peaks due to plastic additive 07 and plastic additive 08 in the chromatogram obtained with reference solution (a).

The chromatogram obtained with test solution S21 only shows peaks due to antioxidants stated in the composition and minor peaks that also appear in the chromatogram obtained with the blank solution.

Calculation of percentage contents:

For plastic additive 07 and/or 08, use the concentration of the corresponding reference substance in reference solutions (d) and/or (e).

Limits:
- *plastic additive 07*: maximum 0.125 per cent;
- *plastic additive 08*: maximum 0.3 per cent.

B. If the substance to be examined contains one or more of the following antioxidants:
- plastic additive 09;
- plastic additive 10;
- plastic additive 11;
- plastic additive 12;
- plastic additive 13;

proceed as described above with the following modifications.

Mobile phase *water for chromatography R, tetrahydrofuran R, acetonitrile R* (10:30:60 *V/V/V*).

Flow rate 1.5 mL/min.

Injection 20 µL of test solution S21, the corresponding blank solution, reference solution (b), reference solution (c) and the reference solutions of the antioxidants in the list above that are stated in the composition.

System suitability:
- *resolution*: minimum 2.0 between the peaks due to plastic additive 09 and plastic additive 10 in the chromatogram obtained with reference solution (b); minimum 2.0 between the peaks due to plastic additive 11 and plastic additive 12 in the chromatogram obtained with reference solution (c).

The chromatogram obtained with test solution S21 only shows peaks due to antioxidants stated in the composition and minor peaks that also appear in the chromatogram obtained with the blank solution.

Calculation of percentage contents:

For plastic additive 09, 10, 11, 12 and/or 13, use the concentration of the corresponding reference substance in reference solutions (g), (h), (i), (j) and/or (f).

Limits:
- *plastic additive 09*: maximum 0.3 per cent;
- *plastic additive 10*: maximum 0.3 per cent;
- *plastic additive 11*: maximum 0.3 per cent;
- *plastic additive 12*: maximum 0.3 per cent;
- *plastic additive 13*: maximum 0.3 per cent.

C. If the substance to be examined contains plastic additive 18, proceed as described for plastic additive 07 and/or plastic additive 08 with the following modifications.

Mobile phase *tetrahydrofuran R, acetonitrile R* (20:80 *V/V*).

Flow rate 1.5 mL/min.

Detection Spectrophotometer at 270 nm.

Injection 20 µL of test solution S22, the corresponding blank solution and reference solution (k).

Identification of peaks Use the chromatogram supplied with *plastic additive 18 CRS* and the chromatogram obtained with reference solution (k) to identify the peaks due to plastic additive 18.

Retention time Of the 2 principal peaks due to plastic additive 18: about 3.3 min and about 6.6 min.

The chromatogram obtained with test solution S22 shows 2 principal peaks due to plastic additive 18. The sum of the areas of these peaks is not less than 50 per cent of the sum of the areas of all the peaks due to plastic additive 18.

System suitability:
- *resolution*: minimum 6.0 between the 2 principal peaks in the chromatogram obtained with reference solution (k).

The chromatogram obtained with test solution S22 only shows peaks due to antioxidants stated in the composition and minor peaks that also appear in the chromatogram obtained with the blank solution.

Calculation of percentage content:
- sum up the areas of all the peaks due to plastic additive 18 eluting between 2.0 min and 9.5 min; use the concentration of *plastic additive 18 CRS* in reference solution (k).

Limit:
- *sum of the areas of the peaks due to plastic additive 18*: maximum 0.1 per cent; disregard any peak with an area less than 0.3 per cent of the total area.

Non-phenolic antioxidants

Thin-layer chromatography (*2.2.27*).

Test solution S23 Evaporate 100 mL of solution S2 to dryness *in vacuo* at 45 °C. Dissolve the residue in 2 mL of *acidified methylene chloride R*.

Reference solution (l) Dissolve 60 mg of *plastic additive 14 CRS* in 10 mL of *methylene chloride R*. Dilute 2 mL of this solution to 10 mL with *acidified methylene chloride R*.

Reference solution (m) Dissolve 60 mg of *plastic additive 15 CRS* in 10 mL of *methylene chloride R*. Dilute 2 mL of this solution to 10 mL with *acidified methylene chloride R*.

Reference solution (n) Dissolve 60 mg of *plastic additive 16 CRS* in 10 mL of *methylene chloride R*. Dilute 2 mL of this solution to 10 mL with *acidified methylene chloride R*.

Reference solution (o) Dissolve 60 mg of *plastic additive 17 CRS* in 10 mL of *methylene chloride R*. Dilute 2 mL of this solution to 10 mL with *acidified methylene chloride R*.

Reference solution (p) Dissolve 60 mg of *plastic additive 16 CRS* and 60 mg of *plastic additive 17 CRS* in 10 mL of *methylene chloride R*. Dilute 2 mL of this solution to 10 mL with *acidified methylene chloride R*.

Plate *TLC silica gel F_{254} plate R*.

Mobile phase A *hexane R*.

Mobile phase B *methylene chloride R*.

Application 20 µL of test solution S23, reference solution (p) and the reference solutions corresponding to all the phenolic and non-phenolic antioxidants mentioned in the type composition of the material to be examined.

Development A Over a path of 18 cm with mobile phase A.

Drying A In air.

Development B Over a path of 17 cm with mobile phase B.

Drying B In air.

Detection Examine in ultraviolet light at 254 nm; spray with *alcoholic iodine solution R* and examine in ultraviolet light at 254 nm after 10-15 min.

System suitability Reference solution (p):
— the chromatogram shows 2 clearly separated spots.

Limit Any spots in the chromatogram obtained with test solution S23 are not more intense than the spots in the corresponding positions in the chromatograms obtained with the reference solutions.

Plastic additive 22

Liquid chromatography (*2.2.29*).

Test solution Evaporate 25 mL of solution S2 to dryness *in vacuo* at 45 °C. Dissolve the residue in 10 mL of *toluene R* and 10 mL of a 10 g/L solution of *tetrabutylammonium hydroxide R* in a mixture of 35 volumes of *toluene R* and 65 volumes of *anhydrous ethanol R*. Boil under a reflux condenser for 3 h. Allow to cool and filter if necessary.

Reference solution Dissolve 30 mg of *plastic additive 22 CRS* in 50 mL of *toluene R*. Add 1 mL of this solution to 25 mL of blank solution S2 and evaporate to dryness *in vacuo* at 45 °C. Dissolve the residue in 10 mL of *toluene R* and 10 mL of a 10 g/L solution of *tetrabutylammonium hydroxide R* in a mixture of 35 volumes of *toluene R* and 65 volumes of *anhydrous ethanol R*. Boil under a reflux condenser for 3 h. Allow to cool and filter if necessary.

Column:
— *size*: l = 0.25 m, Ø = 4.6 mm;
— *stationary phase*: *aminopropylsilyl silica gel for chromatography R* (5 µm).

Mobile phase *anhydrous ethanol R, hexane R* (11:89 *V/V*).

Flow rate 2 mL/min.

Detection Spectrophotometer at 227 nm.

Injection 20 µL.

Run time 10 min.

System suitability:
— *resolution*: minimum 7 between the peaks due to the 'diol' component and to the diluent of the reference solution.

Limit The area of the peak due to the 'diol' component from plastic additive 22 in the chromatogram obtained with the test solution is less than the corresponding peak in the chromatogram obtained with the reference solution.

Amides and stearates

Thin-layer chromatography (*2.2.27*).

Test solution Use test solution S23 described in the test for non-phenolic antioxidants.

Reference solution (q) Dissolve 20 mg of *stearic acid CRS* (plastic additive 19) in 10 mL of *methylene chloride R*.

Reference solution (r) Dissolve 40 mg of oleamide (*plastic additive 20 CRS*) in 20 mL of *methylene chloride R*.

Reference solution (s) Dissolve 40 mg of erucamide (*plastic additive 21 CRS*) in 20 mL of *methylene chloride R*.

Plate *TLC silica gel F_{254} plate R* (2 plates).

A. *Mobile phase*: *anhydrous ethanol R, trimethylpentane R* (25:75 *V/V*).

Application 10 µL of test solution S23 and reference solution (q).

Development Over a path of 10 cm.

Drying In air.

Detection Spray with a 2 g/L solution of *dichlorophenolindophenol, sodium salt R* in *anhydrous ethanol R* and heat in an oven at 120 °C for a few minutes to intensify the spots.

Limit Any spot corresponding to plastic additive 19 in the chromatogram obtained with test solution S23 is similar in position (R_F = about 0.5) but not more intense than the corresponding spot in the chromatogram obtained with reference solution (q).

B. *Mobile phase A*: *hexane R*.

Mobile phase B *methanol R, methylene chloride R* (5:95 *V/V*).

Application 10 µL of the test solution S23 and the reference solutions (r) and (s).

Development A Over a path of 13 cm with mobile phase A.

Drying A In air.

Development B Over a path of 10 cm with mobile phase B.

Drying B In air.

Detection Spray with a 40 g/L solution of *phosphomolybdic acid R* in *anhydrous ethanol R*. Heat in an oven at 120 °C until spots appear.

Limits Any spots corresponding to plastic additive 20 or plastic additive 21 in the chromatogram obtained with test solution S23 are similar in position (R_F = about 0.2) but not more intense than the corresponding spots in the chromatograms obtained with reference solutions (r) and (s).

C. Polyethylene

1. Polyethylene Without Additives for Containers for Parenteral Preparations and for Ophthalmic Preparations

(Ph. Eur. method 3.1.4)

DEFINITION

Polyethylene without additives is obtained by polymerisation of ethylene under high pressure in the presence of oxygen or free-radical-forming initiators as catalyst.

CHARACTERS

Appearance

Beads, granules, powder or, after transformation, translucent sheets of varying thickness or containers.

Solubility

Practically insoluble in water, soluble in hot aromatic hydrocarbons, practically insoluble in anhydrous ethanol, in hexane and in methanol.

It softens at temperatures beginning at 65 °C.

Relative density

0.910 to 0.937.

IDENTIFICATION

If necessary, cut the samples of the material to be examined into pieces of maximum dimension on a side of not greater than 1 cm.

A. Infrared absorption spectrophotometry (*2.2.24*).

Preparation To 0.25 g add 10 mL of *toluene R* and boil under a reflux condenser for about 15 min. Place a few drops of the solution on a sodium chloride slide or on a disc of *potassium bromide R* and evaporate the solvent in an oven at 80 °C.

Alternatively, the spectrum may be recorded directly on a cut piece of suitable size (sheets), granules or hot pressed films by attenuated total reflection (ATR).

Absorption maxima *at some of the following wavenumbers (tolerance: ± 5 cm^{-1})*: at 2915 cm^{-1}, 2848 cm^{-1}, 1471 cm^{-1}, 1465 cm^{-1}, 729 cm^{-1} and 719 cm^{-1}.

The spectrum obtained is identical to that obtained with the material selected for the type sample (see chapter *3.2.2. Plastic containers and closures for pharmaceutical use* for the definition of 'type sample').

B. Additives (see Tests).

TESTS

If necessary, cut the samples of the material to be examined into pieces of maximum dimension on a side of not greater than 1 cm.

Solution S1

Place 25 g in a borosilicate-glass flask with a ground-glass neck. Add 500 mL of *water R* and heat under a reflux condenser for 5 h. Allow to cool and decant. Keep part of the solution for the test for appearance of solution. Filter the rest through a sintered glass filter (16) (*2.1.2*). Use within 4 h of preparation.

Solution S2

Place 2.0 g in a conical borosilicate-glass flask with a ground-glass neck. Add 80 mL of *toluene R* and boil under a reflux condenser with constant stirring for 90 min. Allow to cool to 60 °C and add, with contant stirring, 120 mL of *methanol R*. Filter the solution through a sintered-glass filter (16) (*2.1.2*). Rinse the flask and the filter with 25 mL of a mixture of 40 mL of *toluene R* and 60 mL of *methanol R*, add the rinsings to the filtrate and dilute to 250 mL with the same mixture of solvents. Prepare a blank solution.

Solution S3

Place 100 g in a conical borosilicate-glass flask with a ground-glass neck. Add 250 mL of *0.1 M hydrochloric acid* and boil under a reflux condenser with constant stirring for 1 h. Allow to cool and decant the solution.

Appearance of solution

Solution S1 is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

Acidity or alkalinity

To 100 mL of solution S1 add 0.15 mL of *BRP indicator solution R*. Not more than 1.5 mL of *0.01 M sodium hydroxide* is required to change the colour of the indicator to blue.

To 100 mL of solution S1 add 0.2 mL of *methyl orange solution R*. Not more than 1.0 mL of *0.01 M hydrochloric acid* is required to reach the beginning of the colour change of the indicator from yellow to orange.

Absorbance (*2.2.25*)

Maximum 0.2, determined between wavelengths of 220 nm and 340 nm on solution S1.

Reducing substances

To 20 mL of solution S1 add 1 mL of *dilute sulfuric acid R* and 20 mL of *0.002 M potassium permanganate*. Boil under a reflux condenser for 3 min and cool immediately. Add 1 g of *potassium iodide R* and titrate immediately with *0.01 M sodium thiosulfate*, using 0.25 mL of *starch solution R* as indicator. Carry out a blank titration. The difference between the titration volumes is not more than 0.5 mL.

Additives

Thin-layer chromatography (*2.2.27*).

Test solution Evaporate 50 mL of solution S2 to dryness *in vacuo* at 45 °C. Dissolve the residue in 5 mL of *methylene chloride R*. Prepare a blank solution from the blank solution corresponding to solution S2.

Reference solution Dissolve 20 mg of *plastic additive 15 CRS* and 20 mg of *plastic additive 08 CRS* in *methylene chloride R* and dilute to 10 mL with the same solvent.

Plate *TLC silica gel G plate R.*

Mobile phase A *hexane R.*

Mobile phase B *methanol R, methylene chloride R* (5:95 *V/V*).

Application 10 µL.

Development A Over a path of 13 cm with mobile phase A.

Drying A In air.

Development B Over a path of 10 cm with mobile phase B.

Drying B In air.

Detection Spray with a 40 g/L solution of *phosphomolybdic acid R* in *ethanol (96 per cent) R* and heat at 120 °C until the spots appear in the chromatogram obtained with the reference solution.

System suitability Reference solution:

— the chromatogram shows 2 separated spots.

Limit No spot appears in the chromatogram obtained with the test solution, except for a spot which may be at the solvent front from the first development and which corresponds to oligomers. Disregard any spots corresponding to those obtained in the chromatogram with the blank solution.

Extractable heavy metals (*2.4.8*)

Maximum 2.5 ppm.

Evaporate 50 mL of solution S3 to about 5 mL on a water-bath and dilute to 20 mL with *water R*. 12 mL of solution complies with test A. Prepare the reference solution using 2.5 mL of *lead standard solution (10 ppm Pb) R*.

Sulfated ash (*2.4.14*)
Maximum 0.02 per cent, determined on 5.0 g.

2. Polyethylene With Additives for Containers for Parenteral Preparations and for Opthalmic Preparations

(Ph. Eur. method 3.1.5)

DEFINITION

Polyethylene with additives is obtained by polymerisation of ethylene under pressure in the presence of a catalyst or by copolymerisation of ethylene with not more than 25 per cent of higher alkene homologues (C_3 to C_{10}).

PRODUCTION

Depending on the intended use of the polymers, they may contain additives to optimise their processing or their chemical, physical and mechanical properties. Unless otherwise justified and authorised, these additives are chosen from the following list, which specifies for each substance the maximum permitted content.

Polyethylene may contain at most 3 antioxidants, 1 or several lubricants or antiblocking agents as well as titanium dioxide as an opacifying agent when the material must provide protection from light.

- butylhydroxytoluene (plastic additive 07): maximum 0.125 per cent;
- pentaerythrityl tetrakis[3-(3,5-di-*tert*-butyl-4-hydroxyphenyl)propionate] (plastic additive 09): maximum 0.3 per cent;
- 1,3,5-tris(3,5-di-*tert*-butyl-4-hydroxybenzyl)-*S*-triazine2,4,6(1*H*,3*H*,5*H*)-trione (plastic additive 13): maximum 0.3 per cent;
- octadecyl 3-(3,5-di-*tert*-butyl-4-hydroxyphenyl)propionate (plastic additive 11): maximum 0.3 per cent;
- ethylene bis[3,3-bis[3-(1,1-dimethylethyl)-4-hydroxyphenyl]butanoate] (plastic additive 08): maximum 0.3 per cent;
- dioctadecyl disulfide (plastic additive 15): maximum 0.3 per cent;
- 4,4′,4″-(2,4,6-trimethylbenzene-1,3,5-triyltrismethylene) tris[2,6-bis(1,1-dimethylethyl)phenol] (plastic additive 10): maximum 0.3 per cent;
- 2,2′-bis(octadecyloxy)-5,5′-spirobi[1,3,2-dioxaphosphinane] (plastic additive 14): maximum 0.3 per cent;
- didodecyl 3,3′-thiodipropionate (plastic additive 16): maximum 0.3 per cent;
- dioctadecyl 3,3′-thiodipropionate (plastic additive 17): maximum 0.3 per cent;
- tris [2,4-bis(1,1-dimethylethyl)phenyl] phosphite (plastic additive 12): maximum 0.3 per cent.

The total of antioxidant additives listed above does not exceed 0.3 per cent.

- hydrotalcite: maximum 0.5 per cent;
- alkanamides: maximum 0.5 per cent;
- alkenamides: maximum 0.5 per cent;
- sodium silico-aluminate: maximum 0.5 per cent;
- silica (natural or synthetic, coated or uncoated): maximum 0.5 per cent;
- sodium benzoate: maximum 0.5 per cent;
- fatty acid esters or salts: maximum 0.5 per cent;
- trisodium phosphate: maximum 0.5 per cent;
- liquid paraffin: maximum 0.5 per cent;
- zinc oxide: maximum 0.5 per cent;
- magnesium oxide: maximum 0.2 per cent;
- calcium stearate or zinc stearate or a mixture of both: maximum 0.5 per cent;
- titanium dioxide only for materials for containers for ophthalmic use: maximum 4 per cent.

The supplier of the material must be able to demonstrate that the qualitative and quantitative composition of the type sample (see chapter *3.2.2. Plastic containers and closures for pharmaceutical use* for the definition of 'type sample') is satisfactory for each production batch.

CHARACTERS

Appearance
Powder, beads, granules or, after transformation, translucent sheets of varying thicknesses or containers.

Solubility
Practically insoluble in water, soluble in hot aromatic hydrocarbons, practically insoluble in anhydrous ethanol, in hexane and in methanol.

It softens at temperatures between 70 °C and 140 °C.

Relative density: 0.890 to 0.965.

IDENTIFICATION

If necessary, cut the samples of the material to be examined into pieces of maximum dimension on a side of not greater than 1 cm.

A. Infrared absorption spectrophotometry (*2.2.24*).

Preparation To 0.25 g add 10 mL of *toluene R* and boil under a reflux condenser for about 15 min. Place a few drops of the solution on a sodium chloride slide or on a disc of *potassium bromide R* and evaporate the solvent in an oven at 80 °C.

Alternatively, the spectrum may be recorded directly on a cut piece of suitable size (sheets), granules or hot pressed films by attenuated total reflection (ATR).

Absorption maxima at some of the following wavenumbers (*tolerance:* ± *5 cm^{-1}*): at 2915 cm^{-1}, 2848 cm^{-1}, 1471 cm^{-1}, 1465 cm^{-1}, 729 cm^{-1} and 719 cm^{-1}.

The spectrum obtained is identical to that obtained with the material selected for the type sample.

B. It complies with the supplementary tests corresponding to the additives present (see Tests).

C. *(To be performed only on opacified material.)* In a platinum crucible, mix about 20 mg with 1 g of *potassium hydrogen sulfate R* and heat until completely melted. Allow to cool and add 20 mL of *dilute sulfuric acid R*. Heat gently. Filter the resulting solution. To the filtrate add 1 mL of *phosphoric acid R* and 1 mL of *strong hydrogen peroxide solution R*. If the substance is opacified with titanium dioxide, an orange-yellow colour develops.

TESTS

If necessary, cut the samples of the material to be examined into pieces of maximum dimension on a side of not greater than 1 cm.

Solution S1
Place 25 g in a borosilicate-glass flask with a ground-glass neck. Add 500 mL of *water R* and boil under a reflux condenser for 5 h. Allow to cool and decant. Reserve a portion of the solution for the test for appearance of solution and filter the rest through a sintered-glass filter (16) (*2.1.2*). Use within 4 h of preparation.

Solution S2
Place 2.0 g in a conical borosilicate-glass flask with a ground-glass neck. Add 80 mL of *toluene R* and boil under a reflux

condenser for 90 min with constant stirring. Allow to cool to 60 °C and add with continued stirring 120 mL of *methanol R*. Filter the solution through a sintered-glass filter (16) (*2.1.2*). Rinse the flask and the filter with 25 mL of a mixture of 40 mL of *toluene R* and 60 mL of *methanol R*, add the rinsings to the filtrate and dilute to 250 mL with the same mixture of solvents. Prepare a blank solution.

Solution S3

Place 100 g in a conical borosilicate-glass flask with a ground-glass neck. Add 250 mL of *0.1 M hydrochloric acid* and boil under a reflux condenser for 1 h with constant stirring. Allow to cool and decant the solution.

Appearance of solution

Solution S1 is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

Acidity or alkalinity

To 100 mL of solution S1 add 0.15 mL of *BRP indicator solution R*. Not more than 1.5 mL of *0.01 M sodium hydroxide* is required to change the colour of the indicator to blue.
To 100 mL of solution S1 add 0.2 mL of *methyl orange solution R*. Not more than 1.0 mL of *0.01 M hydrochloric acid* is required to reach the beginning of the colour change of the indicator from yellow to orange.

Absorbance (*2.2.25*)

Maximum 0.2, determined between wavelengths of 220 nm and 340 nm on solution S1.

Reducing substances

To 20 mL of solution S1 add 1 mL of *dilute sulfuric acid R* and 20 mL of *0.002 M potassium permanganate*. Boil under a reflux condenser for 3 min and cool immediately. Add 1 g of *potassium iodide R* and titrate immediately with *0.01 M sodium thiosulfate*, using 0.25 mL of *starch solution R* as indicator. Carry out a blank titration. The difference between the titration volumes is not more than 0.5 mL.

Extractable aluminium

Maximum 1 ppm.

Inductively coupled plasma-atomic emission spectrometry (*2.2.57*).

Test solution Use solution S3.

Reference solutions Prepare the reference solutions using *aluminium standard solution (200 ppm Al) R*, diluting with *0.1 M hydrochloric acid*.

Wavelength Use the emission of aluminium at 396.15 nm, the spectral background being taken as 396.25 nm.

Verify the absence of aluminium in the hydrochloric acid used.

Extractable chromium

Maximum 0.05 ppm.

Inductively coupled plasma-atomic emission spectrometry (*2.2.57*).

Test solution Use solution S3.

Reference solutions Prepare the reference solutions using *chromium standard solution (100 ppm Cr) R*, diluting with a mixture of 2 volumes of *hydrochloric acid R* and 8 volumes of *water R*.

Wavelength Use the emission of chromium at 205.55 nm, the spectral background being taken as 205.50 nm.

Verify the absence of chromium in the hydrochloric acid used.

Extractable titanium

(not for materials opacified with titanium dioxide)

Maximum 1 ppm.

Inductively coupled plasma-atomic emission spectrometry (*2.2.57*).

Test solution Use solution S3.

Reference solutions Prepare the reference solutions using *titanium standard solution (100 ppm Ti) R*, diluting with *0.1 M hydrochloric acid*.

Wavelength Use the emission of titanium at 336.12 nm, the spectral background being taken as 336.16 nm.

Verify the absence of titanium in the hydrochloric acid used.

Extractable vanadium

Maximum 0.1 ppm.

Inductively coupled plasma-atomic emission spectrometry (*2.2.57*).

Test solution Use solution S3.

Reference solutions Prepare the reference solutions using *vanadium standard solution (1 g/L V) R*, diluting with a mixture of 2 volumes of *hydrochloric acid R* and 8 volumes of *water R*.

Wavelength Use the emission of vanadium at 292.40 nm, the spectral background being taken as 292.35 nm.

Verify the absence of vanadium in the hydrochloric acid used.

Extractable zinc

Maximum 1 ppm.

Atomic absorption spectrometry (*2.2.23, Method I*).

Test solution Use solution S3.

Reference solutions Prepare the reference solutions using *zinc standard solution (10 ppm Zn) R*, diluting with *0.1 M hydrochloric acid*.

Source Zinc hollow-cathode lamp.

Wavelength 213.9 nm.

Atomisation device Air-acetylene flame.

Verify the absence of zinc in the hydrochloric acid used.

Extractable zirconium

Maximum 0.1 ppm.

Inductively coupled plasma-atomic emission spectrometry (*2.2.57*).

Test solution Use solution S3.

Reference solutions Prepare the reference solutions using *zirconium standard solution (1 g/L Zr) R*, diluting with a mixture of 2 volumes of *hydrochloric acid R* and 8 volumes of *water R*.

Wavelength Use the emission of zirconium at 343.82 nm, the spectral background being taken as 343.92 nm.

Verify the absence of zirconium in the hydrochloric acid used.

Extractable heavy metals (*2.4.8*)

Maximum 2.5 ppm.

Evaporate 50 mL of solution S3 to about 5 mL on a water-bath and dilute to 20.0 mL with *water R*. 12 mL of the solution complies with test A. Prepare the reference solution using 2.5 mL of *lead standard solution (10 ppm Pb) R*.

Sulfated ash (*2.4.14*)

Maximum 1.0 per cent, determined on 5.0 g. This limit does not apply to material opacified with titanium dioxide.

SUPPLEMENTARY TESTS

These tests are to be carried out, in whole or in part, only if required by the stated composition of the material.

Phenolic antioxidants

Liquid chromatography (*2.2.29*).

Solvent mixture *acetonitrile R, tetrahydrofuran R* (50:50 *V/V*).

Test solution S21 Evaporate 50 mL of solution S2 to dryness *in vacuo* at 45 °C. Dissolve the residue in 5.0 mL of the solvent mixture. Prepare a blank solution from the blank solution corresponding to solution S2.

Of the following reference solutions, only prepare those that are necessary for the analysis of the phenolic antioxidants stated in the composition of the substance to be examined.

Reference solution (a) Dissolve 25.0 mg of *butylhydroxytoluene CRS* (plastic additive 07) and 60.0 mg of *plastic additive 08 CRS* in 10.0 mL of the solvent mixture. Dilute 2.0 mL of this solution to 50.0 mL with the solvent mixture.

Reference solution (b) Dissolve 60.0 mg of *plastic additive 09 CRS* and 60.0 mg of *plastic additive 10 CRS* in 10.0 mL of the solvent mixture. Dilute 2.0 mL of this solution to 50.0 mL with the solvent mixture.

Reference solution (c) *Prepare immediately before use.* Dissolve 60.0 mg of *plastic additive 11 CRS* and 60.0 mg of *plastic additive 12 CRS* in 10.0 mL of the solvent mixture. Dilute 2.0 mL of this solution to 50.0 mL with the solvent mixture.

Reference solution (d) Dissolve 25.0 mg of *butylhydroxytoluene CRS* (plastic additive 07) in 10.0 mL of the solvent mixture. Dilute 2.0 mL of this solution to 50.0 mL with the solvent mixture.

Reference solution (e) Dissolve 60.0 mg of *plastic additive 08 CRS* in 10.0 mL of the solvent mixture. Dilute 2.0 mL of this solution to 50.0 mL with the solvent mixture.

Reference solution (f) Dissolve 60.0 mg of *plastic additive 13 CRS* in 10.0 mL of the solvent mixture. Dilute 2.0 mL of this solution to 50.0 mL with the solvent mixture.

Reference solution (g) Dissolve 60.0 mg of *plastic additive 09 CRS* in 10.0 mL of the solvent mixture. Dilute 2.0 mL of this solution to 50.0 mL with the solvent mixture.

Reference solution (h) Dissolve 60.0 mg of *plastic additive 10 CRS* in 10.0 mL of the solvent mixture. Dilute 2.0 mL of this solution to 50.0 mL with the solvent mixture.

Reference solution (i) Dissolve 60.0 mg of *plastic additive 11 CRS* in 10.0 mL of the solvent mixture. Dilute 2.0 mL of this solution to 50.0 mL with the solvent mixture.

Reference solution (j) *Prepare immediately before use.* Dissolve 60.0 mg of *plastic additive 12 CRS* in 10.0 mL of the solvent mixture. Dilute 2.0 mL of this solution to 50.0 mL with the solvent mixture.

A. If the substance to be examined contains plastic additive 07 and/or plastic additive 08, proceed as follows.

Column:
- *size*: *l* = 0.25 m, Ø = 4.6 mm;
- *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

Mobile phase *water for chromatography R, acetonitrile R* (30:70 *V/V*).

Flow rate 2 mL/min.

Detection Spectrophotometer at 280 nm.

Injection 20 µL of test solution S21, the corresponding blank solution, reference solution (a), and either reference solution (d) or (e), or reference solutions (d) and (e).

Run time 30 min.

System suitability:
- *resolution*: minimum 5.0 between the peaks due to plastic additive 07 and plastic additive 08 in the chromatogram obtained with reference solution (a).

The chromatogram obtained with test solution S21 only shows peaks due to antioxidants stated in the composition and minor peaks that also appear in the chromatogram obtained with the blank solution.

Calculation of percentage contents:

For plastic additive 07 and/or 08, use the concentration of the corresponding reference substance in reference solutions (d) and/or (e).

Limits:
- *plastic additive 07*: maximum 0.125 per cent;
- *plastic additive 08*: maximum 0.3 per cent.

B. If the substance to be examined contains one or more of the following antioxidants:
- plastic additive 09;
- plastic additive 10;
- plastic additive 11;
- plastic additive 12;
- plastic additive 13;

proceed as described above with the following modifications.

Mobile phase *water for chromatography R, tetrahydrofuran R, acetonitrile R* (10:30:60 *V/V/V*).

Flow rate 1.5 mL/min.

Injection 20 µL of test solution S21, the corresponding blank solution, reference solution (b), reference solution (c) and the reference solutions of the antioxidants in the list above that are stated in the composition.

System suitability:
- *resolution*: minimum 2.0 between the peaks due to plastic additive 09 and plastic additive 10 in the chromatogram obtained with reference solution (b); minimum 2.0 between the peaks due to plastic additive 11 and plastic additive 12 in the chromatogram obtained with reference solution (c).

The chromatogram obtained with test solution S21 only shows peaks due to antioxidants stated in the composition and minor peaks that also appear in the chromatogram obtained with the blank solution.

Calculation of percentage contents:

For plastic additive 09, 10, 11, 12 and/or 13, use the concentration of the corresponding reference substance in reference solutions (g), (h), (i), (j) and/or (f).

Limits:
- *plastic additive 09*: maximum 0.3 per cent;
- *plastic additive 10*: maximum 0.3 per cent;
- *plastic additive 11*: maximum 0.3 per cent;
- *plastic additive 12*: maximum 0.3 per cent;
- *plastic additive 13*: maximum 0.3 per cent.

Non-phenolic antioxidants

Thin-layer chromatography (*2.2.27*).

Test solution S22 Evaporate 100 mL of solution S2 to dryness *in vacuo* at 45 °C. Dissolve the residue in 2 mL of *acidified methylene chloride R*.

Reference solution (k) Dissolve 60 mg of *plastic additive 14 CRS* in *methylene chloride R* and dilute to 10 mL with the same solvent. Dilute 2 mL of this solution to 10 mL with *acidified methylene chloride R*.

Reference solution (l) Dissolve 60 mg of *plastic additive 15 CRS* in *methylene chloride R* and dilute to 10 mL

with the same solvent. Dilute 2 mL of this solution to 10 mL with *acidified methylene chloride R*.

Reference solution (m) Dissolve 60 mg of *plastic additive 16 CRS* in *methylene chloride R* and dilute to 10 mL with the same solvent. Dilute 2 mL of this solution to 10 mL with *acidified methylene chloride R*.

Reference solution (n) Dissolve 60 mg of *plastic additive 17 CRS* in *methylene chloride R* and dilute to 10 mL with the same solvent. Dilute 2 mL of this solution to 10 mL with *acidified methylene chloride R*.

Reference solution (o) Dissolve 60 mg of *plastic additive 16 CRS* and 60 mg of *plastic additive 17 CRS* in *methylene chloride R* and dilute to 10 mL with the same solvent. Dilute 2 mL of this solution to 10 mL with *acidified methylene chloride R*.

Plate *TLC silica gel F_{254} plate R*.

Mobile phase A *hexane R*.

Mobile phase B *methylene chloride R*.

Application 20 µL of test solution S22, reference solution (o) and reference solutions corresponding to all the phenolic and non-phenolic antioxidants mentioned in the type composition of the material to be examined.

Development A Over a path of 18 cm with mobile phase A.

Drying A In air.

Development B Over a path of 17 cm with mobile phase B.

Drying B In air.

Detection Examine in ultraviolet light at 254 nm, spray with *alcoholic iodine solution R* and examine in ultraviolet light at 254 nm after 10-15 min.

System suitability Reference solution (o):
— the chromatogram shows 2 clearly separated spots.

Limits Any spots in the chromatogram obtained with test solution S22 are not more intense than the spots in the same positions in the chromatograms obtained with the reference solutions.

Amides and stearates

Thin-layer chromatography (*2.2.27*).

Test solution Use test solution S22 described in the test for non-phenolic antioxidants.

Reference solution (p) Dissolve 20 mg of *stearic acid CRS* (plastic additive 19) in *methylene chloride R* and dilute to 10 mL with the same solvent.

Reference solution (q) Dissolve 40 mg of *plastic additive 20 CRS* in *methylene chloride R* and dilute to 20 mL with the same solvent.

Reference solution (r) Dissolve 40 mg of *plastic additive 21 CRS* in *methylene chloride R* and dilute to 20 mL with the same solvent.

Plates *TLC silica gel F_{254} plate R* (2 plates).

A. *Mobile phase: anhydrous ethanol R, trimethylpentane R* (25:75 *V/V*).

Application 10 µL of test solution S22 and reference solution (p).

Development Over a path of 10 cm.

Drying In air.

Detection Spray with a 2 g/L solution of *dichlorophenolindophenol, sodium salt R* in *anhydrous ethanol R* and heat in an oven at 120 °C for a few minutes to intensify the spots.

Limit Any spot corresponding to plastic additive 19 in the chromatogram obtained with test solution S22 is similar in position (R_F = about 0.5) but not more intense than the corresponding spot in the chromatogram obtained with reference solution (p).

B. *Mobile phase A: hexane R*.

Mobile phase B *methanol R, methylene chloride R* (5:95 *V/V*).

Application 10 µL of test solution S22 and reference solutions (q) and (r).

Development A Over a path of 13 cm with mobile phase A.

Drying A In air.

Development B Over a path of 10 cm with mobile phase B.

Drying B In air.

Detection Spray with a 40 g/L solution of *phosphomolybdic acid R* in *anhydrous ethanol R* and heat in an oven at 120 °C until spots appear.

Limits Any spots corresponding to plastic additive 20 or plastic additive 21 in the chromatogram obtained with the test solution S22 are similar in position (R_F = about 0.2) but not more intense than the corresponding spots in the chromatograms obtained with reference solutions (q) and (r).

D. Polypropylene for Containers and Closures for Parenteral Preparations and Ophthalmic Preparations

(Ph. Eur. method 3.1.6)

DEFINITION

Polypropylene consists of the homopolymer of propylene or of a copolymer of propylene with not more than 25 per cent of ethylene or of a mixture (alloy) of polypropylene with not more than 25 per cent of polyethylene. It may contain additives.

PRODUCTION

Depending on the intended use of the polymers, they may contain additives to optimise their processing or their chemical, physical and mechanical properties. Unless otherwise justified and authorised, these additives are chosen from the following list, which specifies for each substance the maximum permitted content.

Polypropylene may contain at most 3 antioxidants, 1 or several lubricants or antiblocking agents as well as titanium dioxide as an opacifying agent when the material must provide protection from light.

— butylhydroxytoluene (plastic additive 07): maximum 0.125 per cent;
— pentaerythrityl tetrakis[3-(3,5-di-*tert*-butyl-4-hydroxyphenyl)propionate] (plastic additive 09): maximum 0.3 per cent;
— 1,3,5-tris(3,5-di-*tert*-butyl-4-hydroxybenzyl)-*s*-triazine-2,4,6(1*H*,3*H*,5*H*)-trione (plastic additive 13): maximum 0.3 per cent;
— octadecyl 3-(3,5-di-*tert*-butyl-4-hydroxyphenyl)propionate, (plastic additive 11): maximum 0.3 per cent;
— ethylene bis[3,3-bis[3-(1,1-dimethylethyl)-4-hydroxyphenyl]butanoate] (plastic additive 08): maximum 0.3 per cent;

— dioctadecyl disulfide (plastic additive 15): maximum 0.3 per cent;
— 2,2′,2″,6,6′,6″-hexa-*tert*-butyl-4,4′,4″-[(2,4,6-trimethyl-1,3,5-benzenetriyl)trismethylene]triphenol (plastic additive 10): maximum 0.3 per cent;
— 2,2′-bis(octadecyloxy)-5,5′-spirobi[1,3,2-dioxaphosphinane] (plastic additive 14): maximum 0.3 per cent;
— didodecyl 3,3′-thiodipropionate (plastic additive 16): maximum 0.3 per cent;
— dioctadecyl 3,3′-thiodipropionate (plastic additive 17): maximum 0.3 per cent;
— tris(2,4-di-*tert*-butylphenyl) phosphite (plastic additive 12): maximum 0.3 per cent.

The total of antioxidant additives listed above does not exceed 0.3 per cent.
— hydrotalcite: maximum 0.5 per cent;
— alkanamides: maximum 0.5 per cent;
— alkenamides: maximum 0.5 per cent;
— sodium silico-aluminate: maximum 0.5 per cent;
— silica (natural or synthetic, coated or uncoated): maximum 0.5 per cent;
— sodium benzoate: maximum 0.5 per cent;
— fatty acid esters or salts: maximum 0.5 per cent;
— trisodium phosphate: maximum 0.5 per cent;
— liquid paraffin: maximum 0.5 per cent;
— zinc oxide: maximum 0.5 per cent;
— talc: maximum 0.5 per cent;
— magnesium oxide: maximum 0.2 per cent;
— calcium stearate or zinc stearate or a mixture of both: maximum 0.5 per cent;
— titanium dioxide, only for materials for containers for ophthalmic use: maximum 4 per cent.

The supplier of the material must be able to demonstrate that the qualitative and quantitative composition of the type sample (see chapter *3.2.2. Plastic containers and closures for pharmaceutical use* for the definition of 'type sample') is satisfactory for each production batch.

CHARACTERS

Appearance
Powder, beads, granules or, after transformation, translucent sheets of varying thicknesses or containers.

Solubility
Practically insoluble in water, soluble in hot aromatic hydrocarbons, practically insoluble in anhydrous ethanol, in hexane and in methanol.

It softens at temperatures beginning at about 120 °C.

IDENTIFICATION

If necessary, cut the samples of material to be examined into pieces of maximum dimension on a side of not greater than 1 cm.

A. Infrared absorption spectrophotometry (*2.2.24*).

Preparation To 0.25 g add 10 mL of *toluene R* and boil under a reflux condenser for about 15 min. Place a few drops of the solution on a sodium chloride slide or on a disc of *potassium bromide R* and evaporate the solvent in an oven at 80 °C.

Alternatively, the spectrum may be recorded directly on a cut piece of suitable size (sheets), granules or hot pressed films by attenuated total reflection (ATR).

Absorption maxima at some of the following wavenumbers (tolerance: ± 5 cm^{-1}) At 1375 cm^{-1}, 1170 cm^{-1}, 995 cm^{-1} and 973 cm^{-1}.

The spectrum obtained is identical to that obtained with the material selected for the type sample.

B. It complies with the supplementary tests corresponding to the additives present (see Tests).

C. *(To be performed only on opacified material.)* In a platinum crucible, mix about 20 mg with 1 g of *potassium hydrogen sulfate R* and heat until completely melted. Allow to cool and add 20 mL of *dilute sulfuric acid R*. Heat gently. Filter the resulting solution. To the filtrate add 1 mL of *phosphoric acid R* and 1 mL of *strong hydrogen peroxide solution R*. If the substance is opacified with titanium dioxide, an orange-yellow colour develops.

TESTS

If necessary, cut the samples of material to be examined into pieces of maximum dimension on a side of not greater than 1 cm.

Solution S1
Place 25 g in a borosilicate-glass flask with a ground-glass neck. Add 500 mL of *water R* and boil under a reflux condenser for 5 h. Allow to cool and decant. Reserve a portion of the solution for the test for appearance of solution and filter the rest through a sintered-glass filter (16) (*2.1.2*). Use within 4 h of preparation.

Solution S2
Place 2.0 g in a conical borosilicate-glass flask with a ground-glass neck. Add 80 mL of *toluene R* and boil under a reflux condenser for 90 min, with constant stirring. Allow to cool to 60 °C and add, with constant stirring, 120 mL of *methanol R*. Filter the solution through a sintered-glass filter (16) (*2.1.2*). Rinse the flask and the filter with 25 mL of a mixture of 40 mL of *toluene R* and 60 mL of *methanol R*, add the rinsings to the filtrate and dilute to 250 mL with the same mixture of solvents. Prepare a blank solution.

Solution S3
Place 100 g in a conical borosilicate-glass flask with a ground-glass neck. Add 250 mL of *0.1 M hydrochloric acid* and boil under a reflux condenser with constant stirring for 1 h. Allow to cool and decant the solution.

Appearance of solution
Solution S1 is not more opalescent than reference suspension II (*2.2.1*) and is colourless (*2.2.2, Method II*).

Acidity or alkalinity
To 100 mL of solution S1 add 0.15 mL of *BRP indicator solution R*. Not more than 1.5 mL of *0.01 M sodium hydroxide* is required to change the colour of the indicator to blue.
To 100 mL of solution S1 add 0.2 mL of *methyl orange solution R*. Not more than 1.0 mL of *0.01 M hydrochloric acid* is required to reach the beginning of the colour change of the indicator from yellow to orange.

Absorbance (*2.2.25*)
Maximum 0.2, determined between wavelengths of 220 nm to 340 nm on solution S1.

Reducing substances
To 20 mL of solution S1 add 1 mL of *dilute sulfuric acid R* and 20 mL of *0.002 M potassium permanganate*. Boil under a reflux condenser for 3 min and cool immediately. Add 1 g of *potassium iodide R* and titrate immediately with *0.01 M sodium thiosulfate*, using 0.25 mL of *starch solution R* as indicator. Carry out a blank titration. The difference between the titration volumes is not more than 0.5 mL.

Extractable aluminium
Maximum 1 ppm.

Inductively coupled plasma-atomic emission spectrometry (*2.2.57*).

Test solution Use solution S3.

Reference solutions Prepare the reference solutions using *aluminium standard solution (200 ppm Al) R*, diluting with *0.1 M hydrochloric acid*.

Wavelength Use the emission of aluminium at 396.15 nm, the spectral background being taken as 396.25 nm.

Verify the absence of aluminium in the hydrochloric acid used.

Extractable chromium
Maximum 0.05 ppm.

Inductively coupled plasma-atomic emission spectrometry (*2.2.57*).

Test solution Use solution S3.

Reference solutions Prepare the reference solutions using *chromium standard solution (100 ppm Cr) R*, diluting with a mixture of 2 volumes of *hydrochloric acid R* and 8 volumes of *water R*.

Wavelength Use the emission of chromium at 205.55 nm, the spectral background being taken as 205.50 nm.

Verify the absence of chromium in the hydrochloric acid used.

Extractable titanium
Maximum 1 ppm.

Inductively coupled plasma-atomic emission spectrometry (*2.2.57*).

Test solution Use solution S3.

Reference solutions Prepare the reference solutions using *titanium standard solution (100 ppm Ti) R*, diluting with *0.1 M hydrochloric acid*.

Wavelength Use the emission of titanium at 336.12 nm, the spectral background being taken as 336.16 nm.

Verify the absence of titanium in the hydrochloric acid used.

Extractable vanadium
Maximum 0.1 ppm.

Inductively coupled plasma-atomic emission spectrometry (*2.2.57*).

Test solution Use solution S3.

Reference solutions Prepare the reference solutions using *vanadium standard solution (1 g/L V) R*, diluting with a mixture of 2 volumes of *hydrochloric acid R* and 8 volumes of *water R*.

Wavelength Use the emission of vanadium at 292.40 nm, the spectral background being taken as 292.35 nm.

Verify the absence of vanadium in the hydrochloric acid used.

Extractable zinc
Maximum 1 ppm.

Atomic absorption spectrometry (*2.2.23, Method I*).

Test solution Use solution S3.

Reference solutions Prepare the reference solutions using *zinc standard solution (10 ppm Zn) R*, diluting with *0.1 M hydrochloric acid*.

Source Zinc hollow-cathode lamp.

Wavelength 213.9 nm.

Atomisation device Air-acetylene flame.

Verify the absence of zinc in the hydrochloric acid used.

Extractable heavy metals (*2.4.8*)
Maximum 2.5 ppm.

Evaporate 50 mL of solution S3 to about 5 mL on a water-bath and dilute to 20.0 mL with *water R*. 12 mL of the solution complies with test A. Prepare the reference solution using 2.5 mL of *lead standard solution (10 ppm Pb) R*.

Sulfated ash (*2.4.14*)
Maximum 1.0 per cent, determined on 5.0 g. This limit does not apply to material that has been opacified with titanium dioxide.

SUPPLEMENTARY TESTS

These tests are to be carried out, in whole or in part, only if required by the stated composition of the material.

Phenolic antioxidants
Liquid chromatography (*2.2.29*).

Solvent mixture *acetonitrile R, tetrahydrofuran R* (50:50 *V/V*).

Test solution S21 Evaporate 50 mL of solution S2 to dryness *in vacuo* at 45 °C. Dissolve the residue in 5.0 mL of the solvent mixture. Prepare a blank solution from the blank solution corresponding to solution S2.

Of the following reference solutions, only prepare those that are necessary for the analysis of the phenolic antioxidants stated in the composition of the substance to be examined.

Reference solution (a) Dissolve 25.0 mg of *butylhydroxytoluene CRS* (plastic additive 07) and 60.0 mg of *plastic additive 08 CRS* in 10.0 mL of the solvent mixture. Dilute 2.0 mL of this solution to 50.0 mL with the solvent mixture.

Reference solution (b) Dissolve 60.0 mg of *plastic additive 09 CRS* and 60.0 mg of *plastic additive 10 CRS* in 10.0 mL of the solvent mixture. Dilute 2.0 mL of this solution to 50.0 mL with the solvent mixture.

Reference solution (c) *Prepare immediately before use.* Dissolve 60.0 mg of *plastic additive 11 CRS* and 60.0 mg of *plastic additive 12 CRS* in 10 mL of the solvent mixture. Dilute 2.0 mL of this solution to 50.0 mL with the solvent mixture.

Reference solution (d) Dissolve 25.0 mg of *butylhydroxytoluene CRS* (plastic additive 07) in 10.0 mL of the solvent mixture. Dilute 2.0 mL of this solution to 50.0 mL with the solvent mixture.

Reference solution (e) Dissolve 60.0 mg of *plastic additive 08 CRS* in 10.0 mL of the solvent mixture. Dilute 2.0 mL of this solution to 50.0 mL with the solvent mixture.

Reference solution (f) Dissolve 60.0 mg of *plastic additive 13 CRS* in 10.0 mL of the solvent mixture. Dilute 2.0 mL of this solution to 50.0 mL with the solvent mixture.

Reference solution (g) Dissolve 60.0 mg of *plastic additive 09 CRS* in 10.0 mL of the solvent mixture. Dilute 2.0 mL of this solution to 50.0 mL with the solvent mixture.

Reference solution (h) Dissolve 60.0 mg of *plastic additive 10 CRS* in 10.0 mL of the solvent mixture. Dilute 2.0 mL of this solution to 50.0 mL with the solvent mixture.

Reference solution (i) Dissolve 60.0 mg of *plastic additive 11 CRS* in 10.0 mL of the solvent mixture. Dilute 2.0 mL of this solution to 50.0 mL with the solvent mixture.

Reference solution (j) *Prepare immediately before use.* Dissolve 60.0 mg of *plastic additive 12 CRS* in 10.0 mL of the solvent mixture. Dilute 2.0 mL of this solution to 50.0 mL with the solvent mixture.

A. If the substance to be examined contains plastic additive 07 and/or plastic additive 08, proceed as follows.

Column:
— *size*: *l* = 0.25 m, Ø = 4.6 mm;

— *stationary phase*: *octadecylsilyl silica gel for chromatography R* (5 µm).

Mobile phase *water for chromatography R, acetonitrile R* (30:70 *V/V*).

Flow rate 2 mL/min.

Detection Spectrophotometer at 280 nm.

Injection 20 µL of test solution S21, the corresponding blank solution, reference solution (a), and either reference solution (d) or (e), or reference solutions (d) and (e).

Run time 30 min.

System suitability:

— *resolution*: minimum 5.0 between the peaks due to plastic additive 07 and plastic additive 08 in the chromatogram obtained with reference solution (a).

The chromatogram obtained with test solution S21 only shows peaks due to antioxidants stated in the composition and minor peaks that also appear in the chromatogram obtained with the blank solution.

Calculation of percentage contents:

For plastic additive 07 and/or 08, use the concentration of the corresponding reference substance in reference solutions (d) and/or (e).

Limits:

— *plastic additive 07*: maximum 0.125 per cent;

— *plastic additive 08*: maximum 0.3 per cent.

B. If the substance to be examined contains one or more of the following antioxidants:

— plastic additive 09;

— plastic additive 10;

— plastic additive 11;

— plastic additive 12;

— plastic additive 13;

proceed as described above with the following modifications.

Mobile phase *water for chromatography R, tetrahydrofuran R, acetonitrile R* (10:30:60 *V/V/V*).

Flow rate 1.5 mL/min.

Injection 20 µL of test solution S21, the corresponding blank solution, reference solution (b), reference solution (c) and the reference solutions of the antioxidants in the list above that are stated in the composition.

System suitability:

— *resolution*: minimum 2.0 between the peaks due to plastic additive 09 and plastic additive 10 in the chromatogram obtained with reference solution (b); minimum 2.0 between the peaks due to plastic additive 11 and plastic additive 12 in the chromatogram obtained with reference solution (c).

The chromatogram obtained with test solution S21 only shows peaks due to antioxidants stated in the composition and minor peaks that also appear in the chromatogram obtained with the blank solution.

Calculation of percentage contents:

For plastic additive 09, 10, 11, 12 and/or 13, use the concentration of the corresponding reference substance in reference solutions (g), (h), (i), (j) and/or (f).

Limits:

— *plastic additive 09*: maximum 0.3 per cent;

— *plastic additive 10*: maximum 0.3 per cent;

— *plastic additive 11*: maximum 0.3 per cent;

— *plastic additive 12*: maximum 0.3 per cent;

— *plastic additive 13*: maximum 0.3 per cent.

Non-phenolic antioxidants

Thin-layer chromatography (*2.2.27*).

Test solution S22 Evaporate 100 mL of solution S2 to dryness *in vacuo* at 45 °C. Dissolve the residue in 2 mL of *acidified methylene chloride R*.

Reference solution (k) Dissolve 60 mg of *plastic additive 14 CRS* in *methylene chloride R* and dilute to 10 mL with the same solvent. Dilute 2 mL of the solution to 10 mL with *acidified methylene chloride R*.

Reference solution (l) Dissolve 60 mg of *plastic additive 15 CRS* in *methylene chloride R* and dilute to 10 mL with the same solvent. Dilute 2 mL of the solution to 10 mL with *acidified methylene chloride R*.

Reference solution (m) Dissolve 60 mg of *plastic additive 16 CRS* in *methylene chloride R* and dilute to 10 mL with the same solvent. Dilute 2 mL of the solution to 10 mL with *acidified methylene chloride R*.

Reference solution (n) Dissolve 60 mg of *plastic additive 17 CRS* in *methylene chloride R* and dilute to 10 mL with the same solvent. Dilute 2 mL of the solution to 10 mL with *acidified methylene chloride R*.

Reference solution (o) Dissolve 60 mg of *plastic additive 16 CRS* and 60 mg of *plastic additive 17 CRS* in *methylene chloride R* and dilute to 10 mL with the same solvent. Dilute 2 mL of the solution to 10 mL with *acidified methylene chloride R*.

Plate *TLC silica gel F_{254} plate R*.

Mobile phase A *hexane R*.

Mobile phase B *methylene chloride R*.

Application 20 µL of test solution S22, reference solution (o) and reference solutions corresponding to all the phenolic and non-phenolic antioxidants mentioned in the type composition of the material to be examined.

Development A Over a path of 18 cm with mobile phase A.

Drying A In air.

Development B Over a path of 17 cm with mobile phase B.

Drying B In air.

Detection Examine in ultraviolet light at 254 nm; spray with *alcoholic iodine solution R* and examine in ultraviolet light at 254 nm after 10-15 min.

System suitability Reference solution (o):

— the chromatogram shows 2 clearly separated spots.

Limits Any spots in the chromatogram obtained with test solution S22 are not more intense than the spots in the same positions in the chromatograms obtained with the reference solutions.

Amides and stearates

Thin-layer chromatography (*2.2.27*).

Test solution Use test solution S22 described in the test for non-phenolic antioxidants.

Reference solution (p) Dissolve 20 mg of *stearic acid CRS* (plastic additive 19) in *methylene chloride R* and dilute to 10 mL with the same solvent.

Reference solution (q) Dissolve 40 mg of *plastic additive 20 CRS* in *methylene chloride R* and dilute to 20 mL with the same solvent.

Reference solution (r) Dissolve 40 mg of *plastic additive 21 CRS* in *methylene chloride R* and dilute to 20 mL with the same solvent.

Plate *TLC silica gel F_{254} plate R* (2 plates).

A. *Mobile phase*: *anhydrous ethanol R, trimethylpentane R* (25:75 *V/V*).

Application 10 µL of solution S22 and reference solution (p).

Development Over a path of 10 cm.

Drying In air.

Detection Spray with a 2 g/L solution of *dichlorophenolindophenol, sodium salt R* in *anhydrous ethanol R* and heat in an oven at 120 °C for a few minutes to intensify the spots.

Limit Any spot corresponding to plastic additive 19 in the chromatogram obtained with test solution S22 is similar in position (R_F = about 0.5) but not more intense than the corresponding spot in the chromatogram obtained with reference solution (p).

B. *Mobile phase A*: *hexane R.*

Mobile phase B *methanol R, methylene chloride R* (5:95 *V/V*).

Application 10 µL of solution S22 and reference solutions (q) and (r).

Development A Over a path of 13 cm with mobile phase A.

Drying A In air.

Development B Over a path of 10 cm with mobile phase B.

Drying B In air.

Detection Spray with a 40 g/L solution of *phosphomolybdic acid R* in *anhydrous ethanol R*; heat in an oven at 120 °C until spots appear.

Limits Any spots corresponding to plastic additive 20 or plastic additive 21 in the chromatogram obtained with test solution S22 are similar in position (R_F = about 0.2) but not more intense than the corresponding spots in the chromatograms obtained with reference solutions (q) and (r).

E. Poly(Ethylene - Vinyl Acetate) for Containers and Tubing for Total Parenteral Nutrition Preparations

(Ph. Eur. method 3.1.7)

DEFINITION

Poly(ethylene - vinyl acetate), complying with the following requirements, is suitable for the manufacture of containers and tubing for total parenteral nutrition preparations. It is obtained by copolymerisation of mixtures of ethylene and vinyl acetate.

Content of vinyl acetate:

— *material used for containers*: a defined quantity of not more than 25 per cent;

— *material used for tubing*: a defined quantity of not more than 30 per cent.

PRODUCTION

Depending on the intended use of the polymers, they may contain additives to optimise their processing or their chemical, physical and mechanical properties. Unless otherwise justified and authorised, these additives are chosen from the following list, which specifies for each substance the maximum permitted content.

Poly(ethylene - vinyl acetate) may contain not more than 3 of the following antioxidants:

— butylhydroxytoluene (plastic additive 07): maximum 0.125 per cent;

— pentaerythrityl tetrakis[3-(3,5-di-*tert*-butyl-4-hydroxyphenyl)propionate] (plastic additive 09): maximum 0.2 per cent;

— octadecyl 3-(3,5-di-*tert*-butyl-4-hydroxyphenyl)propionate (plastic additive 11): maximum 0.2 per cent;

— tris(2,4-di-*tert*-butylphenyl) phosphite (plastic additive 12): maximum 0.2 per cent;

— 2,2′,2″,6,6′,6″-hexa-*tert*-butyl-4,4′,4″-[(2,4,6-trimethyl-1,3,5-benzenetriyl)trismethylene]triphenol (plastic additive 10): maximum 0.2 per cent.

It may also contain:

— oleamide (plastic additive 20): maximum 0.5 per cent;

— erucamide (plastic additive 21): maximum 0.5 per cent;

— calcium stearate or zinc stearate or a mixture of both: maximum 0.5 per cent;

— calcium carbonate or potassium hydroxide: maximum 0.5 per cent;

— colloidal silica: maximum 0.2 per cent.

The supplier of the material must be able to demonstrate that the qualitative and quantitative composition of the type sample (see chapter *3.2.2. Plastic containers and closures for pharmaceutical use* for the definition of 'type sample') is satisfactory for each production batch.

CHARACTERS

Appearance

Beads, granules or, after transformation, translucent sheets or tubing of varying thickness or samples of finished objects.

Solubility

Practically insoluble in water, soluble in hot aromatic hydrocarbons, practically insoluble in anhydrous ethanol, in methanol and in hexane, which dissolves, however, low molecular mass polymers.

It burns with a blue flame.

The temperature at which the substance softens changes with the vinyl acetate content: from about 100 °C for contents of a few per cent to about 70 °C for contents of 30 per cent.

IDENTIFICATION

If necessary, cut the samples of the material to be examined into pieces of maximum dimension on a side of not greater than 1 cm.

Infrared absorption spectrophotometry (*2.2.24*).

Preparation To 0.25 g add 10 mL of *toluene R* and boil under a reflux condenser for about 15 min. Place a few drops of the solution on a sodium chloride slide or on a disc of *potassium bromide R* and evaporate the solvent in an oven at 80 °C.

Alternatively, the spectrum may be recorded directly on a cut piece of suitable size (sheets), granules or hot pressed films by attenuated total reflection (ATR).

Absorption maxima (tolerance: ± 5 cm^{-1}) At 2920 - 2850 cm^{-1}, 1740 cm^{-1}, 1240 cm^{-1}, 1020 cm^{-1}, 720 cm^{-1} and 610 cm^{-1}.

The spectrum obtained is identical to that obtained with the material selected for the type sample.

TESTS

If necessary, cut the samples of the material to be examined into pieces of maximum dimension on a side of not greater than 1 cm.

Solution S1

Place 2.0 g in a conical borosilicate-glass flask with a ground-glass neck. Add 80 mL of *toluene R* and boil under a reflux condenser for 90 min, with constant stirring. Allow to cool to 60 °C and add, with constant stirring, 120 mL of *methanol R.*

Filter the solution through a sintered-glass filter (16) (*2.1.2*). Rinse the flask and the filter with 25 mL of a mixture of 40 mL of *toluene R* and 60 mL of *methanol R*, add the rinsings to the filtrate and dilute to 250 mL with the same mixture of solvents.

Solution S2

Place 25 g in a borosilicate-glass flask with a ground-glass neck. Add 500 mL of *water R* and boil under a reflux condenser for 5 h. Allow to cool and decant. Reserve a portion of the solution for the test for appearance of solution and filter the rest through a sintered-glass filter (16) (*2.1.2*). Use within 4 h of preparation.

Appearance of solution

Solution S2 is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

Acidity or alkalinity

To 100 mL of solution S2 add 0.15 mL of *BRP indicator solution R*. Not more than 1.0 mL of *0.01 M sodium hydroxide* is required to change the colour of the indicator to blue. To 100 mL of solution S2 add 0.2 mL of *methyl orange solution R*. Not more than 1.5 mL of *0.01 M hydrochloric acid* is required to reach the beginning of the colour change of the indicator from yellow to orange.

Absorbance (*2.2.25*)

Maximum 0.2, determined between wavelengths of 220 nm and 340 nm on solution S2.

Reducing substances

To 20 mL of solution S2 add 1 mL of *dilute sulfuric acid R* and 20 mL of *0.002 M potassium permanganate*. Boil under a reflux condenser for 3 min and cool immediately. Add 1 g of *potassium iodide R* and titrate immediately with *0.01 M sodium thiosulfate*, using 0.25 mL of *starch solution R* as indicator. Carry out a blank titration. The difference between the titration volumes is not more than 0.5 mL.

Amides and stearates

Thin-layer chromatography (*2.2.27*).

Test solution Evaporate 100 mL of solution S1 to dryness *in vacuo* at 45 °C. Dissolve the residue in 2 mL of *acidified methylene chloride R*.

Reference solution (a) Dissolve 20 mg of *stearic acid CRS* (plastic additive 19) in 10 mL of *methylene chloride R*.

Reference solution (b) Dissolve 40 mg of *plastic additive 20 CRS* in 20 mL of *methylene chloride R*.

Reference solution (c) Dissolve 40 mg of *plastic additive 21 CRS* in 20 mL of *methylene chloride R*.

Plates *TLC silica gel F_{254} plate R* (2 plates).

A. *Mobile phase: anhydrous ethanol R, trimethylpentane R* (25:75 *V/V*).

Application 10 µL.

Development Over a path of 10 cm.

Drying In air.

Detection Spray with a 2 g/L solution of *dichlorophenolindophenol, sodium salt R* in *anhydrous ethanol R* and heat in an oven at 120 °C for a few minutes to intensify the spots.

Limit Any spot corresponding to plastic additive 19 in the chromatogram obtained with the test solution is not more intense than the spot in the chromatogram obtained with reference solution (a).

B. *Mobile phase A: hexane R.*

Mobile phase B *methanol R, methylene chloride R* (5:95 *V/V*).

Application 10 µL.

Development A Over a path of 13 cm with mobile phase A.

Drying A In air.

Development B Over a path of 10 cm with mobile phase B.

Drying B In air.

Detection Spray with a 40 g/L solution of *phosphomolybdic acid R* in *anhydrous ethanol R* and heat at 120 °C until spots appear.

Limit Any spots corresponding to plastic additive 21 or plastic additive 20 in the chromatogram obtained with the test solution are not more intense than the spots in the chromatograms obtained with reference solutions (b) and (c) respectively.

Phenolic antioxidants

Liquid chromatography (*2.2.29*).

Solvent mixture *acetonitrile R, tetrahydrofuran R* (50:50 *V/V*).

Test solution (a) Evaporate 50 mL of solution S1 to dryness *in vacuo* at 45 °C. Dissolve the residue in 5.0 mL of the solvent mixture.

Test solution (b) Evaporate 50 mL of solution S1 to dryness *in vacuo* at 45 °C. Dissolve the residue in 5.0 mL of *methylene chloride R*.

Reference solution (a) Dissolve 25.0 mg of *butylhydroxytoluene CRS* (plastic additive 07), 40.0 mg of *plastic additive 10 CRS*, 40.0 mg of *plastic additive 09 CRS* and 40.0 mg of *plastic additive 11 CRS* in 10 mL of the solvent mixture. Dilute 2 mL of the solution to 50.0 mL with the solvent mixture.

Reference solution (b) Dissolve 40.0 mg of *plastic additive 11 CRS* and 40.0 mg of *plastic additive 12 CRS* in 10 mL of *methylene chloride R*. Dilute 2 mL of the solution to 50.0 mL with *methylene chloride R*.

Column:

— *size: l* = 0.25 m; Ø = 4.6 mm;

— *stationary phase: octadecylsilyl silica gel for chromatography R* (5 µm).

Mobile phase *water R, tetrahydrofuran R, acetonitrile R* (10:30:60 *V/V/V*).

Flow rate 1.5 mL/min.

Detection Spectrophotometer at 280 nm.

Injection 20 µL of test solution (a) and reference solution (a).

System suitability Reference solution (a):

— *resolution:* minimum 2.0 between the peaks due to plastic additive 09 and plastic additive 10;

— *number of theoretical plates:* minimum 2500, calculated for the peak due to plastic additive 07.

Limits:

— the chromatogram obtained with test solution (a) shows only principal peaks corresponding to the peaks in the chromatogram obtained with reference solution (a) with a retention time greater than 2 min;

— the areas of the peaks in the chromatogram obtained with test solution (a) are not greater than those of the corresponding peaks in the chromatogram obtained with reference solution (a), except for the last peak eluted in the chromatogram obtained with reference solution (a).

If the chromatogram obtained with test solution (a) shows a peak with the same retention time as the last antioxidant

eluted from reference solution (a), carry out the test as described with the following modifications.

Mobile phase *water R, 2-propanol R, methanol R* (5:45:50 *V/V/V*).

Injection 20 µL of test solution (b) and reference solution (b).

System suitability Reference solution (b):
— *resolution*: minimum 2.0 between the peaks due to plastic additive 11 and plastic additive 12.

Limits:
— the chromatogram obtained with test solution (b) shows only principal peaks corresponding to the peaks in the chromatogram obtained with reference solution (b) with a retention time greater than 3 min;
— the areas of the peaks in the chromatogram obtained with test solution (b) are not greater than those of the corresponding peaks in the chromatogram obtained with reference solution (b).

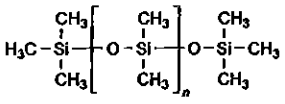
Sulfated ash (*2.4.14*)
Maximum 1.2 per cent, determined on 5.0 g.

ASSAY
Introduce 0.250 g to 1.000 g of the substance to be examined, according to the vinyl acetate content of the copolymer to be examined, into a 300 mL conical flask with a ground-glass neck containing a magnetic stirrer.
Add 40 mL of *xylene R*. Boil under a reflux condenser with stirring for 4 h. Stirring continuously, allow to cool until precipitation begins before slowly adding 25.0 mL of *alcoholic potassium hydroxide solution R1*. Boil again under a reflux condenser with stirring for 3 h. Allow to cool with continued stirring, rinse the condenser with 50 mL of *water R* and add 30.0 mL of *0.05 M sulfuric acid* to the flask. Transfer the contents of the flask into a 400 mL beaker; rinse the flask with 2 quantities, each of 50 mL, of a 200 g/L solution of *anhydrous sodium sulfate R* and 3 quantities, each of 20 mL, of *water R* and add all the rinsings to the beaker containing the initial solution. Titrate the excess sulfuric acid with *0.1 M sodium hydroxide*, determining the end-point potentiometrically (*2.2.20*). Carry out a blank titration.
1 mL of *0.05 M sulfuric acid* is equivalent to 8.609 mg of vinyl acetate.

F. Silicone

1. Silicone Oil Used as a Lubricant

(Ph. Eur. method 3.1.8)



DEFINITION
Silicone oil used as a lubricant is a poly(dimethylsiloxane) obtained by hydrolysis and polycondensation of dichlorodimethylsilane and chlorotrimethylsilane. Different grades exist which are characterised by a number indicating the nominal viscosity placed after the name.

Silicone oils used as lubricants have a degree of polymerisation (n = 400 to 1200) such that their kinematic viscosities are nominally between 1000 $mm^2 \cdot s^{-1}$ and 30 000 $mm^2 \cdot s^{-1}$.

CHARACTERS
Appearance
Clear, colourless liquid of various viscosities.

Solubility
Practically insoluble in water and in methanol, very slightly soluble in anhydrous ethanol, miscible with ethyl acetate, with methyl ethyl ketone and with toluene.

IDENTIFICATION
A. Kinematic viscosity at 25 °C (see Tests).

B. Infrared absorption spectrophotometry (*2.2.24*).

Comparison *silicone oil CRS*.

The region of the spectrum from 850-750 cm^{-1} is not taken into account since it may show slight differences depending on the degree of polymerisation.

C. Heat 0.5 g in a test-tube over a small flame until white fumes begin to appear. Invert the tube over a 2nd tube containing 1 mL of a 1 g/L solution of *chromotropic acid, sodium salt R* in *sulfuric acid R* so that the fumes reach the solution. Shake the 2nd tube for about 10 s and heat on a water-bath for 5 min. The solution is violet.

D. In a platinum crucible, prepare the sulfated ash (*2.4.14*) using 50 mg. The white powder obtained gives the reaction of silicates (*2.3.1*).

TESTS
Acidity
To 2.0 g add 25 mL of a mixture of equal volumes of *anhydrous ethanol R* and *ether R*, previously neutralised to 0.2 mL of *bromothymol blue solution R1*, and shake. Not more than 0.15 mL of *0.01 M sodium hydroxide* is required to change the colour of the solution to blue.

Viscosity (*2.2.10*)
Determine the dynamic viscosity at 25 °C. Calculate the kinematic viscosity taking the relative density to be 0.97. The kinematic viscosity is within the range 95 per cent to 105 per cent of the nominal viscosity stated on the label.

Mineral oils
Place 2 mL in a test-tube and examine in ultraviolet light at 365 nm. The fluorescence is not more intense than that of a solution containing 0.1 ppm of *quinine sulfate R* in *0.005 M sulfuric acid* examined in the same conditions.

Phenylated compounds
The refractive index (*2.2.6*) is not greater than 1.410.

Heavy metals
Maximum 5 ppm.

Solvent mixture *dilute ammonia R2*, 2 g/L solution of *hydroxylamine hydrochloride R* (1:9 *V/V*).

Mix 1.0 g with *methylene chloride R* and dilute to 20 mL with the same solvent. Add 1.0 mL of a freshly prepared 0.02 g/L solution of *dithizone R* in *methylene chloride R*, 0.5 mL of *water R* and 0.5 mL of the solvent mixture. At the same time, prepare the reference solution as follows: to 20 mL of *methylene chloride R* add 1.0 mL of a freshly prepared 0.02 g/L solution of *dithizone R* in *methylene chloride R*, 0.5 mL of *lead standard solution (10 ppm Pb) R* and 0.5 mL of the solvent mixture. Immediately shake each solution vigorously for 1 min. Any red colour in the test solution is not more intense than that in the reference solution.

Volatile matter
Maximum 2.0 per cent, determined on 2.00 g by heating in an oven at 150 °C for 24 h. Carry out the test using a dish 60 mm in diameter and 10 mm deep.

LABELLING

The label states:

— the nominal viscosity by a number placed after the name of the product;

— that the contents are to be used as a lubricant.

2. Silicone Elastomer for Closures and Tubing

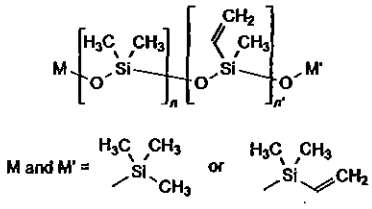
(Ph. Eur. method 3.1.9)

DEFINITION

Silicone elastomer complying with the following requirements is suitable for the manufacture of closures and tubing.

Silicone elastomer is obtained by cross-linking a linear polysiloxane constructed mainly of dimethylsiloxy units with small quantities of methylvinylsiloxy groups; the chain ends are blocked by trimethylsiloxy or dimethylvinylsiloxy groups.

The general formula of the polysiloxane is:



The cross-linking is carried out in the hot state:

— either with:

  — 2,4-dichlorobenzoyl peroxide for extruded products; or

  — 2,4-dichlorobenzoyl peroxide or dicumyl peroxide or *OO*-(1,1-dimethylethyl) *O*-isopropyl monoperoxycarbonate or 2,5-bis[(1,1-dimethylethyl) dioxy]-2,5-dimethylhexane for moulded products;

— or by hydrosilylation by means of polysiloxane with -SiH groups using platinum as a catalyst.

In all cases, appropriate additives are used such as silica and sometimes small quantities of organosilicon additives (α,ω-dihydroxypolydimethylsiloxane).

CHARACTERS

Appearance

Transparent or translucent material.

Solubility

Practically insoluble in organic solvents, some of which, for example cyclohexane, hexane and methylene chloride, cause a reversible swelling of the material.

IDENTIFICATION

A. Infrared absorption spectrophotometry (*2.2.24*) by the multiple reflection method for solids.

Comparison *silicone elastomer CRS.*

B. Heat 1.0 g in a test-tube over a small flame until white fumes begin to appear. Invert the tube over a 2nd tube containing 1 mL of a 1 g/L solution of *chromotropic acid, sodium salt R* in *sulfuric acid R* so that the fumes reach the solution. Shake the 2nd tube for about 10 s and heat on a water-bath for 5 min. The solution is violet.

C. 50 mg of the residue of combustion gives the reaction of silicates (*2.3.1*).

TESTS

If necessary, cut the material into pieces of maximum dimension on a side of not greater than 1 cm.

Solution S

Place 25 g in a borosilicate-glass flask with a ground-glass neck. Add 500 mL of *water R* and boil under a reflux condenser for 5 h. Allow to cool and decant the solution.

Appearance of solution

Solution S is clear (*2.2.1*).

Acidity or alkalinity

To 100 mL of solution S add 0.15 mL of *bromothymol blue solution R1*. Not more than 2.5 mL of *0.01 M sodium hydroxide* is required to change the colour of the indicator to blue. To a further 100 mL of solution S, add 0.2 mL of *methyl orange solution R*. Not more than 1.0 mL of *0.01 M hydrochloric acid* is required to reach the beginning of the colour change of the indicator from yellow to orange.

Relative density (*2.2.5*)

1.05 to 1.25, determined using a density bottle with *anhydrous ethanol R* as the immersion liquid.

Reducing substances

To 20 mL of solution S add 1 mL of *dilute sulfuric acid R* and 20 mL of *0.002 M potassium permanganate*. Allow to stand for 15 min. Add 1 g of *potassium iodide R* and titrate immediately with *0.01 M sodium thiosulfate* using 0.25 mL of *starch solution R* as indicator. Carry out a blank titration using 20 mL of *water R* instead of solution S. The difference between the titration volumes is not more than 1.0 mL.

Substances soluble in hexane

Maximum 3 per cent.

Evaporate 25 mL of the solution obtained in the test for phenylated compounds in a glass evaporating dish on a water-bath and dry in an oven at 100-105 °C for 1 h. The residue weighs not more than 15 mg.

Phenylated compounds

Place 2.0 g in a borosilicate-glass flask with a ground-glass neck and add 100 mL of *hexane R*. Boil under a reflux condenser for 4 h. Cool, then filter rapidly through a sintered-glass filter (16) (*2.1.2*). Collect the filtrate and close the container immediately to avoid evaporation.

At wavelengths from 250 nm to 340 nm, the absorbance (*2.2.25*) is not greater than 0.4.

Mineral oils

Place 2 g in a 100 mL conical flask containing 30 mL of a mixture of 5 volumes of *ammonia R* and 95 volumes of *pyridine R*. Allow to stand for 2 h, shaking frequently. Decant the pyridine solution and examine in ultraviolet light at 365 nm. The fluorescence is not greater than that of a solution containing 1 ppm of *quinine sulfate R* in *0.005 M sulfuric acid* examined in the same conditions.

Volatile matter

Maximum 0.5 per cent for silicone elastomer prepared using peroxides; maximum 2.0 per cent for silicone elastomer prepared using platinum.

Weigh 10.0 g of the substance previously stored for 48 h in a desiccator over *anhydrous calcium chloride R*. Heat in an oven at 200 °C for 4 h, allow to cool in a desiccator and weigh again.

Silicone elastomer prepared using peroxides complies with the following additional test:

Residual peroxides

Maximum 0.08 per cent calculated as dichlorobenzoyl peroxide.

Place 5 g in a borosilicate-glass flask, add 150 mL of *methylene chloride R* and close the flask. Stir with a mechanical stirrer for 16 h. Filter rapidly, collecting the

filtrate in a flask with a ground-glass neck. Replace the air in the container with *oxygen-free nitrogen R*, introduce 1 mL of a 200 g/L solution of *sodium iodide R* in *anhydrous acetic acid R*, close the flask, shake thoroughly and allow to stand protected from light for 30 min. Add 50 mL of *water R* and titrate immediately with *0.01 M sodium thiosulfate*, using 0.25 mL of *starch solution R* as indicator. Carry out a blank titration. The difference between the titration volumes is not greater than 2.0 mL.

Silicone elastomer prepared using platinum complies with the following additional test:

Platinum

Maximum 30 ppm.

In a quartz crucible, ignite 1.0 g of the material to be examined, raising the temperature gradually until a white residue is obtained. Transfer the residue to a graphite crucible. To the quartz crucible add 10 mL of a freshly prepared mixture of 1 volume of *nitric acid R* and 3 volumes of *hydrochloric acid R*, heat on a water-bath for 1-2 min and transfer to the graphite crucible. Add 5 mg of *potassium chloride R* and 5 mL of *hydrofluoric acid R* and evaporate to dryness on a water-bath. Add 5 mL of *hydrofluoric acid R* and evaporate to dryness again; repeat this operation twice. Dissolve the residue in 5 mL of *1 M hydrochloric acid*, warming on a water-bath. Allow to cool and add the solution to 1 mL of a 250 g/L solution of *stannous chloride R* in *1 M hydrochloric acid*, rinse the graphite crucible with a few millilitres of *1 M hydrochloric acid* and dilute to 10.0 mL with the same acid.

Prepare simultaneously the reference solution as follows: to 1 mL of a 250 g/L solution of *stannous chloride R* in *1 M hydrochloric acid*, add 1.0 mL of *platinum standard solution (30 ppm Pt) R* and dilute to 10.0 mL with *1 M hydrochloric acid*.

The colour of the test solution is not more intense than that of the standard.

LABELLING

The label states whether the material was prepared using peroxides or platinum.

G. Plastic Additives

(Ph. Eur. method 3.1.13)

DEFINITION

Plastic additives are chemical substances that are intentionally added to plastic materials to achieve a physical or chemical effect during processing of the plastic or in the final material or container. They may consist of a single chemical substance, a polymeric substance or a defined mixture of different components. Additives are intended to be present in the final material or container.

Substances present that have not been added intentionally are considered to be impurities and include reaction and degradation products, which may be limited by a suitable specification.

GENERAL REQUIREMENTS

Each plastic additive and, if likely to be present in the final material or container, its impurities and reaction and degradation products, must be toxicologically qualified, taking into account the maximum potential exposure as a result of leaching into the contents of the container and product contamination.

Acceptance criteria must be specified for the identity, physico-chemical characteristics, impurities and the assay for each component.

A plastic material contains no more than the strict minimum of additives, at the lowest effective concentration for the intended use and to ensure the stability and quality of the final material or container.

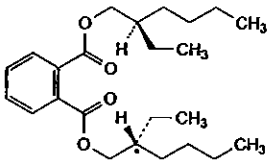
Additives specified for plastic materials described in the European Pharmacopoeia are stated below. Plastic additives other than those described in the Pharmacopoeia may be used subject to agreement on a case-by-case basis by the competent authority.

LIST

NOTE. The nomenclature according to IUPAC rules is given first. The synonym in bold corresponds to the name given in the texts of Chapter 3. The synonym according to Chemical Abstracts (CA) Index rules is also given.

plastic additive 01

$C_{24}H_{38}O_4$. [117-81-7].



its epimer at C* and its enantiomer

bis[(2*RS*)-2-ethylhexyl] benzene-1,2-dicarboxylate

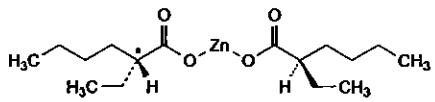
synonyms: — **di(2-ethylhexyl) phthalate,**

— 1,2-benzenedicarboxylic acid, bis(2-ethylhexyl) ester,

— DEHP.

plastic additive 02

$C_{16}H_{30}O_4Zn$. [136-53-8].



its epimer at C* and its enantiomer

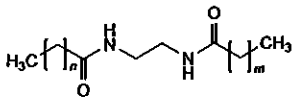
zinc bis[(2*RS*)-2-ethylhexanoate]

synonyms: — **zinc octanoate,**

— hexanoic acid, 2-ethyl, zinc salt (2:1),

— zinc 2-ethylcaproate.

plastic additive 03
[05518-18-3]/[00110-30-5].



N,N′-(ethane-1,2-diyl)dialcanamide (with *n* and *m* = 14 or 16)

synonyms: — ***N,N′*-diacylethylenediamines,**

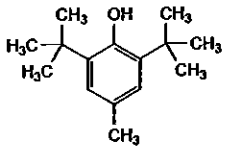
— *N,N′*-diacylethylenediamine (in this context acyl means in particular palmitoyl and stearoyl).

plastic additive 04
[8013-07-8].
epoxidised soya oil

plastic additive 05
[8016-11-3].
epoxidised linseed oil

plastic additive 06
[57455-37-5](TSCA)/[101357-30-6] (EINECS)/Pigment blue 29 (CI 77007)
ultramarine blue

plastic additive 07
$C_{15}H_{24}O$. [128-37-0]



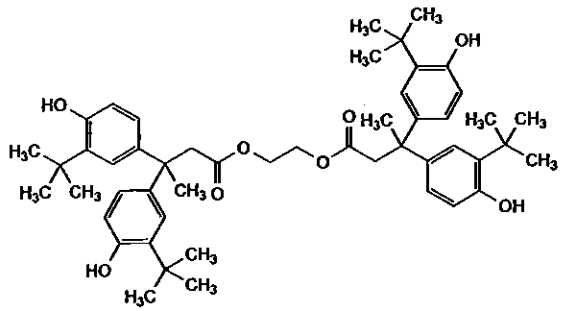
2,6-di-*tert*-butyl-4-methylphenol

synonyms: — **butylhydroxytoluene,**

— phenol, 2,6-bis(1,1-dimethylethyl)-4 methyl-

— 2,6-bis(1,1-dimethylethyl)-4-methylphenol.

plastic additive 08
$C_{50}H_{66}O_8$. [32509-66-3].



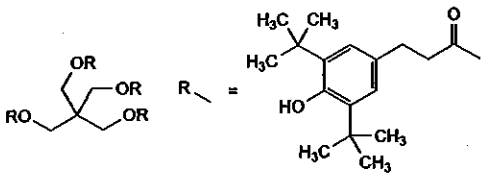
ethane-1,2-diyl bis[3,3-bis(3-*tert*-butyl-4-hydroxyphenyl)butanoate]

synonyms: — **ethylene bis[3,3-bis[3-(1,1-dimethylethyl)-4-hydroxyphenyl] butanoate],**

— benzenepropanoic acid, 3-(1,1-dimethylethyl)-β-[3-(1,1-dimethylethyl)-4-hydroxyphenyl]-4-hydroxy-β-methyl-, 1,1′-(1,2-ethanediyl) ester,

— ethylene bis[3,3-bis(3-*tert*-butyl-4-hydroxyphenyl)butyrate].

plastic additive 09
$C_{73}H_{108}O_{12}$. [6683-19-8].



2,2-bis[[[3-(3,5-di-*tert*-butyl-4-hydroxyphenyl)propanoyl]oxy] methyl]propan-1,3-diyl bis[3-(3,5-di-*tert*-butyl-4-hydroxyphenyl)propanoate]

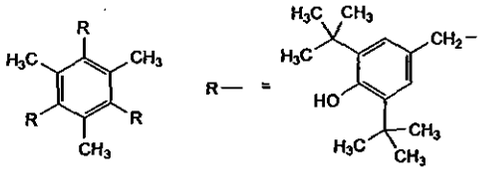
synonyms: — **pentaerythrityl tetrakis[3-(3,5-di-*tert*-butyl-4-hydroxyphenyl)propionate],**

— benzenepropanoic acid, 3,5-bis(1,1-dimethylethyl)-4-hydroxy-, 1,1′-[2,2-bis[[3-[3,5-bis(1,1-dimethylethyl)-4-hydroxyphenyl]-1-oxopropoxy]methyl]-1,3-propanediyl] ester,

— 2,2-bis[[[3-[3,5-bis(1,1-dimethylethyl)-4-hydroxyphenyl]propanoyl]oxy]methyl] propane-1,3-diyl 3-[3,5-bis(1,1-dimethylethyl)-4-hydroxyphenyl]propanoate,

— 2,2-bis(hydroxymethyl)propane-1,3-diol tetrakis[3-(3,5-di-*tert*-butyl-4-hydroxyphenyl)propionate].

plastic additive 10
$C_{54}H_{78}O_3$. [1709-70-2].



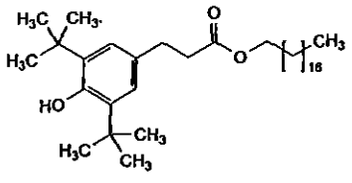
4,4′,4″-[(2,4,6-trimethylbenzene-1,3,5-triyl)tris (methylene)]tris(2,6-di-*tert*-butylphenol)

synonyms: — **2,2′,2″,6,6′,6″-hexa-*tert*-butyl-4,4′,4″-[(2,4,6-trimethyl-1,3,5-benzenetriyl) trismethylene]triphenol,**

— phenol, 4,4′,4″-[(2,4,6-trimethyl-1,3,5-benzenetriyl)tris(methylene)]tris[2,6- bis(1,1-dimethylethyl)-,

— 1,3,5-tris[3,5-di-*tert*-butyl-4-hydroxybenzyl]-2,4,6-trimethylbenzene.

plastic additive 11
$C_{35}H_{62}O_3$. [2082-79-3].

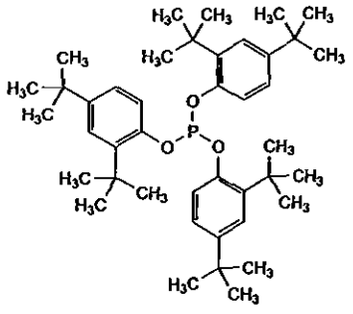


octadecyl 3-(3,5-di-*tert*-butyl-4-hydroxyphenyl)propanoate

synonyms: — **octadecyl 3-(3,5-di-*tert*-butyl-4-hydroxyphenyl)propionate,**

— benzenepropanoic acid, 3,5-bis(1,1-dimethylethyl)-4-hydroxy-, octadecyl ester.

plastic additive 12
$C_{42}H_{63}O_3P$. [31570-04-4].



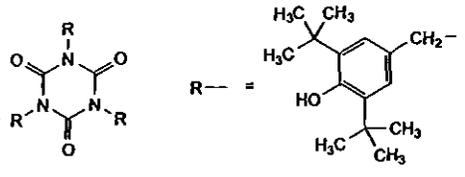
tris(2,4-di-*tert*-butylphenyl) phosphite

synonyms: — **tris(2,4-di-*tert*-butylphenyl) phosphite,**

— phenol, 2,4-bis(1,1-dimethylethyl)-, 1,1′,1″-phosphite,

— 2,4-bis(1,1-dimethylethyl)phenyl, phosphite.

plastic additive 13
$C_{48}H_{69}N_3O_6$. [27676-62-6].

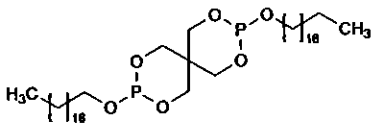


1,3,5-tris[(3,5-di-*tert*-butyl-4-hydroxyphenyl)methyl]-1,3,5-triazine-2,4,6(1*H*,3*H*,5*H*)-trione

synonyms: — **1,3,5-tris(3,5-di-*tert*-butyl-4-hydroxybenzyl)-*s*-triazine-2,4,6-(1*H*,3*H*,5*H*)-trione,**

— 1,3,5-triazine-2,4,6(1*H*,3*H*,5*H*)-trione, 1,3,5-tris[[3,5-bis(1,1-dimethylethyl)-4-hydroxyphenyl]methyl]-.

plastic additive 14
$C_{41}H_{82}O_6P_2$. [3806-34-6].

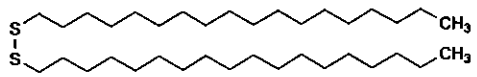


3,9-bis(octadecyloxy)-2,4,8,10-tetraoxa-3,9-diphosphaspiro [5.5]undecane

synonyms: — **2,2′-bis(octadecyloxy)-5,5′-spirobi[1,3,2-dioxaphosphinane],**

— 2,4,8,10-tetraoxa-3,9-diphosphaspiro[5.5] undecane, 3,9-bis(octadecyloxy)-.

plastic additive 15
$C_{36}H_{74}S_2$. [2500-88-1].

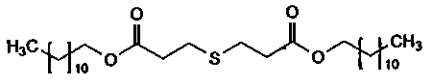


(octadecyldisulfanyl)octadecane

synonyms: — **dioctadecyl disulfide,**

— octadecane, 1,1′-dithio-.

plastic additive 16
$C_{30}H_{58}O_4S$. [123-28-4].



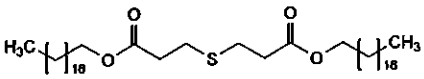
didodecyl 3,3′-sulfanediyldipropanoate

synonyms: — **didodecyl 3,3′-thiodipropionate,**

— propanoic acid, 3,3′-thiobis-, 1,1′-didodecyl ester,

— lauryl thiodipropionate.

plastic additive 17
$C_{42}H_{82}O_4S$. [693-36-7].



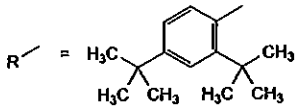
dioctadecyl 3,3′-sulfanediyldipropanoate

synonyms: — **dioctadecyl 3,3′-thiodipropionate,**

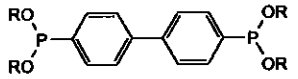
— propanoic acid, 3,3′-thiobis-, 1,1′-dioctadecyl ester,

— stearyl thiodipropionate.

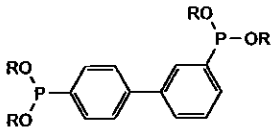
plastic additive 18
[119345-01-6].
mixture of 7 products corresponding to reaction product of di-*tert*-butyl phosphonite with phosphorous trichloride, reaction products with 1,1′-biphenyl and 2,4-di-*tert*-butylphenol:



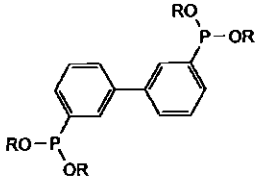
component I



tetrakis(2,4-di-*tert*-butylphenyl) ([1,1′-biphenyl]-4,4′-diyl)bis (phosphonite)
component II

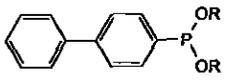


tetrakis(2,4-di-*tert*-butylphenyl) ([1,1′-biphenyl]-3,4′-diyl)bis (phosphonite)
component III



tetrakis(2,4-di-*tert*-butylphenyl) ([1,1′-biphenyl]-3,3′-diyl)bis (phosphonite)

component IV

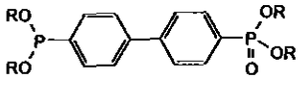


bis(2,4-di-*tert*-butylphenyl) ([1,1′-biphenyl]-4-yl)phosphonite

component V

tris(2,4-di-*tert*-butylphenyl) phosphite

component VI



bis(2,4-di-*tert*-butylphenyl) 4′-[bis(2,4-di-*tert*-butylphenoxy) phosphanyl]([1,1′-biphenyl]-4-yl)phosphonate

component VII

R-OH: 2,4-di-*tert*-butylphenol

plastic additive 19

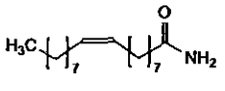
$C_{18}H_{36}O_2$. [57-11-4].

octadecanoic acid

synonyms: — **stearic acid.**

plastic additive 20

$C_{18}H_{35}NO$. [301-02-0].



(9*Z*)-octadec-9-enamide

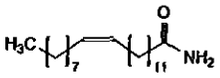
synonyms: — **oleamide,**

— 9-octadecenamide, (9*Z*)-,

— 9-*cis*-oleamide.

plastic additive 21

$C_{22}H_{43}NO$. [112-84-5].



(13*Z*)-docos-13-enamide

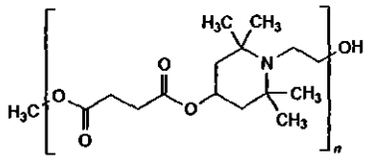
synonyms: — **erucamide,**

— 13-docosenamide, (13*Z*)-,

— 13-*cis*-docosenamide.

plastic additive 22

[65447-77-0].



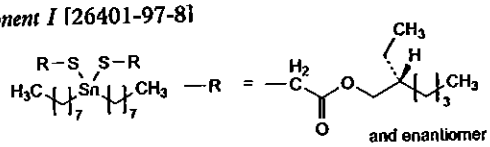
copolymer of dimethyl butanedioate and 1-(2-hydroxyethyl)-2,2,6,6-tetramethylpiperidin-4-ol

synonyms: — **copolymer of dimethyl succinate and (4-hydroxy-2,2,6,6-tetramethylpiperidin-1-yl)ethanol,**

— butanedioic acid, 1,4-dimethyl ester, polymer with 4-hydroxy-2,2,6,6-tetramethyl-1-piperidineethanol.

plastic additive 23
mixture of component I and about 27 per cent of component II

component I [26401-97-8]

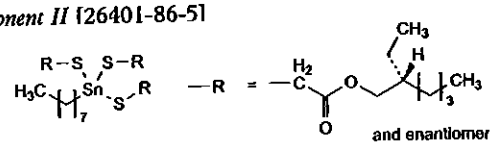


bis[(2*RS*)-2-ethylhexyl][(dioctylstannanetriyl)bis(sulfanediyl)] diacetate

synonyms: — **di(isooctyl) 2,2′-[(dioctylstannylene)bis(thio)]diacetate,**

— acetic acid, 2,2′-[(dioctylstannylene)bis (thio)]bis-, 1,1′-diisooctyl ester.

component II [26401-86-5]

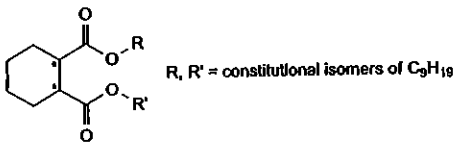


tris[(2*RS*)-2-ethylhexyl][(octylstannanetriyl)tris(sulfanediyl)] triacetate

synonyms: — **tri(isooctyl) 2,2′,2″-[(monooctylstannylidyne)tris(thio)] triacetate,**

— acetic acid, 2,2′,2″-[(octylstannylidyne)tris (thio)]tris-, 1,1′,1″-triisooctyl ester.

plastic additive 24
$C_{26}H_{48}O_4$. [166412-78-8].

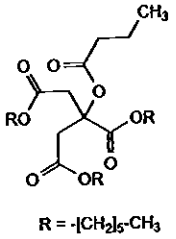


mixture of constitutional isomers of diisononyl (1*E*,2*E*)-cyclohexane-1,2-dicarboxylate.

synonyms: — **cyclohexane 1,2-dicarboxylic acid, diisononyl ester,**

— 1,2-cyclohexanedicarboxylic acid, 1,2-diisononyl ester.

plastic additive 25
$C_{28}H_{50}O_8$. [82469-79-2].

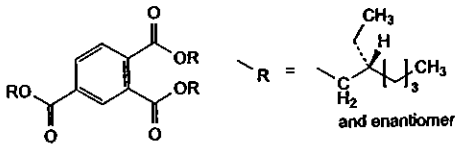


trihexyl 2-(butanoyloxy)propane-1,2,3-tricarboxylate

synonyms: — **butyryl tri-*n*-hexyl citrate,**

— 1,2,3-propanetricarboxylic acid, 2-(1-oxobutoxy)-, 1,2,3-trihexyl ester.

plastic additive 26
$C_{33}H_{54}O_6$. [3319-31-1].

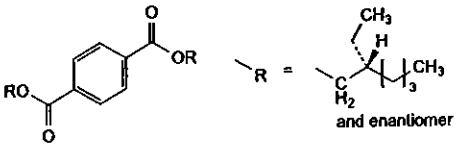


tris[(2*RS*)-2-ethylhexyl] benzene-1,2,4-tricarboxylate

synonyms: — **tris(2-ethylhexyl) trimellitate,**

— 1,2,4-benzentricarboxylic acid, 1,2,4-tris (2-ethylhexyl] estertricarboxylate.

plastic additive 27
$C_{24}H_{38}O_4$. [6422-86-2].



bis[(2*RS*)-2-ethylhexyl] benzene-1,4-dicarboxylate

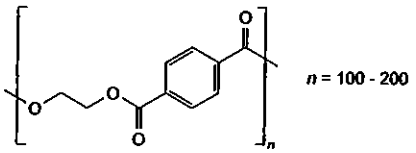
synonyms: — **bis(2-ethylhexyl) terephthalate,**

— 1,4-benzenedicarboxylic acid,

— 1,4-bis(2-ethylhexyl) ester.

H. Polyethylene Terephthalate for Containers for Preparations not for Parenteral Use

(Ph. Eur. method 3.1.15)



DEFINITION

Polyethylene terephthalate is obtained from the polymerisation of terephthalic acid or dimethyl terephthalate with ethylene glycol. Isophthalic acid, dimethyl isophthalate, 1,4-bis(hydroxymethyl)cyclohexane (cyclohexane-1,4-dimethanol) or diethylene glycol may be used in the polymerisation. It may contain not more than 0.5 per cent of silica or silicates and colouring matter approved by the competent authority.

PRODUCTION

The manufacturing process is validated to demonstrate that the residual acetaldehyde content is not greater than 10 ppm in the granules.

CHARACTERS

Appearance

Clear or opaque granules.

Solubility

Practically insoluble in water, in ethanol (96 per cent) and in methylene chloride. It is hydrolysed by strong bases.

IDENTIFICATION

A. Place 0.10 g of the material to be examined into a borosilicate glass flask with a ground-glass neck. Add 25 mL of a 200 g/L solution of *potassium hydroxide R* in a 50 per cent *V/V* solution of *anhydrous ethanol R*. Reflux for 30 min. Allow to cool and dilute to 100 mL with *water R*. Filter if necessary. Dilute 1.0 mL of the filtrate to 100 mL with *water R*. Examined between 210 nm and 330 nm (*2.2.25*), the solution shows an absorption maximum at 240 nm.

B. Dissolve 0.05 g of the material to be examined in 2 mL of *1,1,1,3,3,3-hexafluoropropan-2-ol R*. Apply to a glass plate on a water-bath in a fume cupboard several drops of the solution to produce a film of about 15 mm by 15 mm. Allow the solvent to evaporate and remove the film using a stream of water and a scraper. Dry in an oven at 100-105 °C for 1-2 h. Examine the film by infrared absorption spectrophotometry (*2.2.24*). The spectrum of the material to be examined shows maxima in particular at 1725 cm^{-1}, 1410 cm^{-1}, 1265 cm^{-1}, 1120 cm^{-1}, 1100 cm^{-1}, 1020 cm^{-1}, 875 cm^{-1}, 725 cm^{-1}. The spectrum obtained, in addition, is identical to that of the material selected for the type sample.

TESTS

If necessary, cut out samples for testing to a maximum size of 1 cm per side.

Solution S1

Place 10.0 g of the material to be examined in a borosilicate glass flask with a ground-glass neck. Add 200 mL of *water R* and heat at 50 °C for 5 h. Allow to cool and decant the solution. *Use solution S1 within 4 h of its preparation.*

Solution S2

Place 10 g of the material to be examined in a borosilicate glass flask with a ground-glass neck. Add 100 mL of *ethanol (96 per cent) R* and heat at 50 °C for 5 h. Allow to cool and decant the solution. *Use solution S2 within 4 h of its preparation.*

Solution S3

Place 20 g of the material to be examined in a borosilicate glass flask with a ground-glass neck. Add 50 mL of *0.1 M hydrochloric acid* and heat at 50 °C for 5 h. Allow to cool and decant the solution. *Use solution S3 within 4 h of its preparation.*

Solution S4

Place 20 g of the material to be examined into a borosilicate glass flask with a ground-glass neck. Add 50 mL of *0.01 M sodium hydroxide* and heat at 50 °C for 5 h. Allow to cool and decant. *Use solution S4 within 4 h of its preparation.*

Appearance of solution S1

Solution S1 is clear (*2.2.1*).

Appearance of solution S2

Solution S2 is clear (*2.2.1*) and colourless (*2.2.2, Method II*).

Acidity or alkalinity

To 50 mL of solution S1 add 0.15 mL of *BRP indicator solution R*. The solution turns yellow. Not more than 0.5 mL of *0.01 M sodium hydroxide* is required to change the colour of the indicator to blue. To another 50 mL of solution S1 add 0.2 mL of *methyl orange solution R*. The solution turns yellow. Not more than 0.5 mL of *0.01 M hydrochloric acid* is required to reach the beginning of the colour change of the indicator to orange.

Absorbance of solution S1 (*2.2.25*)

Maximum 0.20 between 220 nm and 340 nm. In addition, for coloured polyethylene terephthalate: maximum 0.05 between 400 nm to 800 nm.

Absorbance of solution S2 (*2.2.25*)

Maximum 0.05 between 400 nm and 800 nm.

Reducing substances

Add 2 mL of *0.5 M sulfuric acid* and 20.0 mL of *0.002 M potassium permanganate* to 20.0 mL of solution S1. Boil for 3 min. Cool immediately to ambient temperature. Add 1 g of *potassium iodide R*, 0.25 mL of *starch solution R* as indicator and titrate with *0.01 M sodium thiosulfate*. Perform a blank titration using 20.0 mL of *water R*. The difference in volume used in the 2 titrations is not greater than 0.5 mL.

Substances soluble in dioxan

Maximum 3 per cent.

Place 2 g of the material to be examined in a borosilicate glass flask with a ground-glass neck. Add 20 mL of *dioxan R* and heat under reflux for 2 h. Evaporate 10 mL of the solution to dryness on a water-bath and then dry the residue at 100-105 °C. The residue weighs a maximum of 30 mg.

Extractable aluminium

Maximum 1 ppm.

Inductively coupled plasma-atomic emission spectrometry (*2.2.57*).

Test solution Solution S3.

Reference solutions Prepare the reference solutions using *aluminium standard solution (200 ppm Al) R*, diluting with *0.1 M hydrochloric acid*.

Wavelength 396.15 nm, the spectral background being taken at 396.25 nm.

Verify the absence of aluminium in the *0.1 M hydrochloric acid* used.

Extractable antimony
Maximum 1 ppm.

Inductively coupled plasma-atomic emission spectrometry (*2.2.57*).

Test solution Solution S4.

Reference solutions Prepare the reference solutions using *antimony standard solution (100 ppm Sb) R*, diluting with *0.01 M sodium hydroxide*.

Wavelength 231.15 nm or 217.58 nm, the spectral background being taken at 231.05 nm.

Extractable barium
Maximum 1 ppm.

Inductively coupled plasma-atomic emission spectrometry (*2.2.57*).

Test solution Solution S3.

Reference solutions Prepare the reference solutions using *barium standard solution (50 ppm Ba) R*, diluting with *0.1 M hydrochloric acid*.

Wavelength 455.40 nm, the spectral background being taken at 455.30 nm.

Verify the absence of barium in the *0.1 M hydrochloric acid* used.

Extractable cobalt
Maximum 1 ppm.

Inductively coupled plasma-atomic emission spectrometry (*2.2.57*).

Test solution Solution S3.

Reference solutions Prepare the reference solutions using *cobalt standard solution (100 ppm Co) R*, diluting with *0.1 M hydrochloric acid*.

Wavelength 228.62 nm, the spectral background being taken at 228.50 nm.

Verify the absence of cobalt in the *0.1 M hydrochloric acid* used.

Extractable germanium
Maximum 1 ppm.

Inductively coupled plasma-atomic emission spectrometry (*2.2.57*).

Test solution Solution S4.

Reference solutions Prepare the reference solutions using *germanium standard solution (100 ppm Ge) R*, diluting with *0.01 M sodium hydroxide*.

Wavelength 206.87 nm or 265.12 nm, the spectral background being taken at 206.75 nm.

Extractable manganese
Maximum 1 ppm.

Inductively coupled plasma-atomic emission spectrometry (*2.2.57*).

Test solution Solution S3.

Reference solutions Prepare the reference solutions using *manganese standard solution (100 ppm Mn) R*, diluting with *0.1 M hydrochloric acid*.

Wavelength 257.61 nm, the spectral background being taken at 257.50 nm.

Verify the absence of manganese in the *0.1 M hydrochloric acid* used.

Extractable titanium
Maximum 1 ppm.

Inductively coupled plasma-atomic emission spectrometry (*2.2.57*).

Test solution Solution S3.

Reference solutions Prepare the reference solutions using *titanium standard solution (100 ppm Ti) R*, diluting with *0.1 M hydrochloric acid*.

Wavelength 323.45 nm or 334.94 nm, the spectral background being taken at 323.35 nm.

Verify the absence of titanium in the 0.1M hydrochloric acid used.

Extractable zinc
Maximum 1 ppm.

Inductively coupled plasma-atomic emission spectrometry (*2.2.57*).

Test solution Solution S3.

Reference solutions Prepare the reference solutions using *zinc standard solution (100 ppm Zn) R*, diluting with *0.1 M hydrochloric acid*.

Wavelength 213.86 nm, the spectral background being taken at 213.75 nm.

Verify the absence of zinc in the *0.1 M hydrochloric acid* used.

Sulfated ash (*2.4.14*)
Maximum 0.5 per cent determined on 1.0 g.

Appendix XXI

A. Abbreviated Titles

In accordance with the General Notices the main title of each monograph may be abbreviated using the following abbreviations. An abbreviated title has the same significance as the main title.

Cations

Alum.	Aluminium
Ammon.	Ammonium
Bism.	Bismuth
Calc.	Calcium
Ferr.	Ferrous
Mag.	Magnesium
Pot.	Potassium
Sod.	Sodium

Anions

Acet.	Acetate
Benz.	Benzoate
Brom.	Bromide
Chlor.	Chloride
Cit.	Citrate
Dihydrochlor.	Dihydrochloride
Fumar.	Fumarate
Hydrobrom.	Hydrobromide
Hydrochlor.	Hydrochloride
Hydrox.	Hydroxide
Iod.	Iodide
Lact.	Lactate
Mal.	Maleate
Methonit.	Methonitrate
Methylsulf.	Methylsulfate
Metilsulf.	Metilsulfate
Nit.	Nitrate
Ox.	Oxide
Phenylprop.	Phenylpropionate
Phos.	Phosphate
Prop.	Propionate
Succin.	Succinate
Sulf.	Sulfate
Tart.	Tartrate

Adjectives

Ammon.	Ammoniated
Arom.	Aromatic
Camph.	Camphorated
Co.	Compound
Conc.	Concentrated
Cryst.	Crystalline
Efferv.	Effervescent
Emulsif.	Emulsifying
Liq.	Liquefied; Liquid
Prep.	Prepared
Simp.	Simple

Preparations

Applic.	Application
Caps.	Capsules
Ext.	Extract
Inf.	Infusion
Inj.	Injection
Lin.	Liniment
Lot.	Lotion
Mixt.	Mixture
Oint.	Ointment
Pess.	Pessaries
Soln.	Solution
Suppos.	Suppositories
Tabs.	Tablets
Tinct.	Tincture

B. Approved Synonyms

Where the English title at the head of a monograph in the European Pharmacopoeia is different from that at the head of the text incorporated into the British Pharmacopoeia or the British Pharmacopoeia (Veterinary), an Approved Synonym (or Approved Synonyms) is created on the recommendation of the British Pharmacopoeia Commission.

In accordance with the General Notice on Titles, the name or names given in the right-hand column of the list below are Approved Synonyms for the name at the head of the monograph of the European Pharmacopoeia given in the left-hand column. Where there is more than one entry in the right-hand column, the first entry is used as the title of the monograph in the British Pharmacopoeia or the British Pharmacopoeia (Veterinary) and the remaining entries are included as subsidiary titles.

Approved Synonyms and subsidiary titles have the same significance as the main title and are thus official titles.

Names made by changing the order of the words in an Approved Synonym, with the addition of a preposition when necessary, are also Approved Synonyms.

Where square brackets are used in a title these may be replaced by round brackets, and *vice versa*. The words 'per cent' may be replaced by the symbol '%'.

Where the word 'Injection' appears in the title or synonym of a monograph in the European Pharmacopoeia, the abbreviation 'Inj.' is declared to be an Approved Synonym for that part of the title.

Monographs included in the British Pharmacopoeia (Veterinary) are identified by means of a superscript number:[1].

EUROPEAN PHARMACOPOEIA TITLE	APPROVED SYNONYM
Medicinal Substances and Formulated Preparations	
β-Acetyldigoxin	Acetyldigoxin
Acetylsalicylic Acid	Aspirin
N-Acetyltryptophan	Acetyltryptophan
N-Acetyltyrosine	Acetyltyrosine
Adrenaline Tartrate	Adrenaline Acid Tartrate / Epinephrine Acid Tartrate
Medicinal Air	Medical Air
Synthetic Medicinal Air	Synthetic Air
Alimemazine Hemitartrate	Alimemazine Tartrate
Hydrated Aluminium Oxide	Dried Aluminium Hydroxide
Hydrated Aluminium Phosphate	Dried Aluminium Phosphate
4-Aminobenzoic Acid	Aminobenzoic Acid
Concentrated Ammonia Solution	Strong Ammonia Solution
Ammonium Glycyrrhizate	Ammonium Glycyrrhizinate
Ammonium Hydrogen Carbonate	Ammonium Bicarbonate
Amphotericin B	Amphotericin
Amprolium Hydrochloride for Veterinary Use	Amprolium Hydrochloride[1]
Refined Arachis Oil	Arachis Oil
Azaperone for Veterinary Use	Azaperone[1]
Benzylpenicillin (Benzathine) Tetrahydrate	Benzathine Benzylpenicillin Tetrahydrate
Refined Borage (Starflower) Oil	Refined Borage Oil Refined Starflower Oil
Butyl Parahydroxybenzoate	Butyl Hydroxybenzoate Butylparaben
Butylhydroxyanisole	Butylated Hydroxyanisole
Butylhydroxytoluene	Butylated Hydroxytoluene
Caffeine Monohydrate	Caffeine Hydrate
D-Camphor	Natural Camphor
Caprylic Acid	Octanoic Acid
Carprofen for Veterinary Use	Carprofen[1]
Cefepime Dihydrochloride Monohydrate	Cefepime Hydrochloride Monohydrate
Cellulose Acetate Phthalate	Cellacefate
Cetirizine Dihydrochloride	Cetirizine Hydrochloride
Chlorhexidine Diacetate	Chlorhexidine Acetate
Chlorhexidine Digluconate Solution	Chlorhexidine Gluconate Solution
Chlorhexidine Dihydrochloride	Chlorhexidine Hydrochloride
Cholecalciferol	Colecalciferol

EUROPEAN PHARMACOPOEIA TITLE	APPROVED SYNONYM
Medicinal Substances and Formulated Preparations	
Cholecalciferol Concentrate (Oily Form)	Colecalciferol Concentrate (Oily Form)
Cholecalciferol Concentrate (Powder Form)	Colecalciferol Concentrate (Powder Form)
Clazuril for Veterinary Use	Clazuril[1]
Clodronate Disodium Tetrahydrate	Sodium Clodronate Tetrahydrate
Closantel Sodium Dihydrate for Veterinary Use	Closantel Sodium Dihydrate[1]
Refined Coconut-oil	Coconut Oil
Codeine Hydrochloride Dihydrate	Codeine Hydrochloride
Codeine Phosphate Hemihydrate	Codeine Phosphate
Cyproheptadine Hydrochloride 1.5-Hydrate	Cyproheptadine Hydrochloride
Cysteine Hydrochloride Monohydrate	Cysteine Hydrochloride
Deferoxamine Mesilate	Desferrioxamine Mesilate
Dembrexine Hydrochloride Monohydrate for Veterinary Use	Dembrexine Hydrochloride Monohydrate[1]
Detomidine Hydrochloride for Veterinary Use	Detomidine Hydrochloride[1]
Dibrompropamidine Diisetionate	Dibrompropamidine Isetionate
Diclazuril for Veterinary Use	Diclazuril[1]
Difloxacin Hydrochloride Trihydrate for Veterinary Use	Difloxacin Hydrochloride Trihydrate[1]
Dihydrocodeine Hydrogen Tartrate	Dihydrocodeine Tartrate
Dihydrostreptomycin Sulfate for Veterinary Use	Dihydrostreptomycin Sulfate[1]
Dipotassium Phosphate	Dipotassium Hydrogen Phosphate
Disodium Phosphate	Disodium Hydrogen Phosphate
Disodium Phosphate Dihydrate	Disodium Hydrogen Phosphate Dihydrate Sodium Phosphate Dihydrate
Disodium Phosphate Dodecahydrate	Disodium Hydrogen Phosphate Dodecahydrate Disodium Hydrogen Phosphate Sodium Phosphate
Dopexamine Dihydrochloride	Dopexamine Hydrochloride
Doxylamine Hydrogen Succinate	Doxylamine Succinate
Emedastine Difumarate	Emedastine Fumarate
Enilconazole for Veterinary Use	Enilconazole[1]
Enrofloxacin for Veterinary Use	Enrofloxacin[1]
Equine Serum Gonadotrophin for Veterinary Use	Serum Gonadotrophin[1]
Erythromycin Ethylsuccinate	Erythromycin Ethyl Succinate
Anhydrous Ethanol	Ethanol Absolute Alcohol Dehydrated Alcohol
Ethyl Parahydroxybenzoate	Ethyl Hydroxybenzoate Ethylparaben
Febantel for Veterinary Use	Febantel[1]
Fenbendazole for Veterinary Use	Fenbendazole[1]

EUROPEAN PHARMACOPOEIA TITLE	APPROVED SYNONYM
Medicinal Substances and Formulated Preparations	
Fipronil for Veterinary Use	Fipronil[1]
Flunixin Meglumine for Veterinary Use	Flunixin Meglumine[1]
Flupentixol Dihydrochloride	Flupentixol Hydrochloride
Fluphenazine Dihydrochloride	Fluphenazine Hydrochloride
Formaldehyde Solution (35 per cent)	Formaldehyde Solution Formalin
Foscarnet Sodium Hexahydrate	Foscarnet Sodium
Solutions for Haemofiltration and Haemodiafiltration	Haemofiltration and Haemodiafiltration Solutions
Low-molecular-mass Heparins	Low-molecular-weight Heparins
Hexamidine Diisetionate	Hexamidine Isetionate
Concentrated Hydrochloric Acid	Hydrochloric Acid
Hydrocodone Hydrogen Tartrate 2.5-Hydrate	Hydrocodone Hydrogen Tartrate Hydrate
Imidacloprid for Veterinary Use	Imidacloprid[1]
Insulin Zinc Injectable Suspension	Insulin Zinc Suspension Insulin Zinc Suspension (Mixed)
Insulin Zinc Injectable Suspension (Amorphous)	Insulin Zinc Suspension (Amorphous)
Insulin Zinc Injectable Suspension (Crystalline)	Insulin Zinc Suspension (Crystalline)
Soluble Insulin Injection	Insulin Injection Neutral Insulin Neutral Insulin Injection Soluble Insulin
Kanamycin Monosulfate	Kanamycin Sulfate
Ketotifen Hydrogen Fumarate	Ketotifen Fumarate
Liquid Lactulose	Lactulose Solution
Levamisole for Veterinary Use	Levamisole[1]
Lufenuron for Veterinary Use	Lufenuron[1]
DL-Lysine Acetylsalicylate	Aspirin Lysine
Macrogolglycerol Hydroxystearate	Hydrogenated Polyoxyl Castor Oil
Macrogolglycerol Ricinoleate	Polyoxyl Castor Oil
Magnesium Aspartate Dihydrate	Magnesium Aspartate
Magnesium Chloride 4.5-Hydrate	Partially Hydrated Magnesium Chloride
Marbofloxacin for Veterinary Use	Marbofloxacin[1]
Meclozine Dihydrochloride	Meclozine Hydrochloride
Metamizole Sodium Monohydrate	Dipyrone
Methyl Parahydroxybenzoate	Methyl Hydroxybenzoate Methylparaben
Methylene Chloride	Dichloromethane
Methylhydroxyethylcellulose	Hydroxyethylmethylcellulose
N-Methylpyrrolidone	Methylpyrrolidone

EUROPEAN PHARMACOPOEIA TITLE	APPROVED SYNONYM
Medicinal Substances and Formulated Preparations	
Milbemycin for Veterinary Use	Milbemycin Oxime[1]
Morantel Hydrogen Tartrate for Veterinary Use	Morantel Tartrate[1]
Moxidectin for Veterinary Use	Moxidectin[1]
Naftidrofuryl Hydrogen Oxalate	Naftidrofuryl Oxalate
Naloxone Hydrochloride Dihydrate	Naloxone Hydrochloride
Nitrofural	Nitrofurazone
Noradrenaline Hydrochloride	Noradrenaline Hydrochloride / Norepinephrine Hydrochloride
Noradrenaline Tartrate	Noradrenaline Acid Tartrate / Norepinephrine Acid Tartrate
Omega-3-Acid Triglycerides	Omega-3 Marine Triglycerides
Orbifloxacin for Veterinary Use	Orbifloxacin[1]
Oxfendazole for Veterinary Use	Oxfendazole[1]
Pancreas Powder	Pancreatic Extract
Pefloxacin Mesilate Dihydrate	Pefloxacin Mesilate
Pentamidine Diisetionate	Pentamidine Isetionate
Pentoxyverine Hydrogen Citrate	Pentoxyverine Citrate
Pepsin Powder	Pepsin
Perindopril *tert*-Butylamine	Perindopril Erbumine
Concentrated Phosphoric Acid	Phosphoric Acid
Pimobendan for Veterinary Use	Pimobendan[1]
Pirenzepine Dihydrochloride Monohydrate	Pirenzepine Hydrochloride
Potassium Hydrogen Carbonate	Potassium Bicarbonate
Primaquine Diphosphate	Primaquine Phosphate
Propyl Parahydroxybenzoate	Propyl Hydroxybenzoate Propylparaben
Racemic Ephedrine Hydrochloride	Racephedrine Hydrochloride
Racemic Menthol	Racementhol
Racemic Phytomenadione	Phytomenadione
Selamectin for Veterinary Use	Selamectin[1]
Selenium Disulfide	Selenium Sulfide
Sodium Ethyl Parahydroxybenzoate	Ethyl Hydroxybenzoate Sodium Ethylparaben Sodium
Sodium Hydrogen Carbonate	Sodium Bicarbonate
Sodium Laurilsulfate	Sodium Lauryl Sulfate Sodium Dodecyl Sulfate
Sodium Methyl Parahydroxybenzoate	Sodium Methyl Hydroxybenzoate Sodium Methylparaben
Hydrated Sodium Perborate	Sodium Perborate
Sodium Propyl Parahydroxybenzoate	Sodium Propyl Hydroxybenzoate Sodium Propylparaben

EUROPEAN PHARMACOPOEIA TITLE	APPROVED SYNONYM
Medicinal Substances and Formulated Preparations	
Sodium Sulfate Decahydrate	Sodium Sulfate Glauber's Salt
Hydrogenated Soya-bean Oil	Hydrogenated Soya Oil Hydrogenated Soyabean Oil
Refined Soya-bean Oil	Refined Soya Oil Refined Soyabean Oil
Spectinomycin Sulfate Tetrahydrate for Veterinary Use	Spectinomycin Sulfate Tetrahydrate[1]
Liquid Sucrose	Syrup
Sulfadimethoxine Sodium for Veterinay Use	Sulfadimethoxine Sodium[1]
Sulfamethoxypyridazine for Veterinary Use	Sulfametoxypyridazine[1]
Talc	Purified Talc
Theophylline—Ethylenediamine	Aminophylline
Theophylline—Ethylenediamine Hydrate	Aminophylline Hydrate
Theophylline Monohydrate	Theophylline Hydrate
Thiopental Sodium and Sodium Carbonate	Thiopental Sodium
Tiamulin for Veterinary Use	Tiamulin[1]
Tiamulin Hydrogen Fumarate for Veterinary Use	Tiamulin Hydrogen Fumarate[1]
all-*rac*-α-Tocopherol	all-*rac*-Alpha Tocopherol
all-*rac*-α-Tocopheryl Acetate	all-*rac*-Alpha Tocopheryl Acetate
α-Tocopheryl Acetate Concentrate (Powder Form)	Alpha Tocopheryl Acetate Concentrate (Powder Form)
DL-α-Tocopheryl Hydrogen Succinate	Alpha Tocopheryl Hydrogen Succinate
RRR-α-Tocopherol	*RRR*-Alpha Tocopherol
RRR-α-Tocopheryl Acetate	*RRR*-Alpha Tocopheryl Acetate
RRR-α-Tocopheryl Hydrogen Succinate	*RRR*-Alpha Tocopheryl Hydrogen Succinate
Tri-*n*-butyl Phosphate	Tributyl Phosphate
Triclabendazole for Veterinary Use	Triclabendazole[1]
Trimetazidine Dihydrochloride	Trimetazidine Hydrochloride
Trolamine	Triethanolamine
Tylosin for Veterinary Use	Tylosin[1]
Tylosin Phosphate Bulk Solution for Veterinary Use	Tylosin Phosphate[1]
Tylosin Phosphate Bulk Solution for Veterinary Use	Tylosin Phosphate Bulk Solution[1]
Tylosin Tartrate For Veterinary Use	Tylosin Tartrate[1]
Undecylenic Acid	Undecenoic Acid
Valnemulin Hydrochloride for Veterinary Use	Valnemulin Hydrochloride[1]
Vedaprofen for Veterinary Use	Vedaprofen[1]
Synthetic Vitamin A Concentrate (Oily Form)	Synthetic Retinol Concentrate (Oily Form)
Synthetic Vitamin A Concentrate (Powder Form)	Synthetic Retinol Concentrate (Powder Form)

EUROPEAN PHARMACOPOEIA TITLE	APPROVED SYNONYM
Medicinal Substances and Formulated Preparations	
Synthetic Vitamin A Concentrate (Solubilisate / Emulsion)	Synthetic Retinol Concentrate, Solubilisate / Emulsion Synthetic Retinol Concentrate (Water-dispersible Form)
Xylazine Hydrochloride for Veterinary Use	Xylazine Hydrochloride[1]
Zinc Acetate Dihydrate	Zinc Acetate
Zinc Undecylenate	Zinc Undecenoate

EUROPEAN PHARMACOPOEIA TITLE	APPROVED SYNONYM
Herbal Drugs	
Abelmoschi Corolla	Abelmoschus Flower
Angelica Sinensis Root	Processed Angelica Sinensis Root
Baical Skullcap Root	Scutellariae Baicalensis Root
Standardised Belladonna Leaf Tincture	Belladonna Tincture
Dried Bilberry Fruit	Dried Bilberry
Fresh Bilberry Fruit	Fresh Bilberry
Caraway Fruit	Caraway
Roman Chamomile Flower	Chamomile Flowers
Narrow-leaved Coneflower Root	Echinacea Angustifolia Root
Pale Coneflower Root	Echinacea Pallida Root
Purple Coneflower Herb	Echinacea Purpurea Herb
Purple Coneflower Root	Echinacea Purpurea Root
Devil's Claw Root	Devil's Claw Harpagophytum
Equisetum Stem	Horsetail
Fourstamen Stephania Root	Stephania Tetrandra Root
Gastrodia Rhizome	Gastrodia Elata Rhizome
Gentian Root	Gentian
Goldenseal Rhizome	Goldenseal Root
Horse-Chestnut	Horse-Chestnut Fruit
Ipecacuanha Root	Ipecacuanha
Liquorice Root	Liquorice
Matricaria Flower	Matricaria Flowers
Melissa Leaf	Lemon Balm
Melissa Leaf Dry Extract	Lemon Balm Dry Extract
Partly Dementholised Mint Oil	Dementholised Mint Oil
Raw Opium	Opium
Bitter-Orange Epicarp and Mesocarp	Dried Bitter-Orange Peel
Bitter-Orange Epicarp and Mesocarp Tincture	Orange Tincture

EUROPEAN PHARMACOPOEIA TITLE	APPROVED SYNONYM
Herbal Drugs	
Wild Pansy (Flowering Aerial Parts)	Wild Pansy
Ribwort Plantain	Plantain
Pygeum Africanum Bark	Pygeum Bark
Sage Leaf (Salvia officinalis)	Sage Leaf
Clary Sage Oil	Sage Oil
Sanguisorba Root	Greater Burnet Root
Common Selfheal Fruit-spike	Selfheal Fruit-Spike
Serratula Coronata Herb	Serratula Herb
Lightyellow Sophora Root	Sophora Flavescens Root
Valerian Root	Valerian
Cut Valerian Root	Cut Valerian

EUROPEAN PHARMACOPOEIA TITLE	APPROVED SYNONYM
Homoeopathic Preparations	
Arsenicum Album for Homoeopathic Preparations	Arsenious Trioxide for Homoeopathic Preparations
Aurum Chloratum Natronatum for Homoeopathic Preparations	Sodium Tetrachloroaurate Dihydrate for Homoeopathic Preparations
Barium Chloratum for Homoeopathic Preparations	Barium Chloride Dihydrate for Homoeopathic Preparations
Cadmium Sulfuricum for Homoeopathic Preparations	Cadmium Sulfate Hydrate for Homoeopathic Preparations
Calcium Iodatum for Homoeopathic Preparations	Calcium Iodide Tetrahydrate for Homoeopathic Preparations
Cocculus for Homoeopathic Preparations	Cocculus Indicus for Homoeopathic Preparations
Cuprum Aceticum for Homoeopathic Preparations	Copper Acetate Monohydrate for Homoeopathic Preparations
Cuprum Metallicum for Homoeopathic Preparations	Copper for Homoeopathic Preparations Copper for Homoeopathic Use
Ferrum Metallicum for Homoeopathic Preparations	Iron for Homoeopathic Preparations Iron for Homoeopathic Use
Kalium Bichromicum for Homoeopathic Preparations	Potassium Dichromate for Homoeopathic Preparations
Magnesium Phosphoricum for Homoeopathic Preparations	Magnesium Phosphate for Homoeopathic Preparations

EUROPEAN PHARMACOPOEIA TITLE	APPROVED SYNONYM
Blood-related Products	
Anticoagulant and Preservative Solutions for Human Blood	Anticoagulant and Preservative Solutions for Blood
Human Albumin Solution	Albumin Solution Albumin Human Albumin
Human Anti-D Immunoglobulin	Anti-D (Rh_0) Immunoglobulin

EUROPEAN PHARMACOPOEIA TITLE	APPROVED SYNONYM
Blood-related Products	
Human Anti-D Immunoglobulin for Intravenous Administration	Anti-D Immunoglobulin for Intravenous Use
Human Antithrombin III Concentrate	Antithrombin III Concentrate
Human Coagulation Factor VII	Dried Factor VII Fraction
Human Coagulation Factor VIIa (rDNA) Concentrated Solution	Factor VIIa (rDNA) Concentrated Solution
Human Coagulation Factor VIII	Dried Factor VIII Fraction
Human Coagulation Factor VIII (rDNA)	Dried Factor VIII (rDNA)
Human Coagulation Factor IX	Dried Factor IX Fraction
Human Coagulation Factor IX (rDNA) Concentrated Solution	Factor IX (rDNA) Concentrated Solution
Human Coagulation Factor XI	Dried Factor XI Fraction
Human Fibrinogen	Dried Fibrinogen
Human Hepatitis A Immunoglobulin	Hepatitis A Immunoglobulin
Human Hepatitis B Immunoglobulin	Hepatitis B Immunoglobulin
Human Hepatitis B Immunoglobulin for Intravenous Administration	Hepatitis B Immunoglobulin for Intravenous Use
Human Measles Immunoglobulin	Measles Immunoglobulin
Human Normal Immunoglobulin for Intramuscular Administration	Normal Immunoglobulin for Intramuscular Administration Normal Immunoglobulin Normal Immunoglobulin Injection
Human Normal Immunoglobulin for Subcutaneous Administration	Normal Immunoglobulin for Subcutaneous Administration
Human Normal Immunoglobulin for Intravenous Administration	Normal Immunoglobulin for Intravenous Use
Human Plasma for Fractionation	Plasma for Fractionation
Human Plasma (Pooled and Treated for Virus Inactivation)	Plasma (Pooled and Treated for Virus Inactivation)
Human Prothrombin Complex	Dried Prothrombin Complex
Human Rabies Immunoglobulin	Rabies Immunoglobulin
Human Rubella Immunoglobulin	Rubella Immunoglobulin
Human Tetanus Immunoglobulin	Tetanus Immunoglobulin
Human Varicella Immunoglobulin	Varicella Immunoglobulin
Human Varicella Immunoglobulin for Intravenous Administration	Varicella Immunoglobulin for Intravenous Use
Human von Willebrand Factor	von Willebrand Factor

EUROPEAN PHARMACOPOEIA TITLE	APPROVED SYNONYM
Immunological Products (Human)	
BCG Vaccine, Freeze-dried	Bacillus Calmette-Guérin Vaccine BCG Vaccine
Diphtheria and Tetanus Vaccine (Adsorbed)	Adsorbed Diphtheria and Tetanus Vaccine
Diphtheria, Tetanus and Pertussis (Acellular Component) Vaccine (Adsorbed)	Adsorbed Diphtheria, Tetanus and Pertussis (Acellular Component) Vaccine
Diphtheria, Tetanus, Pertussis (Acellular Component) and Haemophilus Type b Conjugate Vaccine (Adsorbed)	Adsorbed Diphtheria, Tetanus, Pertussis (Acellular Component) and Haemophilus Type b Conjugate Vaccine
Diphtheria, Tetanus, Pertussis (Acellular Component) and Hepatitis B (rDNA) Vaccine (Adsorbed)	Adsorbed Diphtheria, Tetanus, Pertussis (Acellular Component) and Hepatitis B (rDNA) Vaccine
Diphtheria, Tetanus, Pertussis (Acellular Component) and Poliomyelitis (Inactivated) Vaccine (Adsorbed)	Adsorbed Diphtheria, Tetanus, Pertussis (Acellular Component) and Inactivated Poliomyelitis Vaccine
Diphtheria Vaccine (Adsorbed)	Adsorbed Diphtheria Vaccine
Hepatitis A Vaccine (Inactivated, Adsorbed)	Inactivated Hepatitis A Vaccine
Hepatitis A (Inactivated) and Hepatitis B (rDNA) Vaccine (Adsorbed)	Hepatitis A (Inactivated) and Hepatitis B (rDNA) Vaccine
Influenza Vaccine (Split Virion, Inactivated)	Inactivated Influenza Vaccine (Split Virion)
Influenza Vaccine (Surface Antigen, Inactivated)	Inactivated Influenza Vaccine (Surface Antigen)
Influenza Vaccine (Whole Virion, Inactivated)	Inactivated Influenza Vaccine (Whole Virion)
Measles Vaccine (Live)	Measles Vaccine, Live
Measles, Mumps and Rubella Vaccine (Live)	Measles, Mumps and Rubella Vaccine, Live
Mumps Vaccine (Live)	Mumps Vaccine, Live
Tuberculin for Human Use, Old	Old Tuberculin
Pertussis Vaccine (Acellular Component, Adsorbed)	Adsorbed Pertussis Vaccine (Acellular Component)
Pertussis Vaccine (Acellular, Co-purified, Adsorbed)	Adsorbed Pertussis Vaccine (Acellular, Co-purified)
Poliomyelitis Vaccine (Inactivated)	Inactivated Poliomyelitis Vaccine
Poliomyelitis Vaccine (Oral)	Poliomyelitis Vaccine, Live (Oral)
Rabies Vaccine for Human Use Prepared in Cell Cultures	Rabies Vaccine
Tetanus Antitoxin for Human Use	Tetanus Antitoxin
Tetanus Vaccine (Adsorbed)	Adsorbed Tetanus Vaccine
Tick-borne Encephalitis Vaccine (Inactivated)	Tick-borne Encephalitis Vaccine, Inactivated
Tuberculin Purified Protein Derivative for Human Use	Tuberculin Purified Protein Derivative Tuberculin P.P.D.
Typhoid Vaccine (Live, Oral, Strain Ty 21a)	Typhoid (Strain Ty 21a) Vaccine, Live (Oral)
Yellow Fever Vaccine (Live)	Yellow Fever Vaccine, Live

Immunological Products (Veterinary) ***Monographs included in the British Pharmacopoeia (Veterinary)***	
Anthrax Spore Vaccine (Live) for Veterinary Use	Anthrax Vaccine, Living
Aujeszky's Disease Vaccine (Inactivated) for Pigs	Aujeszky's Disease Vaccine, Inactivated

Immunological Products (Veterinary) ***Monographs included in the British Pharmacopoeia (Veterinary)***	
Aujeszky's Disease Vaccine (Live) for Pigs for Parenteral Administration	Aujeszky's Disease Vaccine, Living
Avian Infectious Bronchitis Vaccine (Live)	Avian Infectious Bronchitis Vaccine, Living
Avian Infectious Bursal Disease Vaccine (Inactivated)	Infectious Bursal Disease Vaccine, Inactivated Gumboro Disease Vaccine, Inactivated
Avian Infectious Bursal Disease Vaccine (Live)	Infectious Bursal Disease Vaccine, Living Gumboro Disease Vaccine, Living
Avian Infectious Encephalomyelitis Vaccine (Live)	Infectious Avian Encephalomyelitis Vaccine, Living Epidemic Tremor Vaccine, Living
Avian Infectious Laryngotracheitis Vaccine (Live)	Laryngotracheitis Vaccine, Living
Avian Paramyxovirus 3 Vaccine (Inactivated) for Turkeys	Avian Paramyxovirus 3 Vaccine for Turkeys, Inactivated
Bovine Parainfluenza Virus Vaccine (Live)	Bovine Parainfluenza Virus Vaccine, Living
Bovine Respiratory Syncytial Virus Vaccine (Live)	Bovine Respiratory Syncytial Virus Vaccine, Living
Infectious Bovine Rhinotracheitis Vaccine (Live)	Infectious Bovine Rhinotracheitis Vaccine, Living
Brucellosis Vaccine (Live) (Brucella Melitensis Rev. 1 Strain) for Veterinary Use	Brucella Melitensis (Strain Rev. 1) Vaccine, Living
Canine Adenovirus Vaccine (Inactivated)	Canine Adenovirus Vaccine, Inactivated
Canine Adenovirus Vaccine (Live)	Canine Adenovirus Vaccine, Living
Canine Distemper Vaccine (Live)	Canine Distemper Vaccine, Living
Canine Parvovirosis Vaccine (Inactivated)	Canine Parvovirus Vaccine, Inactivated
Canine Parvovirosis Vaccine (Live)	Canine Parvovirus Vaccine, Living
Clostridium Botulinum Vaccine for Veterinary Use	Clostridium Botulinum Vaccine Botulinum Vaccine
Clostridium Chauvoei Vaccine for Veterinary Use	Clostridium Chauvoei Vaccine Blackleg Vaccine
Clostridium Novyi (Type B) Vaccine for Veterinary Use	Clostridium Novyi Type B Vaccine Black Disease Vaccine
Clostridium Perfringens Vaccine for Veterinary Use	Clostridium Perfringens Vaccines
Type B	Clostridium Perfringens Type B Vaccine Lamb Dysentery Vaccine
Type C	Clostridium Perfringens Type C Vaccine Struck Vaccine
Type D	Clostridium Perfringens Type D Vaccine Pulpy Kidney Vaccine
Clostridium Septicum Vaccine for Veterinary Use	Clostridium Septicum Vaccine Braxy Vaccine
Distemper Vaccine (Live) for Mustelids	Ferret and Mink Distemper Vaccine, Living
Egg Drop Syndrome '76 Vaccine (Inactivated)	Egg Drop Syndrome 76 (Adenovirus) Vaccine
Equine Herpesvirus Vaccine (Inactivated)	Equine Herpesvirus Vaccine, Inactivated
Equine Influenza Vaccine (Inactivated)	Equine Influenza Vaccine, Inactivated
Feline Calicivirosis Vaccine (Inactivated)	Feline Calicivirus Vaccine, Inactivated
Feline Calicivirosis Vaccine (Live)	Feline Calicivirus Vaccine, Living

Immunological Products (Veterinary) ***Monographs included in the British Pharmacopoeia (Veterinary)***	
Feline Infectious Enteritis (Feline Panleucopenia) Vaccine (Inactivated)	Feline Infectious Enteritis Vaccine, Inactivated Feline Panleucopenia Vaccine, Inactivated
Feline Infectious Enteritis (Feline Panleucopenia) Vaccine (Live)	Feline Infectious Enteritis Vaccine, Living Feline Panleucopenia Vaccine, Living
Feline Leukaemia Vaccine (Inactivated)	Feline Leukaemia Vaccine, Inactivated
Feline Viral Rhinotracheitis Vaccine (Inactivated)	Feline Viral Rhinotracheitis Vaccine, Inactivated
Feline Viral Rhinotracheitis Vaccine (Live)	Feline Viral Rhinotracheitis Vaccine, Living
Foot-and-Mouth Disease (Ruminants) Vaccine (Inactivated)	Foot and Mouth Disease (Ruminants) Vaccine
Fowl-pox Vaccine (Live)	Fowl Pox Vaccine, Living
Furunculosis Vaccine (Inactivated, Oil-adjuvanted, Injectable) for Salmonids	Furunculosis Vaccine for Salmonids, Inactivated
Marek's Disease Vaccine (Live)	Marek's Disease Vaccine, Living Marek's Disease Vaccine (Turkey Herpes Virus) Marek's Disease Vaccine, Living (HVT)
Neonatal Piglet Colibacillosis Vaccine (Inactivated)	Porcine E. Coli Vaccine, Inactivated Porcine Escherichia Coli Vaccine, Inactivated
Neonatal Ruminant Colibacillosis Vaccine (Inactivated)	Ruminant E. Coli Vaccine, Inactivated Ruminant Escherichia Coli Vaccine, Inactivated
Newcastle Disease Vaccine (Inactivated)	Newcastle Disease Vaccine, Inactivated
Newcastle Disease Vaccine (Live)	Newcastle Disease Vaccine, Living
Porcine Actinobacillosis Vaccine (Inactivated)	Porcine Actinobacillosis Vaccine, Inactivated
Porcine Influenza Vaccine (Inactivated)	Swine Influenza Vaccine, Inactivated
Porcine Parvovirosis Vaccine (Inactivated)	Porcine Parvovirus Vaccine, Inactivated
Porcine Progressive Atrophic Rhinitis Vaccine (Inactivated)	Porcine Progressive Atrophic Rhinitis Vaccine, Inactivated
Rabies Vaccine (Inactivated) for Veterinary Use	Rabies Veterinary Vaccine, Inactivated
Swine Erysipelas Vaccine (Inactivated)	Swine Erysipelas Vaccine, Inactivated
Tetanus Antitoxin for Veterinary Use	Clostridium Tetani Antitoxin Tetanus Antitoxin (Veterinary)
Tetanus Vaccine for Veterinary Use	Clostridium Tetani Vaccines Tetanus Toxoids (Veterinary) *The following titles may be used for vaccines with an appropriate potency:* Clostridium Tetani Vaccine for Equidae Tetanus Toxoid for Equidae
Vibriosis (Cold-water) Vaccine (Inactivated) for Salmonids	Cold-water Vibriosis Vaccine for Salmonids, Inactivated
Vibriosis Vaccine (Inactivated) for Salmonids	Vibriosis Vaccine for Salmonids, Inactivated
Enteric Redmouth Disease Vaccine (Inactivated) for Rainbow Trout	Enteric Redmouth Disease Vaccine for Rainbow Trout (Inactivated)

EUROPEAN PHARMACOPOEIA TITLE	APPROVED SYNONYM
Radiopharmaceutical Preparations	
Human Albumin Injection, Iodinated (^{125}I)	Iodinated (^{125}I) Albumin Injection
Technetium (^{99m}Tc) Human Albumin Injection	Technetium (^{99m}Tc) Albumin Injection

When used in the practice of homoeopathy, certain substances that are the subject of a monograph in the European Pharmacopoeia have been known traditionally by a name other than that at the head of the Ph. Eur. monograph. The name or names given in the right-hand column of the list below are Approved Synonyms that may be used only in relation to homoeopathic preparations for the name at the head of a monograph in the European Pharmacopoeia given in the left-hand column.

EUROPEAN PHARMACOPOEIA TITLE	APPROVED SYNONYM FOR HOMOEOPATHIC USE
Substances used in Homoeopathy	
Ammonium Chloride	Ammonium Muriaticum
Atropine Sulfate	Atropinum Sulfuricum
Caraway Fruit	Carum Carvi
Cascara	Cascara Sagrada
Cholesterol	Cholesterinum
Cinchona Bark	China
Cinnamon	Cinnamomum
Eucalyptus Leaf	Eucalyptus
Bitter Fennel	Foeniculum Vulgare
Lithium Carbonate	Lithium Carbonicum
Magnesium Chloride Hexahydrate	Magnesium Muriaticum
Magnesium Sulfate	Magnesium Sulfuricum
Mercuric Chloride	Mercurius Sublimatus Corrosivus Mercurius Corrosivus
Potassium Chloride	Kali Muriaticum
Quinine Sulfate	Chininum Sulfuricum
Rhatany Root	Ratanhia
Rhubarb	Rheum Palmatum
Senega Root	Senega
Silver Nitrate	Argentum Nitricum
Sodium Carbonate Monohydrate	Natrium Carbonicum
Sodium Chloride	Natrium Muriaticum
Anhydrous Sodium Sulfate	Natrium Sulfuricum
Valerian Root	Valeriana
Zinc Sulfate	Zincum Sulfuricum

C. Eye drops

Codes for eye drops in single-dose containers

The following codes are approved for use on single unit doses of eye drops where the individual container may be too small to bear all of the appropriate labelling information (see the general monograph for Eye Drops).

The inclusion of a preparation in this list does not necessarily mean that a monograph is or will be included in the *British Pharmacopoeia*.

Eye Drops	Code	Eye Drops	Code
Adrenaline / Epinephrine	ADN / EPN	Lidocaine and Fluorescein	LIDFLN
Apraclonidine	ACD	Metipranolol	MPR
Atropine Sulfate	ATR	Moxisylyte	MOX
Betamethasone	BET	Neomycin	NEO
Carbachol	CAR	Oxybuprocaine	OXB
Castor Oil	CASOIL	Phenylephrine	PHNL
Chloramphenicol	CPL	Phenylephrine and Cyclopentolate	PHNCYC
Cocaine	CCN	Pilocarpine	PIL
Cyclopentolate	CYC	Povidone-Iodine	PVI
Diclofenac	DICL	Prednisolone	PRED
Fluorescein	FLN	Proxymetacaine	PROX
Dexamethasone Sodium Phosphate	DSP	Proxymetacaine and Fluorescein	PROXFLN
Gentamicin	GNT	Rose Bengal	ROS
Homatropine	HOM	Sodium Chloride	SALINE[1]
Hydrocortisone	HCOR	Sodium Cromoglicate	SCG
Hydroxyethylcellulose and Sodium Chloride	HECL	Sulfacetamide	SULF
		Tetracaine	TET
Hyoscine	HYO	Timolol	TIM
Hypromellose	HPRM	Tropicamide	TRO
Lachesine	LAC	Zinc Sulfate	ZSU

[1] The term 'Saline' indicates the contents of the container are a 0.9% w/v solution of Sodium Chloride.

Appendix XXII

A. Viral Safety

(Ph. Eur. general texts 5.1.7)

This chapter provides general requirements concerning the viral safety of medicinal products whose manufacture has involved the use of materials of human or animal origin. Since viral safety is a complex issue, it is important that a risk assessment is carried out. Requirements to be applied to a specific medicinal product are decided by the competent authority.

Where the risk of viral contamination exists, complementary measures are used as appropriate to assure the viral safety of medicinal products, based on:

- selection of source materials and testing for viral contaminants;
- testing the capacity of the production process to remove and/or inactivate viruses;
- testing for viral contamination at appropriate stages of production.

Where appropriate, one or more validated procedures for removal or inactivation of viruses are applied.

Further detailed recommendations on viral safety, including validation studies, are provided, in particular, by the *Note for guidance on virus validation studies: the design, contribution and interpretation of studies validating the inactivation and removal of viruses (CPMP/BWP/268/95)* of the Committee for Proprietary Medicinal Products, and the *ICH guideline Q5A: Viral safety evaluation of biotechnology products derived from cell lines of human or animal origin* (including any subsequent revisions of these documents).

Requirements concerning immunological products for veterinary use are dealt with in the monographs *Vaccines for veterinary use (0062)* and *Immunosera for veterinary use (0030)* and related general chapters.

Risk assessment

A risk assessment with respect to viral safety is carried out where materials of human or animal origin are used as ingredients of medicinal products or in the manufacture of active substances, excipients or medicinal products.

The principle of the risk assessment is to consider various factors that may influence the potential level of infectious particles in the medicinal product and factors related to the use of the medicinal product that determine or influence the viral risk to the recipients.

The risk assessment takes into consideration relevant factors, for example:

- the species of origin;
- the organ, tissue, fluid of origin;
- the potential contaminants in view of the origin of the raw material and the history of the donor(s), preferably including epidemiological data;
- the potential contaminants from the manufacturing process (for example, from risk materials used during manufacture);
- the infectivity and pathogenicity of the potential contaminants for the intended recipients of the medicinal product, taking account of the route of administration of the medicinal product;
- the amount of material used to produce a dose of medicinal product;
- controls carried out on the donor(s), on the raw material, during production and on the final product;
- the manufacturing process of the product and its capacity to remove and/or inactivate viruses.

The risk assessment can be based mainly on the manufacturing conditions if these include rigorous inactivation steps (for example, for gelatin etc., and products terminally sterilised by steam or dry heat as described in the general texts on sterility (*5.1*)).

B. Minimising the Risk of Transmitting Animal Spongiform Encephalopathy Agents Via Human and Veterinary Medicinal Products

(Ph. Eur. general texts 5.2.8)

This chapter is identical with the Note for Guidance on Minimising the Risk of Transmitting Animal Spongiform Encephalopathy Agents via Human and Veterinary Medicinal Products – Revision 3, (EMA/410/01 rev. 3).

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1 INTRODUCTION

1-1 SCIENTIFIC BACKGROUND

Transmissible Spongiform Encephalopathies (TSEs) are chronic degenerative nervous diseases characterised by the accumulation of an abnormal isoform of a cellular glycoprotein (known as PrP or prion protein). The abnormal

isoform of PrP (PrP^{TSE}) differs from normal PrP (PrPc) in being highly resistant to protease and heat denaturation treatments. PrP^{TSE} is considered to be the infective agent responsible for transmitting TSE disease.

TSE diseases in animals include:
- bovine spongiform encephalopathy (BSE) in cattle,
- scrapie in sheep and goats,
- chronic wasting disease (CWD) in cervids (deer and elk),
- transmissible mink encephalopathy (TME) in farmed mink,
- feline spongiform encephalopathy (FSE) in felids (specifically domestic cats and captive large cats), and
- spongiform encephalopathy of exotic ungulates in zoos.

In humans, spongiform encephalopathies include different forms of Creutzfeldt-Jakob Disease (CJD), Kuru, Gerstmann-Sträussler-Scheinker Syndrome (GSS), and Fatal Familial Insomnia (FFI).

Iatrogenic transmission of spongiform encephalopathies has been reported. In sheep, scrapie has been accidentally transmitted by the use of Louping Ill vaccine prepared from pooled, formaldehyde treated ovine brain and spleen in which material from scrapie-infected sheep had been inadvertently incorporated. Also, transmission of scrapie to sheep and goats occurred following use of a formol-inactivated vaccine against contagious agalactia, prepared with brain and mammary gland homogenates of sheep infected with *Mycoplasma agalactiae.* In man, cases of transmission of CJD have been reported which have been attributed to the parenteral administration of growth hormone and gonadotropin derived from human cadaveric pituitary glands. Cases of CJD have also been attributed to the use of contaminated instruments in brain surgery and with the transplantation of human dura mater and cornea.

Interspecies TSE transmission is restricted by a number of natural barriers, transmissibility being affected by the species of origin, the prion strain, dose, route of exposure and, in some species, the host allele of the PRNP gene. Species barriers can be crossed under appropriate conditions.

BSE was first diagnosed in the United Kingdom in 1986 and a large number of cattle and individual herds have been affected. It is clear that BSE is a food borne disease associated with feed (e.g. meat and bone meal) derived from TSE affected animals. Other countries have experienced cases of BSE, either in animals imported from the United Kingdom or in indigenous animals. There is convincing evidence to show that the variant form of CJD (vCJD) is caused by the agent which is responsible for BSE in cattle. Therefore, a cautious approach continues to be warranted if biological materials from species naturally affected by TSE diseases, especially bovine species, are used for the manufacture of medicinal products.

In the course of active surveillance programs, two previously unrecognized forms of atypical BSE (BSE-L, also named BASE, and BSE-H) have been identified in rare sporadic cases from Europe, North America, and Japan. The 'L' and 'H' identify the higher and lower electrophoretic positions of their protease-resistant PrP^{TSE} isoforms. It is noteworthy that atypical cases have been found in countries that did not experience classical BSE so far, like Sweden, or in which only few classical BSE cases have been found like Canada or USA. The atypical BSE agent has been experimentally transmitted to transgenic mice expressing the human prion protein and to a cynomolgus monkey.

Scrapie occurs worldwide and has been reported in most European countries. It has the highest incidence in Cyprus.

While humans have been exposed to naturally occurring scrapie for over 250 years, there is no epidemiological evidence directly linking scrapie to spongiform encephalopathies in humans[1]. However, there remains a theoretical and currently unquantifiable risk that some BSE-contaminated protein supplement may have been fed to sheep. Further, it should also be assumed that any BSE agent introduced into the small ruminant population via contaminated feed is likely to be recycled and amplified[2].

There is interest in infecting cells with TSE agents to develop assays and for basic scientific reasons. Some success has been reported, usually but not always with neural cell lines. The conditions needed to infect a cell are not well understood and the process is difficult requiring particular combinations of agent and cell. It is not considered appropriate to make specific recommendations in terms of cell substrates to be used for production of biological/biotechnology-derived substances. Nevertheless, the possibility of infection of cell lines with TSE agents should be taken into account in risk assessments.

1-2 REGULATORY COMPLIANCE

Risk assessment

Since the use of animal-derived materials is unavoidable for the production of some medicinal products and that complete elimination of risk at source is rarely possible, the measures taken to manage the risk of transmitting animal TSEs via medicinal products represent risk minimisation rather than risk elimination. Consequently, the basis for regulatory compliance should be based on a risk assessment, taking into consideration all pertinent factors as identified in this chapter (see below).

Legal basis

The note for guidance is published by the European Commission following
- Annex I, part I, module 3, section 3.2: *Content: basic principles and requirements*, point (9) of Directive 2001/83/EC of the European Parliament and of the Council of 6 November 2001 on the Community code relating to medicinal products for human use[3], as amended, and
- Annex I, Title I, part 2, section C *Production and control of starting material* of Directive 2001/82/EC of the European Parliament and of the Council of 6 November 2001 on the Community code relating to veterinary medicinal products[4], as amended.

These directives require that applicants for marketing authorisation for human and veterinary medicinal products must demonstrate that medicinal products are manufactured in accordance with the latest version of this note for guidance published in the *Official Journal of the European Union.* This is a continuing obligation after the marketing authorisation has been granted.

By definition, the principle of Specified Risk Materials as defined in Regulation (EC) No 999/2001 of the European Parliament and of the Council[5] does not apply to medicinal

[1] *This is currently being assessed by EFSA and ECDC. For updated information, please refer to the following link: http://registerofquestions.efsa.europa.eu/roqFrontend/questionsListLoader?mandate=M-2009-0221*

[2] *In January 2005, after confirmation of BSE in a goat in France, additional legislative measures were taken related to monitoring and an increased testing of small ruminants. The increased surveillance did not identify additional cases of BSE in sheep and goats in the EU.*

[3] *OJ L 311, 28.11.2001, p. 67.*

[4] *OJ L 311, 28.11.2001, p. 1.*

[5] *OJ L 147, 31.5.2001, p. 1.*

products. However, Regulation (EC) No 1774/2002 of the European Parliament and of the Council[6], which applies since 1st May 2003, lays down health rules concerning animal by-products not intended for human consumption. As a general rule, and unless properly justified, all animal by-products used as starting materials in the manufacture of medicinal products should be 'Category 3 (i.e. safe) materials or equivalent', as defined in Regulation (EC) No 1774/2002. Justification for the use of substances derived from other, high infectivity materials must follow an appropriate benefit/risk evaluation (see further below).

The note for guidance should be read in conjunction with the various EU legal instruments including Commission decisions progressively implemented since 1991. Where appropriate, references to these decisions are given in the text. Position statements and explanatory notes made by the Committee for Medicinal Products for Human Use (CHMP) and Committee for Medicinal Products for Veterinary Use (CVMP) are still applicable for the purpose of regulatory compliance unless otherwise superseded by the note for guidance.

The general monograph *Products with risk of transmitting agents of animal spongiform encephalopathies* of the European Pharmacopoeia refers to this chapter, which is identical with the note for guidance. The monograph forms the basis for issuing Certificates of Suitability as a procedure for demonstrating TSE compliance for substances and materials used in the manufacture of human and veterinary medicinal products.

Clarification of note for guidance

As the scientific understanding of TSEs, especially the pathogenesis of the diseases, is evolving, from time to time CHMP and its Biologics Working Party in collaboration with CVMP and its Immunologicals Working Party may be required in the future to develop supplementary guidance in the form of position statements or explanatory notes for the purpose of clarifying the note for guidance.

The supplementary guidance shall be published by the Commission and on the website of the European Medicines Agency and taken into consideration accordingly in the scope of the certification of the European Directorate for the Quality of Medicines & HealthCare (EDQM).

2 SCOPE

TSE-RELEVANT ANIMAL SPECIES

Cattle, sheep, goats and animals that are naturally susceptible to infection with transmissible spongiform encephalopathy agents or susceptible to infection through the oral route other than humans[7] and non-human primates are defined as "TSE-relevant animal species"[8].

MATERIALS

This chapter is concerned with materials derived from "TSE-relevant animal species" that are used for the preparation of:

— active substances,

— excipients and adjuvants, and

— raw and starting materials and reagents used in production (e.g. bovine serum albumin, enzymes, culture media including those used to prepare working cell banks, or new master cell banks for medicinal products which are subject to a new marketing authorisation).

This chapter is also applicable to materials that come into direct contact with the equipment used in manufacture of the medicinal product or that come in contact with the medicinal product and therefore have the potential for contamination.

Materials used in the qualification of plant and equipment, such as culture media used in media fill experiments to validate the aseptic filling process, shall be considered in compliance with this chapter provided that the constituent or constituents are derived from tissues with no detectable infectivity (category IC tissues), where the risk of cross-contamination with potentially infective tissues has been considered (see section 3-3) and where the materials are sourced from countries with negligible BSE risk or controlled BSE risk (Categories A and B, respectively – see section 3-2). Such information shall be provided in the dossier for a marketing authorisation and verified during routine inspection for compliance with Good Manufacturing Practice (GMP).

Other materials such as cleaning agents, softeners and lubricants that come into contact with the medicinal product during its routine manufacture or in the finishing stage or in the primary packaging are considered in compliance with this chapter if they are tallow derivatives prepared using the rigorous physicochemical processes as described in section 6.

SEED LOTS, CELL BANKS AND ROUTINE FERMENTATION/PRODUCTION[9]

For the purpose of regulatory compliance, master seeds or master cell banks in marketing authorisation applications lodged after 1 July 2000 (for human medicinal products) or 1 October 2000 (for veterinary medicinal products) shall be covered by the note for guidance.

Master seeds and master cell banks,

— for vaccine antigens,

— for a biotechnology-derived medicinal product as described in the Annex to Regulation (EC) No 726/2004 of the European Parliament and of the Council[10], and

— for other medicinal products using seed lots or cell banking systems in their manufacture,

that have already been approved for the manufacture of a constituent of an authorised medicinal product shall be considered in compliance with the note for guidance even if they are incorporated in marketing authorisation applications lodged after 1 July 2000 (for human medicinal products) or 1 October 2000 (for veterinary medicinal products).

Master cell banks and master seeds established before 1 July 2000 (for human medicinal products) or 1 October 2000 (for veterinary medicinal products), but not yet approved as a constituent of an authorised medicinal product shall demonstrate that they fulfil the requirements of the note for guidance. If, for some raw or starting materials or reagents used for the establishment of these cell banks or seeds, full documentary evidence is no longer available, the

[6] *OJ L 273, 10.10.2002, p. 1. Regulation (EC) 1774/2002 has been repealed by Regulation (EC) 1069/2009 that will apply from 4 March 2011 (OJ L 300, 14.11.2009, p. 1).*

[7] *Regulatory guidance and position papers have been issued by the Committee for Medicinal Products for Human Use and its Biologics Working Party on human tissue derived medicinal products in relation to CJD and vCJD. Such guidance can be found on http://www.ema.europa.eu*

[8] *Pigs and birds, which are animal species of particular interest for the production of medicinal products, are not naturally susceptible to infection via the oral route. Therefore they are not TSE-relevant animal species within the meaning of this chapter. Also dogs, rabbits and fish are non TSE-relevant animal species within the meaning of this chapter.*

[9] *See also: Position paper on the assessment of the risk of transmission of animal spongiform encephalopathy agents by master seed materials used in the production of veterinary vaccines (EMEA/CVMP/019/01-February 2001 adopted by the Committee for Medicinal Products for Veterinary Use (CVMP) in July 2001, (OJ C 286, 12.10.2001, p. 12)).*

[10] *OJ L 136, 30.4.2004, p. 1.*

applicant should present a risk assessment as described in Section 4 of the note for guidance.

Established working seeds or cell banks used in the manufacture of medicinal products authorised before 1 July 2000 (human medicines) or 1 October 2000 (veterinary medicines), which have been subjected to a properly conducted risk assessment by a Competent Authority of the Member States or the European Medicines Agency and declared to be acceptable, shall also be considered compliant.

However, where materials derived from the "TSE-relevant animal species" are used in fermentation/routine production processes or in the establishment of working seeds and working cell banks, the applicant must demonstrate that they fulfil the requirements of the note for guidance.

3 GENERAL CONSIDERATIONS

3-1 SCIENTIFIC PRINCIPLES FOR MINIMISING RISK

When manufacturers have a choice, the use of materials from "non TSE-relevant animal species" or non-animal origin is preferred. The rationale for using materials derived from "TSE-relevant animal species" instead of materials from "non-TSE-relevant species" or of non-animal origin should be given. If materials from "TSE-relevant animal species" have to be used, consideration should be given to all the necessary measures to minimise the risk of transmission of TSE.

Readily applicable diagnostic tests for TSE infectivity *in vivo* are not yet available. Diagnosis is based on post-mortem confirmation of characteristic brain lesions by histopathology and/or detection of PrP^{TSE} by Western blot or immunoassay. The demonstration of infectivity by the inoculation of suspect tissue into target species or laboratory animals is also used for confirmation. However, due to the long incubation periods of all TSEs, results of *in vivo* tests are available only after months or years.

Several immunochemical tests have been developed for the detection of PrP^{TSE} in post-mortem samples and some are now considered to be extremely sensitive. However, their ability to detect an infected animal depends on the timing of sample collection in relation to timing of exposure, the type of tissue collected and infectious dose acquired, together with consequential timing of onset of clinical disease. There is currently insufficient information on how this might be affected by strain variations.

Although screening of source animals by *in vitro* tests may prevent the use of animals at late stages of incubation of the disease and may provide information about the epidemiological status of a given country or region, none of the tests are considered suitable to unambiguously confirm the negative status of an animal.

Minimising the risks of transmission of TSE is based upon three complementary parameters:

— the source animals and their geographical origin,
— nature of animal material used in manufacture and any procedures in place to avoid cross-contamination with higher risk materials,
— production process(es) including the quality assurance system in place to ensure product consistency and traceability.

3-2 ANIMAL SOURCE

The source materials used for the production of materials for the manufacture of medicinal products shall be derived from animals fit for human consumption following ante- and post-mortem inspection in accordance with EU or equivalent (third country) conditions, except for materials derived from live animals, which should be found healthy after clinical examination.

3-2-1 Geographical sourcing

3-2-1-1 Bovine materials

The World Organisation for Animal Health (OIE)[11] lays down the criteria for the assessment of the status of countries in the chapter of the International Animal Health Code on bovine spongiform encephalopathy. Countries or regions are classified as follows:

A. countries or regions with a negligible BSE risk;
B. countries or regions with a controlled BSE risk;
C. countries or regions with an undetermined BSE risk.

As stipulated in Commission Regulation (EC) No 999/2001, as amended[12], the classification of countries or regions thereof according to their BSE risk, based on the rules laid down by OIE, is legally binding in the EU since 1 July 2007. Commission Decision 2007/453/EC[13] as amended, provides the classification of countries or regions according to their BSE risk.

Previously, the European Commission Scientific Steering Committee (SSC)[14] had established a temporary system for classifying the countries according to their geographical BSE risk (GBR)[15].

For the purposes of this chapter the BSE classification based on the OIE rules should be used. If a country, which was previously classified in accordance to the SSC GBR criteria, has not been classified yet according to the OIE rules, the GBR classification can be used until OIE classification has taken place, provided that there is no evidence of significant change in its BSE risk[16].

Where there is a choice, animals should be sourced from countries with the lowest possible BSE risk (negligible BSE risk countries (Category A)) unless the use of material from countries with a higher BSE risk is justified. Some of the materials identified in Section 6, "Specific Conditions" can be sourced from countries with controlled BSE risk (Category B) and, in some cases, from countries with

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[11] *http://www.oie.int/eng/Status/BSE/en_BSE_free.htm*

[12] *Regulation (EC) No 722/2007 (OJ L 164, 26.6.2007, p. 7)*

[13] *OJ L 172, 30.6.2007, p. 84*

[14] *The Scientific Steering Committee established by Commission Decision 97/404/EC (OJ L 169, 27.6.1997, p. 85) shall assist the Commission to obtain the best scientific advice available on matters relating to consumer health. Since May 2003, its tasks have been taken over by the European Food Safety Authority (EFSA): http://www.efsa.europa.eu*

[15] *The European Scientific Steering Committee classification for geographical BSE risk (GBR) gives an indication of the level of likelihood of the presence of one or more cattle clinically or pre-clinically infected with BSE in a given country or region. A definition of the four categories is provided in the following Table.*

GBR level	Presence of one or more cattle clinically or pre-clinically infected with BSE in a geographical region/country
I	Highly unlikely
II	Unlikely but not excluded
III	Likely but not confirmed or confirmed at a lower level
IV	Confirmed at a higher level (≥ 100 cases/1 Million adult cattle per year)

Reports of the GBR assessment of the countries are available on the SSC website (http://ec.europa.eu/food/fs/sc/ssc/outcome_en.html)

[16] *Experts consider that the GBR classification system is stable enough, so that it can continue to be used, during the interim period, for the demonstration of compliance with this chapter.*

undetermined BSE risk (Category C), provided that the controls and requirements as specified in the relevant sections below are applied. Apart from these exceptions, animals must not be sourced from countries with undetermined BSE risk (Category C), and justifications for the use of animals from countries with undetermined BSE risk (Category C) must always be provided.

3-2-1-2. Sheep and goats (small ruminants)

Naturally occurring clinical scrapie cases have been reported in a number of countries worldwide. As BSE in sheep and goats could possibly be mistaken for scrapie, as a precautionary measure, sourcing of materials derived from small ruminants shall take into account the prevalence of both BSE and scrapie in the country and the tissues from which the materials are derived.

The principles related to "BSE negligible risk (closed) bovine herds" (see section 3-2-2) could equally be applied in the context of small ruminants in order to develop a framework to define the TSE status of a flock of small ruminants.
For sheep, because of the concern over the possibility of BSE in sheep, the use of a genotype(s) showing resistance to BSE/scrapie infection could be considered in establishing TSE free flocks[17]. However, the possibility that genotypes resistant to scrapie could be susceptible to BSE (experimental oral exposure) or atypical scrapie (natural cases) should also be taken into account. Goats have not been studied sufficiently with regard to a genotype specific sensitivity.

Material of small ruminant origin should preferably be sourced from countries with a long history of absence of scrapie. Justification shall be required if the material is sourced from some other origin.

3-2-2 BSE negligible risk (closed) bovine herds

The safest sourcing is from countries or regions with a negligible risk (Category A countries). Other countries may have or have had cases of BSE at some point in time and the practical concept of "Negligible risk (closed) bovine herds" has been developed by the SSC and endorsed by the CHMP and CVMP. Criteria for establishing and maintaining a "BSE negligible risk (closed) bovine herd" can be found in the SSC opinion of 22-23 July 1999[18].

For the time being it is not possible to quantify the reduction of the geographical BSE risk for cattle from BSE 'negligible risk (closed) bovine herds'. However, it is expected that this risk reduction is substantial. Therefore, sourcing from such closed bovine herds shall be considered in the risk assessment in conjunction with the OIE classification of the country.

3-3 ANIMAL PARTS, BODY FLUIDS AND SECRETIONS AS STARTING MATERIAL

In a TSE infected animal, different organs and secretions have different levels of infectivity. If materials from 'TSE-relevant animal species' have to be used, consideration should be given to use materials of the lowest category of risk. The tables given in the Annex of this chapter[19] summarise current data about the distribution of infectivity and PrP^{TSE} in cattle with BSE, and in sheep and goats with scrapie[20].

The information in the tables is based exclusively upon observations of naturally occurring disease or primary experimental infection by the oral route (in cattle) but does not include data on models using strains of TSE that have been adapted to experimental animals, because passaged strain phenotypes can differ significantly and unpredictably from those of naturally occurring disease. Because immunohistochemical and/or Western blot detection of misfolded host protein (PrP^{TSE}) have proven to be a surrogate marker of infectivity, PrP^{TSE} testing results have been presented in parallel with bioassay data. Tissues are grouped into three major infectivity categories, irrespective of the stage of disease:

Category IA	**High-infectivity tissues** **central nervous system (CNS) tissues that attain a high titre of infectivity in the later stages of all TSEs, and certain tissues that are anatomically associated with the CNS**
Category IB	**Lower-infectivity tissues** peripheral tissues that have tested positive for infectivity and/or PrP^{TSE} in at least one form of TSE
Category IC	**Tissues with no detectable infectivity** tissues that have been examined for infectivity, without any infectivity detected, and/or PrP^{TSE}, with negative results

Category IA tissues and substances derived from them shall not be used in the manufacture of medicinal products, unless justified (see Section 5).

Although the category of lower risk tissues (category IB tissues) almost certainly includes some (e.g. blood) with a lower risk than others (e.g. lymphoreticular tissues), the data about infectivity levels in these tissues are too limited to subdivide the category into different levels of risk. It is also evident that the placement of a given tissue in one or another category can be disease and species specific, and subject to revision as new data emerge.

For the risk assessment (see section 4), manufacturers and/or marketing authorisation holders/applicants shall take into account the tissue classification tables in the Annex to this chapter.

The categories in the tables are only indicative and it is important to note the following points.

— In certain situations there could be **cross-contamination** of tissues of different categories of infectivity.
The potential risk will be influenced by the circumstances in which tissues were removed, especially by contact of tissues with lower-infectivity tissues or no detectable infectivity (categories IB and IC tissues) with high-infectivity tissues (category IA tissues). Thus, cross-contamination of some tissues may be increased if infected animals are slaughtered by brain stunning (penetrative or non penetrative) or if the brain and/or spinal cord is sawed. The risk of cross-contamination will be decreased if body fluids are collected with minimal damage to tissue and cellular components are removed, and if foetal blood is collected without contamination

[17] *Opinion of the Scientific Panel on Biological Hazards on 'the breeding programme for TSE resistance in sheep': http://www.efsa.europa.eu/EFSA/efsa_locale-1178620753812_1178620775678.htm*

[18] *SSC Scientific Opinion on the conditions related to "BSE Negligible Risk (Closed) Bovine Herds" adopted at the meeting of 22-23 July 1999. http://ec.europa.eu/food/fs/sc/ssc/out56_en.html*

[19] *The tissue classification tables are based upon the most recent WHO Guidelines on Tissue Infectivity Distribution in Transmissible Spongiform Encephalopathies (2010) http://www.who.int/bloddproducts/tablestissueinfectivity.pdf*

[20] *A Scientific opinion on BSE/TSE infectivity in small ruminant tissues is currently being reviewed by EFSA (Question No EFSA-Q-2010-052). For updated information please follow this link: http://registerofquestions.efsa.europa.eu/roqFrontend/questionsListLoader?mandate=M-2010-0041*

from other maternal or foetal tissues including placenta, amniotic and allantoic fluids. For certain tissues, it is very difficult or impossible to prevent cross-contamination with category IA tissues (e.g. skull). This has to be considered in the risk assessment.

— For certain classes of substances the **stunning/slaughtering techniques** used may be important in determining the potential risk[21] because of the likelihood of disseminating the brain particles into the peripheral organs, particularly to the lungs. Stunning/slaughtering techniques should be described as well as the procedures to remove high infectivity tissues. The procedures to collect the animal tissues/organs to be used and the measures in place to avoid cross-contamination with a higher risk material must also be described in detail.

— The risk of contamination of tissues and organs with BSE-infectivity potentially harboured in central nervous material as a consequence of the stunning method used for cattle slaughtering depends on the following factors:
  — the amount of BSE-infectivity in the brain of the slaughtered animal,
  — the extent of brain damage,
  — the dissemination of brain particles in the animal body.

These factors must be considered in conjunction with the OIE/GBR classification of the source animals, the age of the animals in the case of cattle and the *post-mortem* testing of the cattle using a validated method.

The underlying principles indicated above would be equally applicable to sheep and goats.

The risk posed by cross-contamination will be dependent on several complementary factors including:

— measures adopted to avoid contamination during collection of tissues (see above),
— level of contamination (amount of the contaminating tissue),
— amount and type of materials collected at the same time.

Manufacturers or the marketing authorisation holders/applicants should take into account the risk with respect to cross-contamination.

3-4 AGE OF ANIMALS

As the TSE infectivity accumulates in bovine animals over an incubation period of several years, it is prudent to source from young animals.

Presence of infectious material has essentially been reported in the central nervous system and related tissues, as well as in the lymphoreticular system, depending on the TSE agent (BSE in cattle or scrapie in sheep and goat). The exact time course of infectivity in the respective body parts and tissues, from the date of infection, is not known in both species and, as such, it is difficult to give clear guidance on the age above which the various tissues may be infected and should not be collected. The initial recommendation to collect tissues in the youngest age is still valid. In addition, it is noteworthy that the age criteria depend also on the geographical origin. Age is a more important parameter for materials from countries where the risk is higher (Category B and C countries), than from countries with a negligible BSE risk (Category A countries).

3-5 MANUFACTURING PROCESS

The assessment of the overall TSE risk reduction of a medicinal product shall take into account the control measures instituted with respect to:

— sourcing of the raw/starting materials, and
— the manufacturing process.

Controlled sourcing is a very important criterion in achieving acceptable safety of the product, due to the documented resistance of TSE agents to most inactivation procedures.

A quality assurance system, such as ISO 9000 certification, HACCP[22] or GMP, must be put in place for monitoring the production process and for batch delineation (i.e. definition of batch, separation of batches, cleaning between batches). Procedures shall be put in place to ensure traceability as well as self-auditing and to auditing suppliers of raw/starting materials.

Certain production procedures may contribute considerably to the reduction of the risk of TSE contamination, e.g. procedures used in the manufacture of tallow derivatives (see section 6). As such rigorous processing cannot be applied to many products, processes involving physical removal, such as precipitation and filtration to remove prion-rich material, are likely to be more appropriate than chemical treatments. A description of the manufacturing process, including in-process controls applied, shall be presented and the steps that might contribute to reduction or elimination of TSE contamination should be discussed. Whenever different manufacturing sites are involved, the steps performed at each site shall be clearly identified. The measures in place in order to ensure traceability of every production batch to the source material should be described.

Cleaning process

Cleaning of process equipment may be difficult to validate for the elimination of TSE agents. It is reported that after exposure to high titre preparations of TSE agent, detectable infectivity can remain bound to the surface of stainless steel. The removal of all adsorbed protein by the use of 1 M sodium hydroxide or chlorine releasing disinfectants (e.g. 20 000 ppm chlorine for 1 h) have been considered acceptable approaches where equipment that cannot be replaced has been exposed to potentially contaminated material. Milder treatments with limited concentrations of alkali or stabilized bleach, when properly formulated with detergents and used at specified temperatures, have been shown to exhibit similar efficiency for removing prions as did classical NaOH or chlorine treatments. A system based on vaporised hydrogen peroxide also appeared to be efficient for inactivating TSE agents. These new treatments are more compatible with delicate materials and may be suitable for practical use[23].

If risk materials are used in the manufacture of a product, cleaning procedures, including control measures, shall be put in place in order to minimise the risk of cross-contamination between production batches. This is especially important if materials from different risk categories are handled in the same plant with the same equipment. In the case of using

[21] *SSC opinion on stunning methods and BSE risk (The risk of dissemination of brain particles into the blood and carcass when applying certain stunning methods), adopted at the meeting of 10-11 January 2002. http://ec.europa.eu/food/fs/sc/ssc/out245_en.pdf. Report of the EFSA Working group on BSE risk from dissemination of brain particles in blood and carcass. Question No EFSA-Q-2003-122, adopted on 21 October 2004, http://www.efsa.europa.eu/EFSA/efsa_locale-1178620753812_1178620777397.htm*

[22] *Hazard Analysis Critical Control Point.*

[23] *WHO Guidelines on Tissue Infectivity Distribution in Transmissible Spongiform Encephalopathies (2006) http://www.who.int/bloodproducts/tse/WHO%20TSE%20Guidelines%20FINAL-22%20JuneupdatedNL.pdf*

category IA materials in the manufacture of a product, dedicated equipment shall be used, unless otherwise justified.

Further research is needed to develop and validate new decontamination procedures to lower the risk of cross-contamination for material and devices which are not compatible with WHO-recommended procedures.

Removal/Inactivation validation

Validation studies of removal/inactivation procedures for TSEs can be difficult to interpret. It is necessary to take into consideration the nature of the spiked material and its relevance to the natural situation, the design of the study (including scaling-down of processes) and the method of detection of the agent (*in vitro* or *in vivo* assay). Further research is needed to develop an understanding of the most appropriate "spike preparation" for validation studies. Therefore, validation studies are currently not generally required. However, if claims are made for the safety of the product with respect to TSEs based on the ability of manufacturing processes to remove or inactivate TSE agents, they must be substantiated by appropriate investigational studies[24].

In addition to appropriate sourcing, manufacturers are encouraged to continue their investigations into removal and inactivation methods to identify steps/processes that would have benefit in assuring the removal or inactivation of TSE agents. In any event, a production process wherever possible shall be designed taking account of available information on methods which are thought to inactivate or remove TSE agents.

For certain types of products (see section 6-3 Bovine blood and blood derivatives), where validated removal/inactivation is not readily applicable, process evaluation might be required. This should be based on the starting material and any published data on TSE risk.

4 RISK ASSESSMENT OF MATERIALS OR SUBSTANCES USED IN THE MANUFACTURE AND PREPARATION OF A MEDICINAL PRODUCT IN THE CONTEXT OF REGULATORY COMPLIANCE

The assessment of the risk associated with TSE needs careful consideration of all of the parameters as outlined in section 3-1 (Scientific Principles for Minimising Risk).

As indicated in the introduction to this chapter, regulatory compliance is based on a favourable outcome from a risk assessment. The risk assessments, conducted by the manufacturers and/or the marketing authorisation holders or applicants for the different materials or substances from "TSE-relevant animal species" used in the manufacture of a medicinal product shall show that all TSE risk factors have been taken into account and, where possible, risk has been minimised by application of the principles described in this chapter. TSE Certificates of suitability issued by the EDQM may be used by the marketing authorisation holders or applicants as the basis of the risk assessments.

An overall risk assessment for the medicinal product, conducted by the marketing authorisation holders or applicants, shall take into account the risk assessments for all the different materials from "TSE-relevant animal species" and, where appropriate, TSE reduction or inactivation by the manufacturing steps of the active substance and/or finished product.

The final determination of regulatory compliance rests with the competent authority.

It is incumbent upon the manufacturers and/or the marketing authorisation holders or applicants for both human and veterinary medicinal products to select and justify the control measures for a given "TSE-relevant animal species" derivative, taking into account the latest scientific and technical progress.

5 BENEFIT/RISK EVALUATION

In addition to the parameters as mentioned in sections 3 (that may be covered by a TSE Certificate of Suitability issued by the EDQM) and 4, the acceptability of a particular medicinal product containing materials derived from a "TSE-relevant animal species", or which as a result of manufacture could contain these materials, shall take into account the following factors:

- route of administration of the medicinal product,
- quantity of animal material used in the medicinal product,
- maximum therapeutic dosage (daily dose and duration of treatment),
- intended use of the medicinal product and its clinical benefit,
- presence of a species barrier.

High-infectivity tissues (category IA tissues) and substances derived thereof shall not be used in manufacture of medicinal products, their starting materials and intermediate products (including active substances, excipients and reagents), unless justified. A justification why no other materials can be used shall be provided. In these exceptional and justified circumstances, the use of high-infectivity tissues could be envisaged for the manufacture of active substances, when, after performing the risk assessment as described in Section 4 of this chapter, and taking into account the intended clinical use, a positive benefit/risk assessment can be presented by the marketing authorisation applicant. Substances from category IA materials, if their use is justified, must be produced from animals of countries with negligible BSE risk (Category A).

6 SPECIFIC CONSIDERATIONS

The following materials prepared from "TSE-relevant animal species" are considered in compliance with this chapter provided that they meet at least the conditions specified below. The relevant information or a certificate of suitability granted by the EDQM shall be provided by the marketing authorisation applicant/holder.

6-1 COLLAGEN

Collagen is a fibrous protein component of mammalian connective tissue.

For collagen, documentation to demonstrate compliance with this chapter needs to be provided taking into account the provisions listed in sections 3 to 5. In addition, consideration should be given to the following.

- For collagen produced from bones, the conditions specified for gelatin are applicable (see below). Lower inactivation capacity is expected from the collagen manufacturing process than from that of gelatin. Therefore, sourcing becomes a more critical aspect to consider.
- Collagen produced from tissues such as hides, skins, tendons and sinews do not usually present a measurable TSE risk provided that contamination with potentially infected materials, for example spillage of blood and/or central nervous tissues, is avoided during procurement. Therefore, hides represent a safer raw material for human implants derived from collagen. However, cross-contamination with brain material released during the

[24] *Guideline on the investigation of manufacturing process for plasma-derived medicinal products with regard to vCJD risk CPMP/BWP/5136/03*

slaughtering process that may have dried on the surface of hides would be difficult to eliminate. This is another aspect to consider in the evaluation of safety of this source material.

The collagen manufacturing process can have some steps in common with the manufacture of gelatin such as alkaline and sodium sulphate treatment, calcium hydroxide and sodium hydroxide treatments or enzyme treatment. However, even these common steps can differ in duration and pH condition which can result in significant differences in their inactivation capacity. Manufacturers should at least conduct a process evaluation based on the similarities of the collagen processing steps, as compared to known inactivation steps in the manufacture of gelatin, in order to support the safety of the product. In addition to processing, differences also exist in the final use of the material and, consequently, in their risk assessment, while gelatin is widely used for oral administration, many collagen applications are in the form of surgical implants. This aspect should also be considered in the final risk assessment.

6-2 GELATIN

Gelatin is a natural, soluble protein, gelling or non-gelling, obtained by the partial hydrolysis of collagen produced from bones, hides and skins of animals.

For gelatin, documentation to demonstrate compliance with this chapter needs to be provided taking into account the provisions listed in sections 3 to 5. In addition, consideration should be given to the following[25].

The source material used

Gelatin used in medicinal products can be manufactured from bones or hides.

Hides as the starting material On the basis of current knowledge, hides used for gelatin production represent a safer source material as compared to bones. However, it is highly recommended that measures should be put in place to avoid cross-contamination with potentially infected materials during procurement.

Bones as the starting material Where bones are used to manufacture gelatin, the quality of the starting materials needs to be controlled as an additional parameter to ensure the safety of the final product. Therefore, the following should be applied.

1. Skulls and spinal cord shall be removed from the collected bones (raw/starting material) independent of the age or the country of origin of the cattle.
2. Vertebrae shall be removed from the raw/starting materials from cattle over 30 months from countries with a controlled or an undetermined BSE risk (Categories B or C).
3. Gelatin for parenteral use should only be manufactured from bones coming from countries with a negligible or a controlled BSE risk (Category A and B, respectively). Gelatin for oral use can be manufactured from bones from countries with a negligible, a controlled or an undetermined BSE risk (Category A, B and C, respectively).
4. Gelatin shall be manufactured using one of the manufacturing methods described below.

Manufacturing methods

Hides No specific measures with regard to the processing conditions are required for gelatin produced from hides provided that control measures are put in place to avoid cross-contamination both during the procurement of the hides and during the manufacturing process.

Bones Where bones are used as the starting material, the mode of manufacture will be the second parameter that will ensure the safety of gelatin.

- Gelatin can be manufactured from bones from countries with a negligible, a controlled or an undetermined BSE risk (Categories A, B or C) sourced in accordance with the conditions described in section 6-2 under The source material used, using the acid, alkaline or heat/pressure manufacturing process.
- The manufacturing process shall be taken into consideration when performing the risk assessment as described in Section 4 of this chapter. Both the acid and the alkaline manufacturing methods have shown similar overall inactivation/removal of TSE infectivity in the gelatin validation experiments. Studies have shown that an additional alkaline treatment (pH 13, 2 h) of the bones/ossein further increases the TSE inactivation/removal capacity of the manufacturing process. Other processing steps such as filtration, ion-exchange chromatography and UHT sterilisation also contributes to the safety of gelatin.
- For a typical alkaline manufacturing process, bones are finely crushed, degreased with hot water and demineralised with dilute hydrochloric acid (at a minimum of 4 per cent and pH < 1.5) over a period of at least 2 days to produce the ossein. This is followed by an alkaline treatment with saturated lime solution (pH at least 12.5) for a period of at least 20 days.
- Bovine bones may also be treated by an acid process. The liming step is then replaced by an acid pre-treatment where the ossein is treated at pH < 3.5 for a minimum of 10 hours.
- A "flash" heat treatment (sterilisation) step at 138 °C minimum for 4 s at least is applied to both acid and alkaline manufacturing process.
- In the heat/pressure process, the dried degreased crushed bones are autoclaved with saturated steam at a pressure greater than 3 bar and a minimum temperature of 133 °C, for at least 20 min, followed by extraction of the protein with hot water.

The finishing steps are similar for the alkaline, acid and heat/pressure process and include extraction of the gelatine, washing, filtration and concentration.

6-3 BOVINE BLOOD AND BLOOD DERIVATIVES

Foetal bovine serum is commonly used in cell cultures. Foetal bovine serum should be obtained from foetuses harvested in abattoirs from healthy dams fit for human consumption and the womb should be completely removed and the foetal blood harvested in dedicated space or area by cardiac puncture into a closed collection system using aseptic technique.

Newborn calf serum is obtained from calves under 20 days old and calf serum from animals under the age of 12 months. In the case of donor bovine serum, given that it may be derived from animals less than 36 months old, the TSE negative status of the donor herd shall be well defined and documented. In all cases, serum shall be collected according

[25] *Based on the Opinion of the Scientific Panel on Biological Hazards of the European Food Safety Authority on the 'Quantitative assessment of the human BSE risk posed by gelatine with respect to residual BSE risk'. The EFSA Journal, 312, (1-28). http://www.efsa.europa.eu/EFSA/efsa_locale-1178620753812_1178620776107.htm*
The requirements for source material selection and manufacture are appropriate for oral or parenteral gelatin for use in human and veterinary medicinal products.

to specified protocols by personnel trained in these procedures to avoid cross-contamination with higher risk tissues.

For bovine blood and blood derivatives, documentation to demonstrate compliance with this chapter needs to be provided taking into account the provisions listed in sections 3 to 5. In addition, consideration should be given to the following.

Traceability

Traceability to the slaughterhouse must be assured for each batch of serum or plasma. Slaughterhouses must have available lists of farms from which the animals are originated. If serum is produced from living animals, records must be available for each serum batch which assures the traceability to the farms.

Geographical origin

Whilst tissue infectivity of BSE in cattle is more restricted than scrapie, as a precautionary measure bovine blood should be sourced from Category A countries. Bovine blood from Category B countries is also acceptable provided that there is no risk for cross-contamination of blood with brain material from the slaughter of animals over 21 months[26] of age.

Stunning methods

If it is sampled from slaughtered animals, the method of slaughter is of importance to assure the safety of the material. It has been demonstrated that stunning by captive bolt stunner with or without pithing as well as by pneumatic stunner, especially if it injects air, can destroy the brain and disseminate brain material into the blood stream. Non-penetrative stunning is no more considered as an alternative to penetrative stunning because contamination of blood with brain material has been demonstrated[27]. Negligible risk can be expected from electro-narcosis[28], but this even does not provide strict safety because, when unsuccessful, animals may have to be additionally stunned. The stunning methods must therefore be described for the bovine blood collection process.

Whenever a risk of cross-contamination of blood with brain cannot be avoided at routine slaughtering in countries with a controlled BSE risk (Category B), safety measures such as restriction of the age of the cattle and/or reduction of infectious agents during manufacture have to be applied.

Age

For countries with a controlled BSE risk (Category B), a precautionary age limit of 21 months shall apply for bovine blood or blood derivatives where no significant reduction of TSE agents can be assumed from manufacture. An age limit of 30 months is considered sufficient for blood derivatives where significant reduction of TSE agents can be demonstrated as described below.

Reduction of TSE agents during manufacture

For blood derivatives, the capacity of the manufacturing process to reduce/eliminate TSE agents should be estimated from investigational studies. The estimation may be based on published data or in house data whenever it can be shown that such data is relevant to the specific manufacturing process. If it cannot be concluded that the reduction capacity is comparable, it is recommended that manufacturers undertake product-specific investigational studies. Investigations using biochemical assay may be sufficient if there is scientific evidence that this assay correlates with infectivity data. General guidance for investigational studies on reduction of TSE agents has been outlined[29]. Brain-derived spike preparations are appropriate for studies investigating the risk from brain-contaminated blood.

6-4 TALLOW DERIVATIVES

Tallow is fat obtained from tissues including subcutaneous, abdominal and inter-muscular areas and bones. Tallow used as the starting material for the manufacture of tallow derivatives shall be 'Category 3 material or equivalent', as defined in Regulation (EC) No 1774/2002 of the European Parliament and of the Council of 3 October 2002 laying down health rules concerning animal by-products not intended for human consumption.

Tallow derivatives, such as glycerol and fatty acids, manufactured from tallow by rigorous processes are thought unlikely to be infectious and they have been the subject of specific consideration by CHMP and CVMP. For this reason, such materials manufactured under the conditions at least as rigorous as those given below shall be considered in compliance for this chapter, irrespective of the geographical origin and the nature of the tissues from which tallow derivatives are derived. Examples of rigorous processes are:

- trans-esterification or hydrolysis at not less than 200 °C for not less than 20 min under pressure (glycerol, fatty acids and fatty acid esters production),
- saponification with 12 M NaOH (glycerol and soap production):
  - batch process: at not less than 95 °C for not less than 3 h,
  - continuous process: at not less than 140 °C, under pressure for not less than 8 min, or equivalent,
- distillation at 200 °C.

Tallow derivatives manufactured according to these conditions are unlikely to present any TSE risk and shall therefore be considered compliant with this chapter.

Tallow derivatives produced using other conditions must demonstrate compliance with this chapter.

6-5 ANIMAL CHARCOAL

Animal charcoal is prepared by carbonisation of animal tissues, such as bones, using temperatures higher than 800 °C. Unless otherwise justified, the starting material for the manufacture of animal charcoal shall be Category 3 material or equivalent, as defined in Regulation (EC) No 1774/2002 of the European Parliament and of the Council of 3 October 2002 laying down health rules concerning animal by-products not intended for human consumption. Irrespective of the geographical origin and the nature of the tissue, for the purpose of regulatory compliance, animal charcoal shall be considered in compliance with this chapter.

Charcoal manufactured according to these conditions is unlikely to present any TSE risk and shall therefore be considered compliant with this chapter. Charcoal produced using other conditions must demonstrate compliance with this chapter.

[26] *Opinion of the Scientific Panel on Biological Hazards on the assessment of the age limit in cattle for the removal of certain Specified Risk Materials (SRM). Question No EFSA-Q-2004-146, adopted on 28 April 2005*

[27] *The tissue classification tables are based upon the most recent WHO Guidelines on Tissue Infectivity Distribution in Transmissible Spongiform Encephalopathies (2010) http://www.who.int/bloodproducts/tablestissueinfectivity.pdf*

[28] *Report of the EFSA Working Group on BSE risk from dissemination of brain particles in blood and carcass. Question No EFSA-Q-2003-112, adopted on 21 October 2004, http://www.efsa.europa.eu/en/sciencebiohaz/biohaz_opinions/opinion_annexes/733.html*

[29] *Guideline on the investigation of manufacturing process for plasma-derived medicinal products with regard to vCJD risk CPMP/BWP/5136/03.*

Table 5.2.8.-1. – *Concept for acceptance of bovine blood/sera and derivatives*

Product	Foetal bovine serum	Donor calf serum	Adult bovine donor serum	Calf serum	Adult bovine serum / plasma	Adult bovine serum / plasma / serum derivative	Adult bovine serum derivative	Adult bovine serum derivative
Geographical origin of cattle	Cat. A and B	Cat. A and B	Cat. A and B[1]	Cat. A and B	Cat. A	Cat. B	Cat. A	Cat. B
Age of cattle	unborn	< 1 year	< 36 months	< 1 year	No limit	< 21 months[2]	No limit	< 30 months
Slaughtering/cross-contamination of blood with CNS material	No risk of cross-contamination			Risk of cross-contamination				
Demonstration of Prion reduction during manufacture	No			No				Yes[3]

1. When sourced in Category B countries, cattle should be from well-defined and documented herds.
2. A higher age may be allowed if cross-contamination of blood with CNS material can be clearly ruled out (e.g. halal slaughter).
3. Demonstration of prion reduction may not be required if cross-contamination of blood with CNS material can be clearly ruled out (e.g. halal slaughter).

6-6 MILK AND MILK DERIVATIVES

In the light of the current scientific knowledge and irrespective of the geographical origin, bovine milk is unlikely to present any risk of TSE contamination[30].

Certain materials, including lactose, are extracted from whey, the spent liquid from cheese production following coagulation. Coagulation can involve the use of calf rennet, an extract from abomasum, or rennet derived from other ruminants. The CHMP/CVMP have performed a risk assessment for lactose and other whey derivatives produced using calf rennet and concluded that the TSE risk is negligible if the calf rennet is produced in accordance with the process described in the risk assessment report[31].
The conclusion was endorsed by the SSC[32] which has also performed an assessment of the TSE risk of rennet in general[33].

Bovine milk derivatives manufactured according to the conditions described below are unlikely to present any TSE risk and shall therefore be considered compliant with this chapter.

— The milk is sourced from healthy animals in the same conditions as milk collected for human consumption, and
— no other ruminant materials, with the exception of calf rennet, are used in the preparation of such derivatives (e.g. pancreatic enzyme digests of casein).

Milk derivatives produced using other processes or rennet derived from other ruminant species must demonstrate compliance with this chapter.

6-7 WOOL DERIVATIVES

Derivatives of wool and hair of ruminants, such as lanolin and wool alcohols derived from hair shall be considered in compliance with this chapter, provided the wool and hair are sourced from live animals.

Wool derivatives produced from wool which is sourced from slaughtered animals declared "fit for human consumption" and the manufacturing process in relation to pH, temperature and duration of treatment meets at least one of the stipulated processing conditions listed below are unlikely to present any TSE risk and shall therefore be considered compliant with this chapter.

— Treatment at pH $\geq$ 13 (initial; corresponding to a NaOH concentration of at least 0.1 M NaOH) at 60 °C for at least 1 h. This occurs normally during the reflux stage of the organic-alkaline treatment.
— Molecular distillation at $\geq$ 220 °C under reduced pressure.

Wool derivatives produced using other conditions must demonstrate compliance with this chapter.

6-8 AMINO ACIDS

Amino acids can be obtained by hydrolysis of materials from various sources.

Unless otherwise justified, the starting material for the manufacture of amino acids shall be 'Category 3 material or equivalent', as defined in Regulation (EC) No 1774/2002 of the European Parliament and of the Council of 3 October 2002 laying down health rules concerning animal by-products not intended for human consumption.

Amino acids prepared using the following processing conditions are unlikely to present any TSE risk and shall be considered compliant with this chapter:

— amino acids produced from hides and skins by a process which involves exposure of the material to a pH of 1 to 2, followed by a pH of > 11, followed by heat treatment at 140 °C for 30 min at 3 bar,
— the resulting amino acids or peptides must be filtered after production, and
— analysis is performed using a validated and sensitive method to control any residual intact macromolecules, with an appropriate limit set.

Amino acids prepared using other conditions must demonstrate compliance with this chapter.

6-9 PEPTONES

Peptones are partial hydrolysates of protein, achieved by enzymic or acid digestion. They are used in microbiological culture media to support the nutritional requirements of micro-organisms, which might be used as seed stocks or in industrial scale fermentations for the production of human and veterinary medicinal products, including vaccines. There

[30] *For milk and milk derivatives from small ruminants, please see EFSA opinion on Question No EFSA-Q-2008-310, adopted on 22 October 2008, http://www.efsa.europa.eu/en/scdocs/scdoc/849.htm*

[31] *Committee for Medicinal Products for Human Use and its Biologics Working Party conducted a risk and regulatory assessment of lactose prepared using calf rennet. The risk assessment included the source of the animals, the excision of the abomasums and the availability of well-defined quality assurance procedures. The quality of any milk replacers used as feed for the animals from which abomasums are obtained is particularly important. The report can be found on http://www.ema.europa.eu/pdfs/human/press/pus/057102.pdf*

[32] *Provisional statement on the safety of calf-derived rennet for the manufacture of lactose, adopted by the SSC at its meeting of 4-5 April 2002 (http://ec.europa.eu/food/fs/sc/ssc/out255_en.pdf)*

[33] *The SSC issued an opinion on the safety of animal rennet in regard to risks from animal TSE and BSE in particular, adopted at its meeting of 16 May 2002 (http://ec.europa.eu/food/fs/sc/ssc/out265_en.pdf)*

is considerable interest in the use of vegetable protein as an alternative to animal sourced protein. However:

— where gelatin is used as the protein source material, reference is made to section 6-2 Gelatin, of this chapter,
— where casein is used as the protein source material, reference is made to section 6-6 Milk and milk derivatives, of this chapter,
— where tissue of TSE-relevant animal species is the protein source material, the tissue must be sourced from animals fit for consumption (see section 3-2 Source animals, of this chapter) with a maximum age of 30 months old for cattle from countries with a controlled BSE risk (Category B). The age of animals is of minimal concern for animals from countries with a negligible BSE risk (Category A).

ANNEX: MAJOR CATEGORIES OF INFECTIVITY

The tables below are taken from the *WHO Guidelines on Tissue Infectivity Distribution in Transmissible Spongiform Encephalopathies (2010)*.

Data entries are shown as follows:

+	=	Presence of infectivity or PrP^{TSE}
–	=	Absence of detectable infectivity or PrP^{TSE}
NT	=	Not tested
NA	=	Not applicable
?	=	Uncertain interpretation
0	=	Limited or preliminary data
[]	=	Infectivity or PrP^{TSE} data based exclusively on bioassays in transgenic (Tg) mice over-expressing the PrP-encoding gene or PRP^{TSE} amplification methods

Category IA: High-infectivity tissues

Tissue	Cattle BSE		Sheep and goats Scrapie		Elk and deer CWD	
	Infectivity[1]	PrP^{TSE}	Infectivity[1]	PrP^{TSE}	Infectivity[1]	PrP^{TSE}
Brain	+	+	+	+	+	+
Spinal cord	+	+	+	+	NT	+
Retina	+	NT	NT	+	NT	+
Optic nerve[2]	+	NT	NT	+	NT	+
Spinal ganglia	+	+	+	+	NT	+
Trigeminal ganglia	+	+	NT	+	NT	-
Pituitary gland[3]	–	NT	+	+	NT	+
Dura mater[3]	NT	NT	NT	NT	NT	NT

Category IB: Lower-infectivity tissues

Tissue	Cattle BSE		Sheep and goats Scrapie		Elk and deer CWD	
	Infectivity[1]	PrP^{TSE}	Infectivity[1]	PrP^{TSE}	Infectivity[1]	PrP^{TSE}
Peripheral nervous system						
Peripheral nerves	[+]	+	+	+	NT	+
Autonomic ganglia[4]	NT	+	NT	+	NT	+
Lymphoreticular tissues						
Spleen	–	–	+	+	NT	+
Lymph nodes	–	–	+	+	NT	+
Tonsil	+	–	+	+	NT	+
Nictitating membrane	+	–	[+]	+	NT	+
Thymus	–	NT	+	+	NT	–
Alimentary tract[5]						
Oesophagus	–	NT	[+]	+	NT	+
Fore-stomach[6] (ruminants only)	–	NT	[+]	+	NT	+
Stomach/abomasum	–	NT	[+]	+	NT	+
Duodenum	–	–	[+]	+	NT	+
Jejunum[7]	–	+	[+]	+	NT	NT
Ileum[7]	+	+	+	+	NT	+
Appendix	NA	NA	NA	NA	NA	NA
Colon/caecum[7]	–	–	+	+	NT	+
Rectum	NT	NT	NT	+	NT	+
Reproductive tissues						
Placenta[8]	–	NT	+	+	NT	–
Ovary[3]	–	NT	–	–	NT	–
Uterus[3]	–	NT	–	–	NT	–
Other tissues						
Mammary gland/udder[9]	–	NT	–	+	NT	NT
Skin[3, 10]	–	NT	–	+	[+]	[+]
Adipose tissue	–	NT	NT	NT	[+]	NT
Heart/pericardium	–	NT	–	NT	NT	+
Lung	–	NT	–	–	NT	+
Liver[3]	–	NT	+	–	NT	–
Kidney[3, 11]	–	–	[+]	+	NT	+
Adrenal	[+]	+	+	–	NT	+
Pancreas[3]	–	NT	+	NT	NT	+
Bone marrow[12]	[+]	NT	+	NT	NT	–
Skeletal muscle[13]	[+]	NT	[+]	+	[+]	–
Tongue[14]	–	NT	[+]	+	NT	–
Blood vessels	–	NT	NT	+	NT	–
Nasal mucosa[15]	–	NT	+	+	NT	+
Salivary gland	–	NT	+	NT	–	–
Cornea[16]	NT	NT	NT	NT	NT	NT
Body fluids, secretion and excretions						
CSF	–	NT	+	–	NT	NT
Blood[17]	–	?	+	?	+	?
Saliva	NT	NT	–	NT	+	[–]
Milk[18]	–	–	+	[+]	NT	NT
Urine[19]	–	NT	–	–	–[+]	[+]
Feces[19]	–	NT	–	NT	–[+]	NT

Category IC: Tissues with no detectable infectivity

Tissue	Cattle BSE		Sheep and goats Scrapie		Elk and deer CWD	
	Infectivity[1]	PrP^{TSE}	Infectivity[1]	PrP^{TSE}	Infectivity[1]	PrP^{TSE}
Reproductive tissues						
Testis	–	NT	–	–	NT	–
Prostate/Epididymis/ Seminal vesicle	–	NT	–	–	NT	–
Semen	–	NT	–	–	NT	NT
Placenta fluids	–	NT	NT	NT	NT	NT
Foetus[20]	–	NT	–	–	NT	(–)
Embryos[20]	–	NT	?	NT	NT	NT
Musculo-skeletal tissues						
Bone	–	NT	NT	NT	NT	NT
Tendon	–	NT	NT	NT	NT	NT
Other tissues						
Gingival tissues	NT	NT	NT	NT	NT	NT
Dental pulp	NT	NT	NT	NT	NT	NT
Trachea	–	NT	NT	NT	NT	–
Thyroid gland	NT	NT	–	NT	NT	–
Body fluids, secretions and excretions						
Colostrum[21]	(–)	–	(?)	NT	NT	NT
Cord blood[21]	–	NT	NT	NT	NT	NT
Sweat	NT	NT	NT	NT	NT	NT
Tears	NT	NT	NT	NT	NT	NT
Nasal mucus	NT	NT	NT	NT	NT	NT
Bile	NT	NT	NT	NT	NT	NT

1. Infectivity bioassays of human tissues have been conducted in either primates or mice (or both), bioassays of cattle tissues have been conducted in either cattle or mice (or both), and most bioassays of sheep and/or goat tissues have been conducted only in mice. In regard to sheep and goats not all results are consistent for both species, for example, two goats (but no sheep) have contracted BSE naturally [Eurosurveillance, 2005, Jeffrey et al., 2006]. Similarly, most of the results described for CWD were derived from studies in deer, and findings may not be identical in elk or other cervids.

2. In experimental models of TSE, the optic nerve has been shown to be a route of neuroinvasion, and contains high titres of infectivity.

3. No experimental data about infectivity in pituitary gland or dura mater in humans with all forms of human TSE have been reported, but cadaveric dura mater patches, and growth hormone derived from cadaveric pituitaries have transmitted disease to hundreds of people and therefore must be included in the category of high-risk tissues. PrP^{TSE} was detected by immunoblot in the dura mater of a vCJD patient who died in the US after an unusually long incubation period (see also Table IB for other positive tissues: skin, kidney, liver, pancreas, ovary and uterus) [Notari et al., 2010]. It must be mentioned that earlier studies of numerous cases examined in the UK reported all of these tissues to be negative [Ironside et al., 2002, Head et al., 2004].

4. In cattle, PrP^{TSE} is reported to be inconsistently present in the enteric plexus in the distal ileum, but immunohistochemical examination of tissues from a single 'fallen stock' case of BSE in Japan suggested (albeit equivocally) involvement of myenteric plexuses throughout the small and large intestine [Kimura and Haritani, 2008].

5. In vCJD, PrP^{TSE} is limited to gut-associated lymphoid and nervous tissue (mucosa, muscle, and serosa are negative).

6. Ruminant fore stomachs (reticulum, rumen, and omasum) are widely consumed, as is the true stomach (abomasum). The abomasum of cattle (and sometimes sheep) is also a source of rennet.

7. When a large BSE oral dose was used to infect cattle experimentally, infectivity was detected in the jejunum and the ileo-caecum junction in Tg mice overexpressing PrP [courtesy of Dr M Groschup]. PrP^{TSE} was detected at low incidence in lymphoid tissue of ileum [Terry et al., 2003] and has been detected at an even lower frequency in jejunal lymphoid tissue of cattle similarly infected by the oral route [EFSA, 2009].

8. A single report of transmission of sporadic CJD infectivity from human placenta has never been confirmed and is considered improbable.

9. PrP^{TSE} has been detected in scrapie-infected sheep with chronic mastitis, but not from infected sheep without mastitis [Ligios et al., 2005].

10. Studies in hamsters orally infected with scrapie revealed that PrP^{TSE} deposition in skin was primarily located within small nerve fibres. Also, apical skin 'velvet' from the antlers of CWD-infected deer is reported to contain PrP^{TSE} and infectivity [Angers et al., 2009].

11. PrP^{TSE} detected by immunocytochemistry in the renal pelvis of scrapie-infected sheep [Siso et al., 2006], and in lymphoid follicles within connective tissue adjacent to the renal pelvis in CWD-infected mule deer [Fox et al., 2006].

12. A single positive marrow in multiple transmission attempts from cattle orally dosed with BSE-infected brain [Wells et al., 1999, Wells et al., 2005, Sohn et al., 2009].

13. Muscle homogenates have not transmitted disease to primates from humans with sporadic CJD, or to cattle from cattle with BSE. However, intra-cerebral inoculation of a semitendinosus muscle homogenate (including nervous and lymphatic elements) from a single cow with clinical BSE has transmitted disease to transgenic mice that overexpress PrP at a rate indicative of trace levels of infectivity [Buschmann and Groschup, 2005]. Also, recent published and unpublished studies have reported the presence of PrP^{TSE} in skeletal muscle in experimental rodent models of scrapie and vCJD [Beekes et al., 2005], in experimental and natural scrapie infections of sheep and goats [Andreoletti et al., 2004], in sheep orally dosed with BSE [Andreoletti, unpublished data], and in humans with sporadic, iatrogenic, and variant forms of CJD [Glatzel et al., 2003, Kovacs et al., 2004, Peden et al., 2006]. Bioassays of muscle in transgenic mice expressing cervid PrP have documented infectivity in CWD-infected mule deer [Angers et al., 2006], and experiments are underway to determine whether detectable PrP^{TSE} in other forms of TSE is also associated with infectivity.

14. In cattle, bioassay of infectivity in the tongue was negative, but the presence of infectivity in palatine tonsil has raised concern about possible infectivity in lingual tonsillar tissue at the base of the tongue that may not be removed at slaughter [Wells et al., 2005, EFSA, 2008]. In sheep naturally infected with scrapie, 7 of 10 animals had detectable PrP^{TSE} in the tongue [Casalone et al., 2005, Corona et al., 2006].

15. Limited chiefly to regions involved in olfactory sensory reception.

16. Because only one case of iatrogenic CJD has been certainly attributed to a corneal transplant among hundreds of thousands of recipients (one additional case is considered probable, and another case only possible), cornea has been categorized as a lower-risk tissue, other anterior chamber tissues (lens, aqueous humour, iris, conjunctiva) have been tested with a negative result both in vCJD and other human TSEs, and there is no epidemiological evidence that they have been associated with iatrogenic disease transmission.

17. A wealth of data from studies of blood infectivity in experimental rodent models of TSE have been extended by recent studies documenting infectivity in the blood of sheep with naturally occurring scrapie and in sheep transfused with blood from BSE-infected cattle [Houston et al., 2008], of deer with naturally occurring CWD [Mathiason et al., 2006], and (from epidemiological observations) in the red cell fraction (which includes significant amounts of both plasma and leukocytes) of four blood donors in the pre-clinical phase of vCJD infections [reviewed in Brown, 2006, Hewitt et al., 2006]. Plasma Factor VIII administration has also been potentially implicated in a subclinical case of vCJD in a haemophilia patient [Peden et al., 2010]. Blood has not been shown to transmit disease from humans with any form of 'classical' TSE [Dorsey et al., 2009], or from cattle with BSE (including fetal calf blood). A number of laboratories using new, highly sensitive methods to detect PrP^{TSE} are reporting success in a variety of animal and human TSEs. However, several have experienced difficulty obtaining reproducible results in plasma, and it is not yet clear that positive results imply a potential for disease transmissibility, either because of false positives, or of 'true' positives that are due to sub-transmissible concentrations of PrP^{TSE}. Because of these considerations (and the fact that no data are yet available on blinded testing of specimens from naturally infected humans or animals) the expert group felt that it was still too early to evaluate the validity of these tests with sufficient confidence to permit either a negative or positive conclusion.

18. Evidence that infectivity is not present in milk from BSE-infected bovines includes temporo-spatial epidemiologic observations failing to detect maternal transmission to calves suckled for long periods, clinical observations of over a hundred calves suckled by infected cows that have not developed BSE, and experimental observations that milk from infected cows reared to an age exceeding the minimum incubation period has not transmitted disease when administered intra-cerebrally or orally to mice [Middleton and Barlow, 1993, Taylor et al., 1995]. Also, PrP^{TSE} has not been detected in milk from cattle incubating BSE following experimental oral challenge [SEAC, 2005]. However, low levels (µg to ng/L) of normal PrP have been detected in milk from both animals and humans [Franscini et al., 2006]. PrP^{TSE} has been detected in the mammary glands of scrapie-infected sheep with chronic mastitis [Ligios et al., 2005], and very recently it has been reported that milk (which in some cases also contained colostrum) from scrapie-infected sheep transmitted disease to healthy animals [Konold et al., 2008, Lacroux et al., 2008].

19. A mixed inoculum of urine and faeces from naturally infected CWD deer did not transmit disease during an 18-month observation period after inoculation of healthy deer with a heterozygous (96 G/S) PRNP genotype [Mathiason et al., 2006]. However, recent bioassays in Tg mice have transmitted disease from both urine [Haley et al., 2009] and faeces [Tamgüney et al., 2009]. In addition, mice with lymphocytic nephritis that were experimentally infected with scrapie shed both PrP^{TSE} and infectivity in urine, when bioassayed in Tg mice [Seegeret al., 2005]. Very low levels of infectivity have also been detected in the urine (and histologically normal kidneys) of hamsters experimentally infected with scrapie [Gregori and Rohwer, 2007, Gonzalez-Romero et al., 2008]. Finally, in an experimental scrapie-hamster model, oral dosing resulted in infectious faeces when bioassayed in Tg mice over-expressing PrP [Safar et al., 2008].

20. Embryos from BSE-affected cattle have not transmitted disease to mice, but no infectivity measurements have been made on fetal calf tissues other than blood (negative mouse bioassay) [Fraser and Foster, 1994]. Calves born of dams that received embryos from BSE- affected cattle have survived for observations periods of up to seven years, and examination of the brains of both the unaffected dams and their offspring revealed no spongiform encephalopathy or PrP^{TSE} [Wrathall et al., 2002].

21. Early reports of transmission of sporadic CJD infectivity from human cord blood and colostrum have never been confirmed and are considered improbable. A bioassay from a cow with BSE in transgenic mice over-expressing bovine PrP gave a negative result [Buschmann and Groschup, 2005], and PrP^{TSE} has not been detected in colostrum from cattle incubating BSE following experimental oral challenge [SEAC, 2005].

Appendix XXIII

Weights and Measures

B. Conversion Tables for Commonly Used Units

The following tables are included for the convenience of users. Table 23-5 gives the conversions from parts per million, Table 23-6 gives commonly used concentrations and their conversions.

Table 23-5 Parts Per Million (ppm) Conversions

Parts per billion (ppb)	Parts per million (ppm)	µg/g	Fraction	Decimal	Percentage w/w
1	0.001	0.001	1/1 000 000 000	0.000000001	0.0000001%
10	0.01	0.01	1/100 000 000	0.00000001	0.000001%
100	0.1	0.1	1/10 000 000	0.0000001	0.00001%
1000	1	1	1/1 000 000	0.000001	0.0001%
5000	5	5	5/1 000 000	0.000005	0.0005%
10 000	10	10	1/100 000	0.00001	0.001%
	20	20	2/100 000	0.00002	0.002%
	50	50	5/100 000	0.00005	0.005%
	100	100	1/10 000	0.0001	0.01%
	200	200	2/10 000	0.0002	0.02%
	1000	1000	1/1000	0.001	0.1%
	5000	5000	5/1000	0.005	0.5%

Table 23-6 Concentration Conversions

µg/ml	mg/ml	g/ml	g/100 ml (% w/v)
1	0.001	0.000001	0.0001
10	0.01	0.00001	0.001
20	0.02	0.00002	0.002
50	0.05	0.00005	0.005
100	0.1	0.0001	0.01
200	0.2	0.0002	0.02
500	0.5	0.0005	0.05
1000	1	0.001	0.1
10000	10	0.01	1

Appendix XXIV

Abbreviations

ATCC*	American Type Culture Collection.
BPCRS	British Pharmacopoeia Chemical Reference Substance (see Appendix I E).
BRP	Biological reference preparation (see Appendix I E).
BS	British Standard.
$CCID_{50}$	Cell culture infective dose (the dose of the micro-organism that infects 50% of the cell cultures inoculated).
CIP*	Collection de Bactéries de l'Institut Pasteur.
CRS	Chemical reference substance (see Appendix I E).
DNA	Deoxyribonucleic acid.
EID_{50}	Egg embryos infective dose (the dose of the micro-organism that infects 50% of the embryonated eggs inoculated).
EPBRP	European Pharmacopoeia Biological Reference Preparation (see Appendix I E).
EPCRS	European Pharmacopoeia Chemical Reference Substance (see Appendix I E).
FIP	International Pharmaceutical Federation.
g	Acceleration due to gravity.
HIV	Human immunodeficiency virus
ID_{50}	Infective dose 50 (the dose of the micro-organism that infects 50% of the animals inoculated).
IMI*	Commonwealth Mycological Institute.
IP*	Collection Nationale de Cultures de Microorganismes (CNCM).
ISO	International Organization for Standardization.
IU	International Unit
IUPAC	International Union of Pure and Applied Chemistry.
LD_{50}	Lethal dose 50 (the dose of the preparation or organism that kills 50% of the animals inoculated).
MID	Minimum infective dose.
MLD	Minimum lethal dose.
MRI	Magnetic resonance imaging.
NCIMB*	National Collection of Industrial and Marine Bacteria.
NCPF*	National Collection of Pathogenic Fungi.
NCTC*	National Collection of Type Cultures.
NCYC*	National Collection of Yeast Cultures.
PD_{50}	Protective dose 50 (the dose of the preparation that protects 50% of the animals inoculated).
ppm	Parts per million by weight.
SI	International System of Units.
SN_{50}	Serum neutralising dose 50 (the amount of serum that will protect 50% of the cultures against the specified amount of virus).
SSI	Statens Serum Institut (Copenhagen).
THM	Traditional Herbal Medicine.
THMP	Traditional Herbal Medicinal Product.
VS	Volumetric solution (see Appendix I B).
µkat	Microkatal: the enzyme activity that, under defined conditions, produces 1 micromole of the reaction product per second or consumes one micromole of the reaction substrate per second.

*Strains of the micro-organisms referred to in the Pharmacopoeia may be obtained from:

ATCC	*American Type Culture Collection, 10801 University Boulevard, Manassas, Virginia 20110-2209, USA.*
CIP	*Collection de Bactéries de l'Institut Pasteur, BP 52, 25 Rue du Dr-Roux, F-75724, Paris Cedex 15, France.*
IMI	*International Mycological Institute, Bakeham Lane, Surrey TW20 9TY, England.*
IP	*Service de la Collection Nationale de Culture de Microorganismes (CNCM), Institut Pasteur, 25 Rue de Dr Roux, F-75724, Paris Cedex 15 France.*
NCIMB	*National Collection of Industrial and Marine Bacteria Ltd, 23 St Machar Drive, Aberdeen, AB24 3RY, Scotland.*
NCPF	*National Collection of Pathogenic Fungi, London School of Hygiene and Tropical Medicine, Keppel Street, London WC1E 7HT, England.*
NCTC	*National Collection of Type Cultures, Central Public Health Laboratory, Colindale Avenue, London NW9 5HT, England.*
NCYC	*National Collection of Yeast Cultures, AFRC Food Research Institute, Colney Lane, Norwich NR4 7UA, England.*

Appendix XXV

Names, Symbols and Atomic Weights of Elements

The following atomic weights are those published in 2001 by the International Union of Pure and Applied Chemistry (*Pure Appl. Chem.* 2003, 75, 1107).

Element	Symbol	Atomic Weight	Element	Symbol	Atomic Weight
Actinium	Ac	[227]	Molybdenum	Mo	95.94
Aluminium	Al	26.981538	Neodymium	Nd	144.24
Americium	Am	[243]	Neon	Ne	20.1797
Antimony	Sb	121.760	Neptunium	Np	[237]
Argon	Ar	39.948	Nickel	Ni	58.6934
Arsenic	As	74.92160	Niobium	Nb	92.90638
Astatine	At	[210]	Nitrogen	N	14.0067
Barium	Ba	137.327	Nobelium	No	[259]
Berkelium	Bk	[247]	Osmium	Os	190.23
Beryllium	Be	9.012182	Oxygen	O	15.9994
Bismuth	Bi	208.98038	Palladium	Pd	106.42
Bohrium	Bh	[264]	Phosphorus	P	30.973761
Boron	B	10.811	Platinum	Pt	195.078
Bromine	Br	79.904	Plutonium	Pu	[244]
Cadmium	Cd	112.411	Polonium	Po	[209]
Caesium	Cs	132.90545	Potassium	K	39.0983
Calcium	Ca	40.078	Praseodymium	Pr	140.90765
Californium	Cf	[251]	Promethium	Pm	[145]
Carbon	C	12.0107	Protactinium	Pa	231.03588
Cerium	Ce	140.116	Radium	Ra	[226]
Chlorine	Cl	35.453	Radon	Rn	[222]
Chromium	Cr	51.9961	Rhenium	Re	186.207
Cobalt	Co	58.933200	Rhodium	Rh	102.90550
Copper	Cu	63.546	Roentgenium	Rg	[272]
Curium	Cm	[247]	Rubidium	Rb	85.4678
Darmstadtium	Ds	[281]	Ruthenium	Ru	101.07
Dubnium	Db	[262]	Rutherfordium	Rf	[261]
Dysprosium	Dy	162.500	Samarium	Sm	150.36
Einsteinium	Es	[252]	Scandium	Sc	44.955910
Erbium	Er	167.259	Seaborgium	Sg	[266]
Europium	Eu	151.964	Selenium	Se	78.96
Fermium	Fm	[257]	Silicon	Si	28.0855
Fluorine	F	18.9984032	Silver	Ag	107.8682
Francium	Fr	[223]	Sodium	Na	22.989770
Gadolinium	Gd	157.25	Strontium	Sr	87.62
Gallium	Ga	69.723	Sulfur	S	32.065
Germanium	Ge	72.64	Tantalum	Ta	180.9479
Gold	Au	196.96655	Technetium	Tc	[98]
Hafnium	Hf	178.49	Tellurium	Te	127.60
Hassium	Hs	[277]	Terbium	Tb	158.92534
Helium	He	4.002602	Thallium	Tl	204.3833
Holmium	Ho	164.93032	Thorium	Th	232.0381
Hydrogen	H	1.00794	Thulium	Tm	168.93421
Indium	In	114.818	Tin	Sn	118.71
Iodine	I	126.90447	Titanium	Ti	47.867
Iridium	Ir	192.217	Tungsten	W	183.84
Iron	Fe	55.845	Ununbium	Uub	[285]
Krypton	Kr	83.798	Ununhexium	Uuh	
Lanthanum	La	138.9055	Ununoctium	Uuo	
Lawrencium	Lr	[262]	Ununquadium	Uuq	[289]
Lead	Pb	207.2	Uranium	U	238.02891
Lithium	Li	[6.941]	Vanadium	V	50.9415
Lutetium	Lu	174.967	Xenon	Xe	131.293
Magnesium	Mg	24.3050	Ytterbium	Yb	173.04
Manganese	Mn	54.938049	Yttrium	Y	88.90585
Meitnerium	Mt	[268]	Zinc	Zn	65.409
Mendelevium	Md	[258]	Zirconium	Zr	91.224
Mercury	Hg	200.59			

Supplementary Chapters

Introduction

Supplementary Chapters contain no standards, tests or assays nor any other mandatory specifications with respect to any Pharmacopoeial article. They comprise explanatory and other ancillary texts and are provided for the assistance and information of users of the Pharmacopoeia.

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Supplementary Chapter I

Basis of Pharmacopoeial Requirements

Introduction

This chapter provides explanatory text and guidance on the pharmacopoeial approach to a range of subjects.

Separate, lettered sections of this chapter explain the current approach to a particular aspect of pharmacopoeial control and, where appropriate, give an indication of future developments. The British Pharmacopoeia Commission's policies continue to evolve and these sections will be updated as and when necessary to reflect further developments.

While these texts outline general policies that are adopted in the Pharmacopoeia, each monograph is considered individually. Departures from the general rule are accepted where justified and are accommodated by appropriate statements in the individual monographs.

In providing these texts to users of the Pharmacopoeia it is emphasised that the specifications of the Pharmacopoeia are one facet of the overall control of the quality of medicinal products and their constituents. Those concerned with the manufacture of medicinal substances and those responsible for their incorporation into pharmaceutical dosage forms must also pay due attention to the requirements and recommendations of other competent authorities. Within the European Community the information required for marketing authorization is laid down in the relevant Directives, Notice to Applicants and associated Notes for Guidance available from the Commission of the European Communities as the series 'The Rules Governing Medicinal Products in the European Community'.

Dialogue with users is an essential element of pharmacopoeial development and the British Pharmacopoeia Commission hopes that this chapter will provide insight into certain features of pharmacopoeial requirements.
The Commission places great value on the assistance it receives from manufacturers and others with the necessary knowledge to assist it in its work. It welcomes suggestions for improvement of published texts and constructive comment on any issues of interest and concern to users.

General considerations

1. A proper understanding of the basis on which the requirements of the Pharmacopoeia are established is essential to the correct interpretation of the requirements.

2. The Pharmacopoeia contributes significantly to the overall control of the quality of medicinal products and provides a publicly available statement concerning the quality that a product or a component of a product is expected to meet at any time during its period of use. Pharmacopoeial specifications are used within licensing systems and by manufacturers, suppliers, purchasers and those acting on behalf of consumers of medicinal products.

3. A manufacturer must recognise that a product or material may be challenged at any time during its claimed period of use by the methods of the Pharmacopoeia and that it must then comply with the pharmacopoeial requirements. These requirements allow for acceptable levels of change that may occur during storage and distribution and reject articles showing unacceptable levels of change. Frequently a manufacturer will need to apply more stringent test limits at the time of release of a batch of the product or material in order to ensure compliance. As stated in the General Notices, a manufacturer may assure himself that the requirements of the Pharmacopoeia will be met by means other than routinely performing all of the tests prescribed in the Pharmacopoeia. It is emphasised that the circumstances under which, and the frequency with which, tests of the Pharmacopoeia should be performed by a manufacturer as part of his overall quality assurance are ultimately matters for agreement between the manufacturer and the competent authority.

4. The requirements included in a monograph, other than any instructions given under the side-heading Production, are designed to provide the means by which an independent judgement can be made as to the overall quality of a particular article. A manufacturer in possession of detailed knowledge of the manufacturing process may have no need to carry out certain tests. The example of some impurity tests in monographs for formulated preparations is discussed in more detail in section A of this chapter. The methods described in the Pharmacopoeia must be robust because they are intended to be used by analysts in a wide range of laboratories, sometimes on an infrequent basis.
Understandably, a manufacturer may wish to use other methods that may be more suitable for frequent use or automation and is entitled to do so. However in the event of any doubt or dispute as to whether or not a material is of pharmacopoeial quality, as the General Notice on Assays and Tests makes clear, the methods of the Pharmacopoeia alone are authoritative.

5. This view of pharmacopoeial requirements is also significant when considering the size of sample to be taken for test. In an overall programme designed to give assurance of quality of a manufactured product, the statistical validity of any sampling programme must be beyond doubt.
The standards of the Pharmacopoeia, on the other hand, are intended to apply to the sample available, perhaps the container of dispensed tablets provided to a patient in accordance with a prescription. The Pharmacopoeia requires that twenty of those tablets should meet the test for Uniformity of weight. A manufacturer establishing a sampling and testing protocol designed to ensure ultimate compliance with the pharmacopoeial requirements will need to operate at a level designed to show with an acceptable degree of confidence that any twenty tablets, taken at random from a given batch, will meet the requirements.

6. Pharmacopoeial methods and limits are set with the intention that they should be used as compliance requirements and not as requirements to guarantee total quality assurance. An article is not of pharmacopoeial quality if any sample of the size stipulated in the monograph taken at any time during storage, distribution and use within the accepted shelf-life fails to meet all of the requirements.

7. Arising from this it may be useful to underline that compliance of a product with pharmacopoeial requirements demands that the product meets *all* mandatory aspects of the appropriate monograph and that those requirements shall be interpreted in the light of any relevant General Notices.
In certain cases individual requirements of particular tests may seem to be incompatible with those of other tests; where this is apparently the case such requirements have been framed intentionally. For example, the requirement for the overall content of active ingredient in a tablet preparation, as determined on a powdered sample of twenty tablets, might be 95.0 to 105.0% of the stated amount. Thus an assay result of 96.0% would indicate compliance. For the Uniformity of content test a further ten tablets might be

individually examined, each tablet being required to contain between 85 and 115% of the mean value, with the possibility of a single exception between 75 and 125%. Thus if nine out of ten tablets fall within the range (assuming the mean to be 96.0%) 81.6 and 110.4% and the tenth falls within the range 72.0 to 120.0% then the tablets examined comply with that requirement. For the Dissolution test each tablet examined might be required to yield at least 70% of the labelled claim into solution within 45 minutes. It has been suggested that since a single outlier tablet might contain as little as 72.0% of the labelled claim and yet still fall within the acceptance limits for content, the requirements for dissolution should be relaxed to take this into account. In framing requirements, however, the view is taken that it is neither realistic nor profitable to attempt to compound the results of various tests in this way. Each test in a pharmacopoeial monograph and the acceptance limit is therefore framed as an individual entity with requirements based on values encountered in practice; compliance with the monograph requires compliance with each and every test.

8. The philosophy outlined above has an important bearing on the construction of a monograph for the Pharmacopoeia. To achieve maximum benefit from the examination of a product the recommended approach is that, wherever possible, a variety of different analytical techniques should be employed. The monographs of the British Pharmacopoeia are, therefore, usually constructed to use fundamentally different procedures for assay and for the examination for impurities. For a medicinal substance the general approach has been to employ spectrophotometric or other appropriate techniques for identification, chromatographic techniques for the control of impurities and a precise, albeit non-specific method for assay. It has been held that this approach confers greater confidence in the verification of the identity and quality of the substance and in the detection of unexpected impurities than would be the case in using, for example, a single stability-indicating liquid chromatographic method for all three purposes. The British Pharmacopoeia Commission recognises, however, that as chromatographic methods become more precise it will become increasingly possible to use them for assay purposes thereby combining precision with specificity and economising upon analytical effort and time. For dosage forms this concept has already been adopted and more specific assay methods, such as those employing liquid chromatography, are being employed increasingly.

9. A discussion of the basis of pharmacopoeial requirements would be incomplete without reminding users that any article described by a name at the head of a monograph in the current edition of the Pharmacopoeia, *whether or not it is referred to as 'BP'*, must comply with that monograph. The name at the head of a monograph is to be interpreted in accordance with the General Notice on Titles. In particular, a formulated preparation that is labelled with a title that includes the full nonproprietary name of the active ingredient, where this is not included in the title of the monograph, must also comply with the monograph. Thus, for example, a preparation labelled Labetalol Hydrochloride Tablets must comply with the monograph for Labetalol Tablets.

A. Control of Impurities

This section provides a guide to the pharmacopoeial approach to the control of impurities in medicinal substances and formulated preparations. Other guidance relevant to manufacturers is provided in, for example, ICH guidelines on impurities in new drug substances (Q3A) and new drug preparations (Q3B) and VICH guidelines on impurities in new veterinary drug substances (GL10) and new veterinary medicinal products (GL11).

1. This section relates primarily to totally synthetic organic medicinal substances and those substances obtained by synthetic modification of a naturally-produced precursor. It is not necessarily applicable to other organic substances (e.g., those of plant or animal origin), inorganic substances and excipients.

Certain additional information of specific relevance to impurity control in the formulated preparation monographs of the British Pharmacopoeia is also provided.

2. The control provided by chemical tests limiting the levels of particular impurities or classes of impurities is often augmented by physical tests such as absorbance, specific optical rotation, melting point and clarity and colour of solution and, for a liquid, refractive index, boiling point range and weight per mL.

3. Tests such as sulfated ash and loss on drying are non-specific but they contribute to an assurance of the general quality of the material, the use of good pharmaceutical manufacturing practice in its production, the avoidance of contamination especially by inorganic substances and the removal of volatile solvents. Typical limits are 0.1% for sulfated ash and 0.5% for loss on drying.

4. Tests for purity are intended to provide appropriate limitation of known potential or actual impurities rather than to provide against all possible impurities. The tests are not necessarily designed to detect any adventitious contaminants or adulteration. Material found to contain an impurity not detectable by means of the prescribed tests is not of pharmacopoeial quality if the nature or amount of the impurity found is incompatible with good pharmaceutical practice.

5. Some medicinal substances are mixtures of closely related compounds. Where these components have similar activity they are not usually regarded as impurities and may indeed contribute to the result obtained in the assay. Examples include Erythromycin, Gentamicin Sulfate and Sodium Lauryl Sulfate. It may however be appropriate to control the relative amounts of such components, e.g., gentamicins C_1, C_{1a}, C_2, C_{2a} and C_{2b} in Gentamicin Sulfate in order to ensure batch to batch consistency for material from one manufacturer and uniformity between supplies of the same substance from different manufacturers.

6. Many medicinal substances already on the market have been made available as racemic mixtures with little or nothing known about the biological activities of the separate isomers. This has been reflected in the monograph in the Pharmacopoeia and a test to show that the substance is the racemic mixture has not usually been included unless it was known that at least one of the separate enantiomers was also available commercially. Nevertheless, with increasing concern by regulatory authorities for substances to be made available as single isomers, tests for enantiomeric composition will become more common. When a medicinal substance is a racemate, an indication is given by means of the graphic formula [see also Supplementary Chapter I K; Stereochemistry].

Related Substances

7. It is usual to include a test for related substances in a monograph for a medicinal substance. These may be manufacturing impurities (intermediates or by-products) or degradation products or both. When preparation of a monograph is initiated the manufacturer is asked to provide information concerning the nature of such impurities, the reason for their presence, the amounts that may be encountered in material prepared under conditions of good pharmaceutical manufacturing practice and the manner in which proportions may vary on storage, together with an indication of the toxicity of any impurities in relation to that of the substance itself. Where there is only one manufacturer of a substance, pharmacopoeial limits are set in the knowledge that the level of impurities in production batches of the substance will have been accepted by the registration authority after a full consideration of the toxicity studies and clinical trials carried out before the granting of a licence. Such studies and trials will have been carried out on material with an impurity profile that is qualitatively and quantitatively similar to that of subsequent production batches.
Any subsequent changes to the manufacturing process by the original manufacturer or the introduction of material from another manufacturer utilising a different route of synthesis will be subject to the need to demonstrate essential similarity or to provide equivalent data to the relevant registration authority. In some cases a change in production or source may give rise to impurities that are not adequately controlled by the published pharmacopoeial monograph. Appropriate revision of the monograph will be carried out provided that the pharmacopoeial authority is notified of the need and that it is supplied with the relevant information [see paragraph 28 below].

8. Tests for related substances may be specific or general.

9. **Specific tests for named impurities** A specific test is included where a particular impurity arising from the manufacturing process or from degradation needs to be limited on grounds of toxicity or for another special reason. Where an impurity is known to be particularly toxic, this is taken into account in setting the limit; for example, a limit of 1 ppm is specified for hydrazine in the monograph for Povidone.

9.1 Such specific tests usually employ a chromatographic or colorimetric comparison with a sample of the named substance, for example, 4-chloroaniline in Chlorhexidine Irrigation Solution and 4-aminophenol in Benorilate Tablets.

9.2 Where a specimen of the impurity is required in the test, this will be made available as a Chemical Reference Substance unless it is known that specimens of the requisite quality can readily be obtained through the usual suppliers of chemical reagents.

9.3 Specific control may be included within a more general test controlling other impurities; an example is 4-epianhydrotetracycline in the monograph for Lymecycline.

9.4 In other cases, an absolute method is more appropriate. Such a test may be for a group of potentially toxic impurities, e.g., polycyclic aromatic hydrocarbons in Liquid Paraffin.

9.5 Sometimes an impurity may be named in the Pharmacopoeia because it is necessary to use a named substance in its control for analytical reasons, such as different response factors in the specified test method. Examples are iminodibenzyl in Imipramine Tablets, and dibenzosuberone in Amitriptyline Tablets. Typical wording is as follows:

Any spot corresponding to [x] in the chromatogram obtained with solution (1) is not more intense than the principal spot in the chromatogram obtained with solution (2). [Solution (1) contains the substance being examined and solution (2) contains a named impurity [x].]

10. **General tests for unnamed impurities** It is unusual for the Pharmacopoeia to require the absence of a visible spot in a thin-layer chromatogram or the absence of a peak in a liquid chromatogram. Reasons for this include the difficulty of interpreting and defining absence that is a consequence of variations in the sensitivity of a method when performed in different laboratories by different analysts. It is more usual to limit the levels of impurities. This may be done in a simple test by comparison with a spot or peak obtained with a dilute solution of the substance being examined. An example is Methyl Nicotinate:

Any secondary spot in the chromatogram obtained with solution (1) is not more intense than the spot in the chromatogram obtained with solution (2). [Solutions (1) and (2) contain the substance being examined at high and low concentrations respectively.]

10.1 In the absence of evidence that the limit for a particular impurity needs to be set on the basis of its toxicity, control is often provided by a two-level test requiring, say, not more than one related substance at a nominal concentration of up to 0.5% and any others at nominal concentrations of up to 0.1%. The actual limits may be chosen on the basis of batch data for material manufactured in accordance with good pharmaceutical manufacturing practice and will take account of a number of factors, including the dose regimen of the substance and the number of impurities commonly present. An example is Oxetacaine:

Any secondary spot in the chromatogram obtained with solution (1) is not more intense than the spot in the chromatogram obtained with solution (2) (0.5%) and not more than one such spot is more intense than the spot in the chromatogram obtained with solution (3) (0.1%).

Another example, with different limits, is Phenindione:

Any secondary spot in the chromatogram obtained with solution (1) is not more intense than the spot in the chromatogram obtained with solution (2) (2%) and not more than one such spot is more intense than the spot in the chromatogram obtained with solution (3) (0.5%).

10.2 Where it is known that several impurities are likely to be present at significant concentrations, a three-level test may be appropriate. An example is Pentazocine Injection:

By each method of visualisation, in the chromatogram obtained with solution (1) any secondary spot is not more intense than the spot in the chromatogram obtained with solution (2) (1%), not more than one such spot is more intense than the spot in the chromatogram obtained with solution (3) (0.5%) and not more than four such spots are more intense than the spot in the chromatogram obtained with solution (4) (0.25%).

10.3 General tests with an 'open' design such as those described above have the great advantage that they provide a means of limiting the levels of related substances that may arise from modified or alternative synthetic routes not in use at the time the test was elaborated. In this context, thin-layer chromatography

has the advantage over liquid chromatography and gas chromatography that it allows detection of impurities completely retained or those not retained at all by the stationary phase.

11. **Total impurity limits** In gas chromatographic and liquid chromatographic tests, it is increasingly common to limit the total areas of peaks due to related substances. In monographs currently in force the limit for the sum is commonly in the range 1 to 2% but these values are applicable at the end of the shelf-life. This procedure is rarely adopted in thin-layer chromatographic tests, because of the semi-quantitative nature of estimating individual spots and resulting imprecision in expression of results for the totals. This apparent drawback to the use of thin-layer chromatography is largely overcome by means of two- and three-level tests as described in paragraphs 10.1 and 10.2, above.

12. In response to requests from users of the Pharmacopoeia, statements of the approximate real or nominal levels of impurities controlled by tests for impurities have been widely introduced, where appropriate, for information [see paragraphs 14 and 15 below]. Conformity with the requirements will still be determined on the basis of compliance or otherwise with the stated test.

Test design and expression of limits

13. For **identified impurities**, several aspects are taken into account in designing the test. These include the nature of the impurity, its toxicity and the levels likely to be found in routine production. Analytical considerations such as the correction factor (defined in paragraph 16) for the impurity and practical issues such as availability of the impurity as a reference material or reagent also influence the test design.

14. If a major and/or toxic impurity in a material is known to have a significantly different response (more than ±25%) from that of the main peak in the substance being examined in the conditions of the test, the preferred manner of limiting this impurity is to use a reference substance of the impurity. If this is not possible, a reference solution of the substance being examined containing a known amount of the impurity may be used. Using either of these approaches, the concentration limit indicated in the monograph for information in parentheses expresses the approximate limit as a real percentage of the impurity in question. This is also referred to as % w/w. When neither of these approaches is possible, a dilution of the solution of the substance being examined may be used as a reference solution. This approach is also commonly used in tests where an impurity that is known (but not named within the test) has a response within ±25% of that of the main peak in the substance being examined.

15. Unless explicitly stated otherwise, an indication of the approximate concentration limit provided in any test where a dilution of the substance being examined is used as the reference solution should be interpreted as an expression in terms of a nominal percentage of the substance being examined (in accordance with the General Notices) rather than as a real percentage of the impurity.

16. No reference is made in a test to a **correction factor** for an identified impurity unless unavoidable. When used, the term is defined as follows:

> The correction factor ($1/k$) is a relative term, being the reciprocal of the peak response of equal concentrations of one substance relative to that of another in the conditions described in the test.

In the context of a related substances test where a correction factor is quoted for an impurity, unless otherwise stated, this is the expected correction for that impurity in relation to a response of unity for the substance being examined. The way in which a correction factor is to be used in any subsequent calculation is stated in the monograph.

17. Correction factors of less than 0.2 or more than 5 are not generally used. If the difference between the response of an impurity and that of the substance being examined is outside these limits, a different method of determination, such as a different detection wavelength (λ) or a different method of visualisation, is used.

18. For a correction factor quoted in a pharmacopoeial test, the following points are observed:

(a) the peak to which the factor applies is identified unambiguously, recognising the difficulties associated with such identification in chromatograms showing peaks of similar retention times [the use of a sample containing an unquantified amount of the relevant impurity can sometimes be used to assist such identification],

(b) the correction factor quoted is a confirmed value and preferably is based on relative peak areas of equal concentrations of the impurity and the sample under the conditions of the test [alternatively the reciprocal of an absolute figure based on the A(1%, 1 cm) for the detection wavelength used (or similar for other methods of detection) of the impurity and the sample may be used],

(c) the correction factor is stated in a way that does not imply an unrealistic or misleading degree of precision [declaration to several significant figures is not meaningful when placed in context of the nature of the test and the amount of the impurity likely to be present (typically, less than 0.5%) (e.g., 1.4, 2.5, 3.0, 0.5) or 1 decimal place (e.g., 1.4, 7.5) are generally appropriate],

(d) older monographs of the BP may contain the term response factor. The term will be revised to correction factor as these monographs are updated.

19. Identification of peaks is generally not based on absolute retention times since these may be too 'system dependent'; however, advice such as 'the principal peak has a retention time of about x minutes' may be given. In some cases, for example, where a simple chromatogram is expected to show a limited number of impurities, an expected *relative* retention may be given to designate impurities. In other cases where potential impurities have similar retention, a sample containing the components of interest and a sample chromatogram may be provided.

20. **Unidentified impurities** may be limited by reference to a dilution of the solution of the substance being examined used as a reference solution together with an open design of statement limiting 'any' or 'any other' *secondary peak* or *spot*. Such a reference solution may be used in addition to those containing named impurities (any other secondary peak/spot) or, in some simple tests, control of unspecified and specified (but unnamed) impurities may be exerted by means of a comparison between the sample solution and a dilution of this solution (any secondary peak/spot).

21. An indication of the approximate limit concentration for an unidentified impurity would only be given in terms of a nominal percentage of the substance being examined (see paragraph 15) since no assumption can be made about the response of an unidentified impurity. While such nominal

limits are not quantitatively fully transparent and caution is needed in attempting to use them to set a total impurity limit, their use is less misleading than quoting an arbitrary/assumed correction factor of 1 for an unidentified impurity.

Formulated Preparations

22. Many monographs for formulated preparations in the British Pharmacopoeia and the British Pharmacopoeia (Veterinary) also include tests for impurities. In general, wherever possible a test for impurities based on that in the monograph for the active ingredient is included with any necessary modification.

23. Wider limits and/or additional controls may be required for impurities arising on manufacture or storage of the dosage form.

24. Tests for impurities in monographs for formulated preparation are used to control not only degradation products but also by-products of the synthetic route used for manufacture of the active ingredient. It has been argued, for example in the ICH guideline Q3B, that by-products of synthesis have been controlled already during examination of the substance before formulation and that further testing for these impurities is unnecessary. Clearly it would be repetitious and wasteful of resources for tests, often complex in nature, to be repeated routinely simply to demonstrate acceptably low levels of impurities that could arise only during synthesis (as opposed to degradation) of the active ingredient. However, this information is available only to those who know the detailed attributes of the active raw material that has been used. For an analyst who has access only to the dosage form, the profile of synthesis-related impurities offers one means of establishing whether or not the dosage form has been prepared from an active ingredient of pharmacopoeial quality. It is for this reason that such tests are included in British Pharmacopoeia and British Pharmacopoeia (Veterinary) monographs for formulated preparations [see paragraph 3 of General considerations in the introduction to Supplementary Chapter I].

Current and future developments

25. **Transparency** A statement giving the identities of impurities that are known to be limited by the specifications is being added to appropriate monographs for medicinal substances and formulated preparations. For example the monograph for Diamorphine Hydrochloride contains the following information at the end of the monograph:

> *IMPURITIES The impurity limited by the this monograph is:* 6-O-acetylmorphone

This increase in the transparency of pharmacopoeial specifications is of assistance to licensing authorities and others when considering whether the standards in the monograph are appropriate to a new source of supply. It is emphasised that other, unnamed impurities may also be limited. The Commission is actively seeking information that will allow the statements to be extended in future.

26. **System suitability** In chromatographic tests increasing use is being made of system suitability tests to enable the analyst to confirm that performance of the chosen column or plate is satisfactory under the chosen conditions. In liquid chromatographic and gas chromatographic tests, peak separation between impurities and the substance being examined is generally considered to offer the best indication of performance of the system.

27. **Residual solvents** A general test for residual solvents has been included in the European Pharmacopoeia (Appendix VIII L) together with guidelines on its application (Supplementary Chapter IV D). At present, a test for solvent residues is included in a specific monograph only where variation in levels of known solvents requires control, e.g., methanol in Gentamicin Sulfate.

28. The British Pharmacopoeia Commission welcomes suggestions for improving the monographs. In particular, where it is found that significant impurities are not controlled by the monograph, the Commission would be glad to receive details of validated methods that can be considered for adoption.

B. Polymorphism

1. Polymorphism (the occurrence of more than one morphic form) is a function of the internal structure of crystalline solids. Its occurrence cannot be predicted and, as it can be induced in many materials in appropriate conditions, its absence is difficult to demonstrate using a single, specific test. Different polymorphs may exhibit different physicochemical properties such as melting point, dissolution rate and infrared absorption spectrum. In some cases these may affect the handling characteristics of the material, the stability of formulated preparations and bioavailability. Control of morphic form by a manufacturer is necessary during processing of active ingredients and excipients and during production of a formulated product to ensure the correct physical characteristics of the product. Its main importance to the control of medicinal products is in the areas of bioavailability and stability.

2. The morphic form of a readily soluble starting material that is incorporated into a solution, for example, an injection, an oral solution or eye drops, is not usually important. (An exception to this statement might be if the concentration of the solution is such that it is close to the limit of solubility of one of the possible polymorphs.) The morphic form may be important when the material is included in a solid dosage form or as a suspension in a liquid dosage form when the characteristics of the different polymorphs are such as to affect the bioavailability of the material.

3. **Pharmaceutical and medicinal substances** For most substances it will not usually be appropriate or necessary for the monograph to control the morphic form. There are, however, a few monographs that restrict the substance to a single form. This may be done by permitting no deviation from the spectrum/form of the reference substance prescribed or by restricting the melting point range. An example is Carbamazepine where the infrared spectrum is determined without prior treatment of the sample and the melting range permitted excludes the polymorph of lower melting point.

4. Where it is known that the substance exists in more than one morphic form a statement is frequently included in the monographs of the Pharmacopoeia under Characteristics, for example, the monographs for Dextropropoxyphene Napsilate and Spironolactone. In the absence of such a statement, the existence of polymorphism may sometimes be deduced from the tests of the monograph. The most common example is found in infrared identification tests where the analyst may be instructed to prepare a solution spectrum or recrystallise the sample if the spectrum obtained is not concordant with the reference spectrum or spectrum of a reference substance, for example, Prednisolone Sodium Phosphate. This procedure recognises polymorphism but does not limit the form permitted.

5. It is intended to extend the inclusion of explicit statements in monographs for pharmaceutical and medicinal substances as information on the occurrence of polymorphism becomes available. The Commission would welcome information from users of the Pharmacopoeia in order that such statements can be added in appropriate cases.

6. **Formulated preparations** Polymorphism is a potential problem in solid dosage forms, such as tablets, or in liquid or semi-solid preparations where the active ingredient is present as a solid, for example, in oral suspensions. In most cases it is difficult to demonstrate that the material contains the desired polymorph as the process of extracting a sufficient sample of the material in question for analysis may itself change the form of the material. For a solid dosage form where the active ingredient is known to exist in forms with significant differences in solubility, a dissolution test may be the method of choice.

7. Where the active ingredient is known to exist in more than one morphic form and the choice of polymorph is critical with regard to bioavailability and/or stability, the method of manufacture should ensure the presence of the correct amount of the desired polymorph in the preparation.
In future the side heading 'Production' will be used to draw attention to control of morphic form during manufacture in cases where control of morphic form is known to be important.

8. Such Production statements might also include reference to the need during product development to examine drug sensitivity to polymorphic change due to granulation conditions or compressional forces and to make appropriate processing adjustments to control potential variation.

9. The Commission would find it helpful to be advised of instances where control of morphic form is important so that a suitable Production statement may be added. In such cases it would also appreciate receiving details of any validated test methods that will appropriately limit the undesirable form.

C. Bacterial Endotoxin Testing

This section provides an exposition of the Commission's policy and information on its implementation. The guidelines of the European Pharmacopoeia are included as an Annex.

The test for bacterial endotoxins of the European Pharmacopoeia (Ph Eur) is included as Appendix XIV C of the British Pharmacopoeia. This text has been prepared in collaboration with the Japanese Pharmacopoeia and the United States Pharmacopeia.

1. This *in vitro* test is being progressively applied in appropriate monographs of both the British and European Pharmacopoeia in place of the *in vivo* test for pyrogens.

2. Methods for the detection of Gram-negative bacterial endotoxins are based on the use of a lysate of amoebocytes from the horseshoe crab *(Limulus polyphemus* or *Tachypleus tridentatus)*. Addition of endotoxin to this lysate may result in gelation, precipitation or turbidity. The method used in the monographs of the European and British Pharmacopoeias is, unless otherwise stated, that using a gelation end-point.

3. A European Pharmacopoeia Biological Reference Preparation (BRP) of Endotoxin calibrated in International Units (IU) has been established for use in this test.
The current BRP (batch 5) was established following an international collaborative study. It consists of endotoxin from the same bulk as the Third International Standard established by the World Health Organization and the current standard established by the Food and Drug Administration and the United States Pharmacopeia for use in the United States of America (EC-7). Following adoption of the recommendations in the report of the collaborative study[1] global harmonisation of endotoxin unitage has been maintained[2], that is, the FDA/USP Endotoxin Unit (EU) is equivalent to the International Unit (IU).

General policy

4. In any individual monograph only one test is required, either that for pyrogens or that for bacterial endotoxins.

5. In the absence of evidence to the contrary, the test for bacterial endotoxins is preferred, since it is considered usually to provide equal or better protection to the patient.

6. Before including a test for bacterial endotoxins in a monograph, evidence is required that a test, as described in Appendix XIV C, can be applied satisfactorily to the item in question.

7. The necessary information is sought from manufacturers. Companies are invited to provide any validation data that they have concerning the applicability of the test for bacterial endotoxins to the substances and formulations of interest.

7.1 Such data should include details of sample preparation and of any procedures necessary to eliminate interfering factors.

7.2 In addition, any available parallel data for rabbit pyrogen testing that would contribute to an assurance that the replacement of a rabbit pyrogen test by the test for bacterial endotoxin is appropriate, should be provided.

7.3 For formulated preparations, a distinction should be made between any sample treatment necessitated by excipients included in a particular product and any required due to the nature of the active constituent. Attention should also be drawn to any manipulation normally required for the active constituent that is rendered redundant by the composition of the formulation, for example, pH adjustment.

8. In order to set an appropriate limit for bacterial endotoxins it is necessary to know the intended route of parenteral administration (in particular, if the substance may be administered intrathecally) together with the maximum dose as recommended in relevant product data sheets.
The limit for a given material or preparation is expressed as the endotoxin limit (EL) or endotoxin limit concentration (EL). The EL may be expressed in IU of endotoxin per millilitre *for a defined solution* of the material or preparation, or, for some medicinal substances, in IU of endotoxin in relation to a defined quantity of the material (that is, per milligram or, for biologically assayed materials, per IU) of material. The limit, which is stated in the monograph, is usually established on the following basis:

$EL = K/M$

8.1 K, sometimes referred to as the minimum pyrogenic dose, is the maximum number of IU of endotoxin which the patient may receive without suffering toxic

[1] *Poole, S., Dawson, P., Gaines Das, R.E. (1997). Second international standard for endotoxin: calibration in an international collaborative study. Journal of Endotoxin Research 4 (3), 221–231.*

[2] *Poole, S. et al. (2012). WHO international standard for endotoxin: report of an international collaborative study to evaluate three preparations of endotoxin for their suitability to serve as the third international standard for bacterial endotoxin. World Health Organisation.*

Type of product	Route of administration	*K*	
		per person	per kg
All parenteral preparations	Intrathecal[1]	14	0.2
Radiopharmaceuticals	Intravenous	175	2.5
All parenteral preparations except intrathecal	All parenteral routes except radiopharmaceuticals	350	5.0

[1]Where a product can be administered both intrathecally and by another parenteral route, the more stringent value of *K* for the intrathecal route will be taken as the basis of establishing the ELC.

reactions. The appropriate value for *K* will be taken from the table below.

8.2 *M* is the maximum dose of the drug substance per person (or per kg) per hour. This is interpreted as the maximum amount that might be administered within one hour. For subcutaneous (SC), intramuscular (IM) or bolus intravenous (IV) injections this will be an entire single dose. For intravenous infusions given over a prolonged period it is the proportion of the dose that would be infused during one hour and depends upon the rate of infusion. The value used is the maximum dose recommended by the manufacturer and stated in the relevant product data sheet. It is accepted that in exceptional circumstances this dose may be exceeded at the discretion of the physician; such use is outside the scope of the Pharmacopoeia.

Implementation

9. The British Pharmacopoeia Commission is seeking to replace the test for pyrogens by that for bacterial endotoxins wherever possible in the Pharmacopoeia. The European Pharmacopoeia Commission has a similar policy and has indicated that, for monographs already published, the change will be carried out, where appropriate, whenever the monograph in question is revised.

9.1 The test for bacterial endotoxins is now specified in the European Pharmacopoeia monograph for Water for Injections and in many other monographs including a range of antibiotics (for example, Doxorubicin Hydrochloride and Oxytetracycline Hydrochloride), biological materials (for example, Somatropin and Heparin) and radiopharmaceutical preparations.

9.2 Revision of the European Pharmacopoeia monograph for Parenteral Preparations to permit the use of the test for bacterial endotoxins in defined circumstances opened the way for the test for pyrogens to be replaced by that for bacterial endotoxins in individual monographs for parenteral preparations in the British Pharmacopoeia. The necessary change has already been made in a wide range of monographs including those for certain biological formulations such as Protamine Sulfate Injection and in the monographs for a number of widely used intravenous infusions such as Sodium Chloride Intravenous Infusion. Appropriate data are being sought from manufacturers for the remaining monographs for which a test for pyrogens is specified.

Annex

Guidelines concerning the test for Bacterial endotoxins have been published as an Annex to the method text 2.6.14 in the European Pharmacopoeia and are reproduced here.

The following section is published for information.

Guidelines for using the Test for Bacterial Endotoxins

(Ph. Eur. general texts 5.1.10)

1. INTRODUCTION

Endotoxins from gram-negative bacteria are the most common cause of toxic reactions resulting from contamination of pharmaceutical products with pyrogens; their common pyrogenic activity is much higher than that of other known pyrogenic substances. These endotoxins are lipopolysaccharides. Although there are a small number of pyrogens that possess a different structure, the conclusion is generally justified that the absence of bacterial endotoxins in a substance or product implies the absence of pyrogenic components, provided the presence of non-endotoxin pyrogenic substances can be ruled out. The monocyte-activation test (*2.6.30*) is a suitable method to use to rule out the presence of non-endotoxin pyrogens in substances or products.

The presence of endotoxins in a substance or product may be masked by factors interfering with the reaction between the endotoxins, the test reagents and the amoebocyte lysate. Also, the ability to detect endotoxins may be affected by storage conditions or storage time. Hence, the analyst who wishes to implement a test for bacterial endotoxins or to replace the pyrogen test by a test for bacterial endotoxins has to demonstrate that a valid test can be carried out on the substance or product concerned; this may entail a procedure for removing interference.

As indicated in general chapter *2.6.14. Bacterial endotoxins*, information must be available on the following 2 aspects before a test on a sample can be regarded as valid.

— The suitability of the material to be used for the test has to be established. The absence of endotoxins in the water for BET (water for bacterial endotoxins test) and in the other reagents and consumables must be assured and the sensitivity of the amoebocyte lysate must be checked to confirm the sensitivity declared by the manufacturer.

— As the substance or product to be examined may interfere with the test, the sensitivity of the amoebocyte lysate is determined in the presence and in the absence of the substance or product to be examined. There must be no difference between the 2 sensitivity values.

General chapter *2.6.14. Bacterial endotoxins* indicates methods for removing interfering factors; in the case of interference, another test must be carried out after such a method has been applied to check whether the interference has indeed been neutralised or removed.

This general chapter explains the reasons for the requirements in the test for bacterial endotoxins, then deals with reading and interpretation of the results.

Replacement of the rabbit pyrogen test required in a pharmacopoeial monograph by an amoebocyte lysate test, or by other methods such as the monocyte-activation test or a test using recombinant factor C reagent as a replacement for the amoebocyte lysate, constitutes the use of an alternative method of analysis and hence requires demonstration that the method is appropriate for the given substance or product and gives a result consistent with that obtained with the prescribed method as described in the General Notices (see also section 13).

The prescribed method for bacterial endotoxins may be stated in the monograph on a given substance or product. The use of a method other than the method prescribed in the monograph is considered as the use of an alternative method. Where no method is stated, any of methods A to F of general chapter *2.6.14. Bacterial endotoxins* can be used.

2. METHOD AND ACCEPTANCE CRITERIA

2-1 METHODS AND PRECAUTIONS TO BE TAKEN

The addition of endotoxins to amoebocyte lysate may result in turbidity, precipitation or gelation (gel-clot); initially only the gel-clot method was used in the Pharmacopoeia as an evaluation criterion in the test for bacterial endotoxins. The advantage was the simplicity of basing the decision to pass or fail the substance or product to be examined on the absence or presence of a gel-clot, visible with the naked eye. The quantitative methods C, D, E and F were developed later: they require more instrumentation, but they are easier to automate for the regular testing of large numbers of samples of the same substance or product.

Endotoxins may be adsorbed onto the surface of tubes or pipettes made from certain plastics or types of glass. Interference may appear due to the release of substances from plastic materials. Hence, the materials used must be checked.

2-2 ENDOTOXIN LIMIT CONCENTRATION

The decision to use the test for bacterial endotoxins as a limit test implies firstly that an endotoxin limit concentration must be defined for the substance or product to be examined, and secondly that the objective of the test is to know whether the endotoxin concentration in the sample to be examined is below or above this limit. The quantitative methods C, D, E and F make it possible to determine the endotoxin concentration in the sample to be examined, but for compliance with the Pharmacopoeia and in routine quality control the final question is whether or not this concentration exceeds a defined limit.

The dose of the substance or product to be examined must be taken into account in setting the endotoxin limit concentration: the limit is set so as to ensure that, as long as the endotoxin concentration in the substance or product remains below this limit, even the maximal dose administered by the intended route per hour does not contain sufficient endotoxin to cause a toxic reaction.

When the endotoxin concentration in the substance or product exactly equals the endotoxin limit concentration, gelation will occur, as is the case when the endotoxin concentration is much higher, and the substance or product will fail the test, because the all-or-none character of the test makes it impossible to differentiate between a concentration exactly equal to the endotoxin limit concentration and one that is higher. It is only when no gelation occurs that the analyst may conclude that the endotoxin concentration is below the endotoxin limit.

For substances or products in the solid state, this endotoxin limit concentration per mass unit or per International Unit (IU) of substance or product has to be converted into a concentration of endotoxin per millilitre of solution to be examined, as the test can only be carried out on a solution. The case of substances or products that already exist in the liquid state (such as infusion fluids) is discussed below.

2-3 CALCULATION OF THE ENDOTOXIN LIMIT

The endotoxin limit for active substances administered parenterally, defined on the basis of dose, is equal to:

$$\frac{K}{M}$$

K = threshold pyrogenic dose of endotoxin per kilogram of body mass;

M = maximum recommended bolus dose of product per kilogram of body mass.

When the product is to be injected at frequent intervals or infused continuously, M is the maximum total dose administered per hour.

The endotoxin limit depends on the product and its route of administration and may be stated in the monograph. Values for K are suggested in Table 5.1.10.-1.

For other routes, the acceptance criterion for bacterial endotoxins is generally determined on the basis of results obtained during the development of the preparation.

Table 5.1.10.-1

Route of administration	*K*
Intravenous	5.0 IU of endotoxin per kilogram of body mass
Intravenous for radiopharmaceuticals	2.5 IU of endotoxin per kilogram of body mass
Intrathecal	0.2 IU of endotoxin per kilogram of body mass
Parenteral formulations administered per square metre of body surface	100 IU of endotoxin/m^2

2-4 CONSIDERATIONS WHEN ESTABLISHING AN ENDOTOXIN LIMIT FOR A SPECIFIC SUBSTANCE OR PRODUCT

The endotoxin limit for a substance or product is established with consideration of the following aspects.

Calculated endotoxin limit

The endotoxin limit is calculated as described in section 2-3. This represents a safety limit not to be exceeded if the product is to be administered to humans.

Limit prescribed in an individual substance monograph

The limit stated in an individual substance monograph frequently reflects what is achievable in a controlled production environment. The limit prescribed in a monograph can therefore be lower than the calculated endotoxin limit. However a manufacturer may specify a limit that is more stringent than that stated in the monograph.

Process capability

The capability of the process to reduce or remove bacterial endotoxins during manufacture might result in lower endotoxin limits for specific processes.

Additional safety requirements

Precautions are taken in consideration of patient population (such as paediatric use, malnourished or cachectic patients, etc.), specific local requirements (e.g. countries might wish to operate with a lower average body weight of 60 kg instead of 70 kg frequently employed in Europe) or any additional safety margins requested by the competent authority.

Formulation of the product
The limit must take into consideration any theoretical bacterial endotoxin load introduced by any other components used for reconstitution and/or dilution of the product (e.g. water for injections) or introduced by starting materials and/or raw materials.

2-5 MAXIMUM VALID DILUTION
Which dilution of the substance or product is to be used in the test to obtain maximal assurance that a negative result means that the endotoxin concentration of the substance or product is less than the endotoxin limit and that a positive result means that the lysate detected an endotoxin concentration equal to or greater than the endotoxin limit? This dilution depends on the endotoxin limit and on the sensitivity of the lysate; it is called the maximum valid dilution (MVD) and its value may be calculated using the following expression:

$$\frac{\textit{endotoxin limit} \times \textit{concentration of test solution}}{\lambda}$$

Concentration of test solution:
— in mg/mL if the endotoxin limit is specified by mass (IU/mg);
— in Units/mL if the endotoxin limit is specified by unit of biological activity (IU/Unit);
— in mL/mL if the endotoxin limit is specified by volume (IU/mL).

λ = the labelled lysate sensitivity in the gel-clot technique (IU/mL) or the lowest concentration used in the standard curve of the turbidimetric or chromogenic techniques.

When the value of the MVD is not a whole number, a convenient whole number smaller than the MVD may be used for routine purposes (which means preparing a solution of the substance or product that is less diluted than the MVD indicates). In this case, a negative result indicates that the endotoxin concentration of the substance or product lies below the limit value. However, when the endotoxin concentration of the substance or product in such a test is less than the endotoxin limit but high enough to make the reaction with the lysate result in a clot, the test may be positive under these conditions. Hence, when a test with this 'convenient' dilution factor is positive, the substance or product is diluted to the MVD and the test is repeated. In any case of doubt or dispute, the MVD must be used.

This stresses the importance of the confirmation of the sensitivity of the lysate.

Example

A 50 mg/mL solution of phenytoin sodium (intended for intravenous injection) has to be tested. Determine the MVD, given the following variables:

M = maximum human dose = 15 mg per kilogram of body mass;
c = 50 mg/mL;
K = 5 IU of endotoxin per kilogram of body mass;
λ = 0.4 IU of endotoxin per millilitre.

$$MVD = \frac{5 \times 50}{15} \times \frac{1}{0.4} = 41.67$$

For routine tests on this product, it may be expedient to dilute 1 mL of the solution to be examined to 20 mL (MVD/2 rounded to the next lower whole number). However, if this test result is positive the analyst will have to dilute 1 mL to 41.67 mL and repeat the test. A dilution to 41.67 mL is also necessary when the test is performed to settle a dispute.

3. RISK ASSESSMENT

As stated in section 1 of this general chapter, the conclusion is generally justified that the absence of bacterial endotoxins in a substance or product implies the absence of pyrogenic components, provided the presence of non-endotoxin pyrogenic substances can be ruled out. To rule out the presence of non-endotoxin pyrogens in substances or products, the use of the monocyte-activation test (*2.6.30*) is recommended at release or during development of the production process; if any changes are made to the production process that could influence the quality of the product regarding pyrogenicity, the monocyte-activation test is repeated. Examples of such changes include the use of different raw materials, a different production site and different process parameters.

The decision to use the test for bacterial endotoxins as the sole pyrogenicity test is to be made after careful evaluation of the risk of the substance or product containing non-endotoxin pyrogens. The risk assessment is made with consideration given to any factor that could result in the inclusion of pyrogens not detected by the test for bacterial endotoxins. The items below constitute a non-exhaustive list of factors to be considered in the risk assessment.

Production process
(chemical synthesis, fermentation, biotechnological method). For products of fermentation, the expression system is to be considered (prokaryotic, eukaryotic) and, for a prokaryotic expression system, whether gram-positive or gram-negative bacteria are used. Also, the culture media components are examined with consideration given to their origin (synthetic, animal, plant).

Bioburden
The potential presence of gram-positive bacteria and fungi as contaminants of the active substance, excipients or starting materials and raw materials used in the production of the medicinal product, and the origin of the raw materials (synthetic, animal, plant) have to be taken into consideration. The quality of the water plays an important role on the overall evaluation.

Capability of the downstream process
It must be verified whether bacterial endotoxin removal steps are part of the downstream process.

Safety
The target population and the route of administration (e.g. intravenous, intrathecal) have to be taken into account in the risk assessment.

Stability of the detectable endotoxins
It has to be considered that the ability to detect endotoxins can be affected by interaction with certain components, storage conditions or storage time, temperature and handling of the test sample. Procedures that demonstrate stability of the detectable endotoxin content have to be established for storing, handling and mixing of samples.

4. REFERENCE MATERIAL

Endotoxin standard BRP is intended for use as the reference preparation. It has been assayed against the WHO International Standard for Endotoxin and its potency is expressed in International Units of endotoxin per vial. The International Unit of endotoxin is defined as the specific activity of a defined mass of the International Standard.

For routine purposes, another preparation of endotoxin may be used, provided it has been assayed against the

International Standard for Endotoxin or the BRP and its potency is expressed in International Units of endotoxin.

NOTE: 1 International Unit (IU) of endotoxin is equal to 1 Endotoxin Unit (EU).

5. WATER FOR BET

Water for BET is sterile water that is free of detectable levels of endotoxin. Usually it is commercially available and certified.

General chapter *2.6.14. Bacterial endotoxins* indicates that methods other than triple distillation may be used to prepare water for BET. Reverse osmosis has been used with good results; some analysts may prefer to distil the water more than 3 times. Whatever method is used, the resultant product must be free of detectable bacterial endotoxins.

6. PH OF THE MIXTURE

In the test for bacterial endotoxins, optimum gel-clot occurs for a mixture at pH 6.0-8.0. However, the addition of the lysate to the sample may result in a lowering of the pH.

7. VALIDATION OF THE LYSATE

It is important to follow the manufacturer's instructions for the preparation of the solutions of the lysate.

The positive end-point dilution factors in gel-clot methods A and B are converted to logarithms. The reason is that if the frequency distribution of these logarithmic values is plotted, it usually approaches a normal distribution curve much more closely than the frequency distribution of the dilution factors themselves; in fact it is so similar that it is acceptable to use the normal frequency distribution as a mathematical model and to calculate confidence limits with Student's *t*-test.

8. PRELIMINARY TEST FOR INTERFERING FACTORS

Some substances or products cannot be tested directly for the presence of bacterial endotoxins because they are not miscible with the reagents, they cannot be adjusted to pH 6.0-8.0 or they inhibit or activate enzymatic reaction (such as β-D-glucans).

Therefore a preliminary test is required to check for the presence of interfering factors; when these are found the analyst must demonstrate that the procedure to remove them has been effective and that by applying this procedure, any bacterial endotoxins present have not been removed.

The object of the preliminary test is to test the null hypothesis that the sensitivity of the lysate in the presence of the substance or product to be examined does not differ significantly from the sensitivity of the lysate in the absence of the product. A simple criterion is used in methods A and B: the null hypothesis is accepted when the sensitivity of the lysate in the presence of the product is at least 0.5 times and not more than twice the sensitivity of the lysate by itself.

The test for interfering factors in gel-clot methods A and B requires the use of a sample of the substance or product in which no endotoxins are detectable. This presents a theoretical problem when an entirely new product has to be tested. Hence, a different approach was designed for quantitative methods C, D, E and F.

Note that methods D and E, which use a chromogenic peptide, require reagents that are absent in methods A, B, C and F, and hence compliance of methods A, B, C or F with the requirements for interfering factors cannot be extrapolated to method D or method E without further testing.

9. REMOVAL OF INTERFERING FACTORS

The procedures to remove interfering factors must not increase or decrease (for example, by adsorption) the amount of endotoxin in the substance or product to be examined. The correct way of checking this is to apply the procedures to a spiked sample of the substance or product to be examined, that is, a sample to which a known amount of endotoxin has been added, and then to measure the recovery of the endotoxin after the removal process has been conducted.

Methods C and D

If the nature of the product to be examined results in an interference that cannot be removed by classical methods (e.g. dilution or centrifugation), it may be possible to determine the standard curve in the same type of substance or product freed from endotoxins by appropriate treatment or by dilution of the substance or product. The endotoxins test is then carried out by comparison with this standard curve.

Ultrafiltration with cellulose triacetate asymmetric membrane filters has been found to be suitable in most cases. The filters must be properly validated, because under some circumstances cellulose derivatives (β-D-glucans) can cause false positive results.

Another option to remove interfering factors is a 2-step procedure in which 1) endotoxin within the interfering sample is fixed on a solid phase, and 2) after removal of the interfering substance (e.g. by washing) the endotoxin is detected unimpaired under suitable testing conditions.

10. THE PURPOSE OF THE CONTROLS

The purpose of the control made up with water for BET and the reference preparation of endotoxin at twice the concentration of the labelled lysate sensitivity is to verify the activity of the lysate at the time and under the conditions of the test (for method A and B). The purpose of the negative control is to verify the absence of a detectable concentration of endotoxin in the water for BET.

The positive control, which contains the product to be examined at the concentration used in the test, is intended to show the absence of inhibiting factors at the time and under the conditions of the test.

11. READING AND INTERPRETATION OF RESULTS

Minute amounts of bacterial endotoxin in the water for BET, or in any other reagent or material to which the lysate is exposed during the test, may escape detection as long as they do not reach the sensitivity limit of the lysate. However, they may raise the amount of bacterial endotoxin in the solution containing the substance or product to be examined to just above the sensitivity limit and cause a positive reaction.

The risk of this happening may be reduced by testing the water for BET and the other reagents and materials with the most sensitive lysate available, or at least one that is more sensitive than the one used in the test on the product. Even then, the risk of such a 'false positive result' cannot be ruled out completely.

12. IMPLEMENTATION OF METHODS DESCRIBED IN THE PH. EUR.

As stated in the General Notices, the test methods given in monographs and general chapters have been validated in accordance with accepted scientific practice and current recommendations on analytical validation. The methods described in general chapters *2.6.14. Bacterial endotoxins*, *2.6.30. Monocyte-activation test* and *2.6.32. Test for bacterial*

endotoxins using recombinant factor C therefore do not have to be re-validated *per se*, other than in consideration of their use for a specific substance or product in a specific analytical environment.

The procedure and the materials and reagents used in the method must be validated as described for the test concerned.

The absence of interfering factors (and, if necessary, the procedure for removing them) is verified on samples of at least 3 production batches.

As stated in general chapter *2.6.30. Monocyte-activation test*, the monocyte-activation test is primarily intended as a replacement of the rabbit pyrogen test. Guidelines on which methods to use (A, B or C) and on how to validate the monocyte-activation test are described in general chapter *2.6.30. Monocyte-activation test*.

13. REPLACEMENT OF A METHOD PRESCRIBED IN A MONOGRAPH

13-1 REPLACEMENT BY ANOTHER METHOD DESCRIBED IN THE PH. EUR.

Replacement of a method prescribed in a monograph by another method described in the Ph. Eur. is to be regarded as the use of an alternative method in the replacement of a pharmacopoeial test, as described in the General Notices.

The analyst has to demonstrate that a valid test can be carried out on the substance or product concerned.

The alternative method does not have to be re-validated *per se*, other than in consideration of its use for a specific substance or product in a specific analytical environment and of its equivalence to the prescribed method.

13-2 REPLACEMENT BY A METHOD NOT DESCRIBED IN THE PH. EUR.

Replacement of a method prescribed in a monograph by a method not described in the Ph. Eur. is to be regarded as the use of an alternative method in the replacement of a pharmacopoeial test, as described in the General Notices.

D. Excipients

1. The General Notice on Excipients states that 'any substances added in preparing an official preparation shall... not interfere with the assays and tests of the Pharmacopoeia'.

2. The British Pharmacopoeia Commission wishes to stress that any preparation described by a name at the head of a monograph in the current edition of the Pharmacopoeia, *whether or not it is referred to as BP*, is not of pharmacopoeial quality unless it meets all of the requirements of the monograph when tested by the methods set down.

3. It is recognised that new formulations of existing preparations may from time to time be developed and that the excipients and other ingredients used might result in interference with the official assays and tests. In such cases the Commission is prepared to consider modification of methods to overcome the difficulties thus caused.

4. When seeking modification manufacturers are invited to submit details of the nature of the interference together with proposals for change that will allow the valid testing, by an independent analyst, not only of the proposed new formulation *but also of all similar preparations already on the market*. Practical evaluation in the Laboratory will usually be necessary before any amendments to a British Pharmacopoeia monograph can be considered. It is therefore in the interests of a manufacturer to submit any proposals at the earliest possible date.

E. Dissolution Testing of Solid Oral Dosage Forms

This section provides information on the pharmacopoeial dissolution test and guidance on its function and application in individual monographs of the British Pharmacopoeia for tablets and capsules.

1. Harmonisation

1.1 The dissolution test for solid oral dosage forms in Appendix XII B1 of the British Pharmacopoeia is that of the European Pharmacopoeia (*Ph. Eur. method 2.9.3*). It is a harmonised text prepared by the Pharmacopoeial Discussion Group (PDG). The PDG is comprised of representatives of the European Pharmacopoeia, Japanese Pharmacopoeia and the United States Pharmacopeia. Although the test is largely harmonised, some regional differences remain.

1.2 The British Pharmacopoeia has committed to modernising Monographs containing dissolution acceptance criteria that are not currently harmonised with the test in Appendix XII B1. Appendix XII B1 contains a section entitled *Monographs of the British Pharmacopoeia* which provides requirements for monographs for tablets and capsules of the BP that are yet to be updated. Taking account of permissible assay ranges and content uniformity, this pharmacopoeial (that is, shelf-life) dissolution requirement is considered to offer an acceptable degree of assurance of 'complete dissolution'. The choice of a time is, of necessity, somewhat arbitrary but 45 minutes is considered satisfactory for the majority of conventional-release (non-modified-release) products.

2. Apparatus

2.1 Four types of apparatuses are now described in the British and European Pharmacopoeias; the basket, the paddle, the reciprocating cylinder and the flow-through cell. The descriptions are concordant with those published in the United States Pharmacopeia (USP).

2.2 Of the two established apparatuses (basket and paddle) the paddle is now the apparatus of choice for many preparations. However, where a published test uses the basket, work to validate a change to the paddle method is not contemplated. The reciprocating cylinder is useful for pH profiling studies while the flow-through cell may be appropriate for preparations of poorly soluble active ingredients (see Appendix XII B Annex).

3. Test conditions and acceptance criteria

3.1 **Test conditions** The harmonised test conditions included in Appendix XII B1 will be applied to all monographs of the British Pharmacopoeia over time. Where an individual monograph prescribes the use of the requirements stated under *Monographs of the British Pharmacopoeia* in Appendix XII B1, the following conditions using the basket or paddle apparatus are preferred.

— rotation speed:100 rpm (basket), 50 rpm (paddle)
— dissolution medium volume: 900 mL
— dissolution medium composition: aqueous, commonly 0.1M *hydrochloric acid* or phosphate buffers of pH 6.8 to 7.6
— number of units tested: 6 (plus 6, if a retest is required).
— The number of units tested is specified in Appendix XII B1; other conditions are specified in the relevant individual monographs.

In situations where it has been demonstrated that the harmonised criteria are not applicable (e.g. low solubility preparations, 'coning' of material in the vessel, low concentration of analyte), modifications may be made to the test conditions, such as, adding a surfactant, increasing the paddle rotation speed or using a modified vessel and reducing the volume of dissolution medium used.

3.2 **Acceptance criteria** For monographs for conventional-release preparations, unless otherwise stated, the harmonised acceptance criteria ("Q" values) in Appendix XII B1 should be applied.
For monographs that are yet to be updated to "Q" values, the established BP criteria using either the basket or the paddle apparatus are specified under 'Monographs of the British Pharmacopoeia' in Appendix XII B1.

3.3 Standardised conditions and limits are considered appropriate for a pharmacopoeial test that is intended to apply to monographs covering products from different manufacturers. It might be argued that non-standardised conditions and limits would be more discriminatory but 'tailor-made' test conditions and limits may introduce product bias and may discriminate unnecessarily between products that are equally acceptable from a clinical view-point. Similarly with sufficient manipulation of the test conditions, dissolution of almost any product can be achieved. Ideally the test should reflect clinically significant differences in bioavailability arising from differences in dissolution in such a way that clinically acceptable formulations will pass whereas clinically unacceptable formulations will fail.

3.4 Another issue that has been considered in relation to test conditions and criteria is that of multiple-point dissolution profiles as opposed to single-point dissolution tests. It has been concluded that for conventional-release preparations such an extension of testing is not generally necessary or appropriate for pharmacopoeial purposes.

4. Function

4.1 The ultimate objective of dissolution testing may be described as ensuring adequate and reproducible bioavailability without recourse to routine *in-vivo* testing. On some occasions, this may be achieved by dissolution testing of a particular product for which *in vitro/in vivo* correlation has been demonstrated.

4.2 A more common objective of dissolution testing is to obtain information about the drug release characteristics of a particular formulation or batch of product under standardised *in vitro* testing conditions.

4.3 Dissolution testing may also be carried out during product development studies and is a useful tool in optimising formulation and manufacturing parameters. It is usually required by the Competent Authority as part of a marketing authorisation application.

4.4 Once a product is licensed, dissolution testing may be required routinely as part of quality control to demonstrate consistency of manufacture before the release of each batch of the finished product or, when necessary, to provide evidence to support changes in manufacture such as minor changes in formulation or process, changes in site or changes in immediate packaging materials.

5. Application

5.1 The British Pharmacopoeia Commission has not adopted a policy of universal application of the dissolution test and, therefore, a dissolution requirement will not be included automatically in every capsule and tablet monograph. The International Conference on Harmonisation (ICH) guideline on Test Procedures and Acceptance Criteria for New Drug Substances and New Drug Products (ICH Q6A) contains guidance on the application of dissolution testing to drug products and this will be used as a basis for deciding whether to include a dissolution test in an individual monograph.

5.2 As a *general guideline*, it is expected that all new monographs for conventional-release capsules and tablets will contain a dissolution requirement except (i) where the solubility of the active ingredient at 37 ± 0.5° is high throughout the physiological pH range (dose/solubility volume <250 mL at pH 1.2 to 6.8); (ii) where the dissolution of the dosage form is greater than 80% in 15 minutes at pH 1.2, 4.0 and 6.8; (iii) where a relationship has been determined between disintegration and dissolution, or when disintegration has been observed to be more discriminatory than dissolution.
In circumstances where conditions (i) to (iii) have been satisfied, the dissolution test may be replaced by the *disintegration test for tablets and capsules*, Appendix XII A1.

5.3 If challenged, a product would be expected to comply with the test for dissolution specified in the individual monograph, or if none is specified, with the Test conditions and acceptance criteria outlined in section 3.

5.4 Dissolution tests have been added to a considerable number of monographs in the British Pharmacopoeia. While the objective is to include a 'standard' pharmacopoeial test wherever appropriate, the circumstances for each preparation are considered individually in consultation with the manufacturers. It should be appreciated, however, that the retrospective addition of dissolution tests is not without its difficulties. The problems are most acute for those well-established preparations that are manufactured by a wide range of companies, each with its own dissolution specification. A pragmatic approach is being taken to developing compromise test procedures in these circumstances.

6. Bioavailability and Bioequivalence

6.1 Compliance with the standard British Pharmacopoeia requirement for dissolution provides an assurance that most of the active ingredient will be dissolved in a reasonable amount of time when the preparation is subjected to mild agitation.

6.2 It should be noted that compliance with the pharmacopoeial dissolution test does not by itself guarantee bioavailability and is not necessarily an adequate basis for judging bioequivalence between preparations. Preparations having similar dissolution characteristics may not be bioequivalent and *vice versa*.

7. Application of harmonised dissolution limits ("Q" values)

7.1 Since the harmonisation of the dissolution test through the Pharmacopoeial Discussion Group and the adoption of harmonised dissolution criteria ("Q values") between the European, United States and Japanese Pharmacopoeias, the BP Commission has received

many queries regarding the application of limits to dissolution tests.

7.2 As an aid to analysts several worked examples are provided as an illustrative guide below.

7.3 An immediate-release tablet formulation has a limit of Q = 75%. Six units are tested.

Unit	Result (% of stated amount)
1	83
2	87
3	80
4	88
5	90
6	82

In order to pass S1, all units must be greater than or equal to (Q + 5)% (i.e. 80%). Hence, the above batch passes at S1.

7.4 An immediate-release tablet formulation has a limit of Q = 75%. Six units are tested.

Unit	Result (% of stated amount)
1	77
2	87
3	70
4	88
5	90
6	82

Since units 1 and 3 are less than (Q+5)% (i.e. 80%), the batch fails at S1. A further six units must then be tested.

Unit	Result (% of stated amount)
7	78
8	85
9	79
10	84
11	87
12	85

In order to pass S2, the mean of twelve units must be greater than or equal to Q and no unit can be less than (Q – 15)% (i.e. 60%). The mean of the twelve units is 83% so this passes at S2 and, units 1 and 3, whilst failing at S1, are not less than (Q – 15)%, so this batch passes at S2.

7.4 An immediate-release tablet formulation has a limit of Q = 75%. Six units are tested.

Unit	Result (% of stated amount)
1	77
2	87
3	70
4	88
5	76
6	79

Since units 1, 3, 5 and 6 are less than (Q+5)% (i.e. 80%), the batch fails at S1. A further six units must then be tested

Unit	Result (% of stated amount)
7	73
8	85
9	79
10	84
11	87
12	58

Because unit 12 is less than (Q – 15)% (i.e. 60%) the batch fails at S2. A further twelve units must then be tested.

Unit	Result (% of stated amount)
13	79
14	83
15	67
16	84
17	80
18	82
19	78
20	85
21	84
22	81
23	83
24	88

In order to pass S3, the mean of 24 units must be greater than or equal to Q , not more than two units can be less than (Q – 15)% (i.e. 60%) and no unit can be less than (Q – 25)% (i.e. 50%). The mean of the 24 units is 80% so this passes at S3. Only unit 12 is less than (Q – 15)% and none are less than (Q – 25)% so this batch passes at S3.

7.6 An immediate-release capsule formulation has a limit of Q = 75%. Six units are tested.

Unit	Result (% of stated amount)
1	83
2	82
3	80
4	79
5	49
6	77

Since unit 5 is less than (Q – 25)% (i.e. 50%), the batch would fail at S3. Testing further units will not enable this batch to pass the test.

7.7 An immediate-release capsule formulation has a limit of Q = 75%. 24 units have been tested.

Unit	Result (% of stated amount)
1	76
2	82
3	80
4	79
5	70
6	77
7	65
8	69
9	77
10	73
11	68
12	71
13	79
14	65
15	70
16	72
17	67
18	83
19	63
20	68
21	74
22	71
23	75
24	70

All the units are greater than (Q – 15)% (i.e. 60%), however; because the mean (73%) is less than Q, this batch fails at S3.

F. Declaration of Content

This section describes the way in which the content of active substance in a preparation is declared and the method adopted to express the content of the medicinal substances themselves. A proper understanding of such statements is essential to their correct interpretation.

Medicinal substances

1. The purpose of the assay in monographs for medicinal substances, taken in conjunction with the tests for impurities, is to determine the purity of the medicinal substance and the limits are therefore usually stated in terms of the molecular entity (salt, ester, etc.) and calculated with reference to the anhydrous or dried substance as appropriate (depending on whether the monograph includes a test for water or for loss on drying).

2. One advantage of this form of expression in 'parent' monographs is that it gives an indication 'at a glance' of the purity of the substance. For example (albeit an extreme example) the purity of Amitriptyline Embonate is stated as not less than 98.5% of $(C_{20}H_{23}N)_2,C_{23}H_{16}O_6$ calculated with reference to the anhydrous substance rather than as not less than 57.9% of $C_{20}H_{23}N$ calculated with reference to the anhydrous substance.

3. The mode of expression chosen for the 'parent' monograph in no way circumscribes that which may be used in the monograph for a preparation. There is no reason why the two should be the same and there is frequently good reason why they should be different. In the example of Amitriptyline Embonate, the assay limits for the preparation Amitriptyline Oral Suspension are stated in terms of amitriptyline, $C_{20}H_{23}N$ in the BP 2000.

Formulated preparations

4. The purpose of the assay in monographs for formulated preparations is to determine whether the content of the active ingredient is within acceptable limits of the labelled claim and the limits are therefore of necessity stated in terms of the moiety declared on the label as established by the manufacturer.

5. Every effort is made in the British Pharmacopoeia to achieve internal consistency within monographs, that is, to use the same terms for content statement, assay and label. The British Pharmacopoeia Commission, however, has no means of achieving external (inter-monograph) consistency since, unless it perceives there to be a potentially serious risk, it would not seek to obtain a change in a manufacturer's established practice. Problems arise when a manufacturer is not consistent or does not state clearly to what the strength refers and, in particular, when different manufacturers of the same preparation express the content in different terms.

6. Ideally in many cases where several salts or hydrated forms of the same drug substance are available, the label and dose (and therefore all monograph statements) should be in terms of the anhydrous free base or acid, that is, the active moiety, in order to facilitate comparison and equivalent dosage.

7. Implementation of such a policy would clearly require that each case should be judged on its merits since there would be instances when, for example, a different salt is considered as a different active moiety or where it would be misleading to suggest that two different forms are therapeutically equivalent. Nevertheless it is strongly recommended that as a general rule *for new drug substances*, doses and strengths of preparations should be expressed in terms of the active moiety.

8. Meanwhile, for established materials the Pharmacopoeia will continue to reflect current practice. In this respect it should be noted that the labelling requirements of the Pharmacopoeia are not comprehensive. Thus a monograph requirement to state the content of active ingredient in terms of the entire drug substance molecule does not preclude an additional indication of the content expressed in terms of the active moiety where such an indication is considered desirable.

G. Labelling

This section provides guidance on the status and interpretation of pharmacopoeial labelling sections.

1. The General Notice on Labelling distinguishes between the mandatory status of those Pharmacopoeial labelling statements that are necessary to demonstrate compliance with the monograph and the advisory status of other labelling statements included in the Pharmacopoeia.

2. This distinction, which is consistent with the approach adopted in the European Pharmacopoeia, is made in recognition of the complexity of the statutory and advisory framework within which the labelling of medicines is determined. It is hoped that by thus restricting mandatory pharmacopoeial labelling statements to those that are essential for pharmacopoeial purposes, the potential for conflict between pharmacopoeial and other statutory provisions will be minimised.

3. Within the context of any particular monograph, it should be apparent which of the labelling statements are necessary to demonstrate compliance with the monograph and are thus mandatory. As guidance, a labelling statement is considered essential for a medicinal substance

(i) where a test or assay requirement is expressed in relation to a declared value, the 'labelled claim' (for example, the apparent viscosity of Carmellose Sodium or the potency of Calcitonin (Salmon));

(ii) where different test requirements or limits apply to materials derived from different sources (for example, the requirements for matter insoluble in 5M ammonia depend on the botanical source of Podophyllum Resin), or intended for different purposes (for example, the sterility of Benzylpenicillin Sodium when intended for use in the manufacture of a parenteral dosage form without a further sterilisation process);

(iii) in other special circumstances.

4. Likewise a labelling statement is considered essential for a formulated preparation

(i) where a test requirement is expressed in relation to a declared value, the 'labelled claim';

(ii) where the content of active ingredient is required to be expressed in terms other than the weight of the official medicinal substance used in making the formulation (for example, Primaquine Tablets contain Primaquine Phosphate but the content is expressed in terms of the equivalent amount of primaquine base);

(iii) where different test requirements or limits apply to the formulated preparation manufactured from different bulk drug substances (for example, Heparin Injection manufactured from Heparin Calcium or Heparin Sodium) or intended for different purposes (for example, Isosorbide Dinitrate Tablets intended to be

chewed before swallowing or allowed to dissolve in the mouth);

(iv) in other special circumstances.

5. Advisory labelling statements for formulated preparations may be included either in the general monograph or in the monograph for the individual preparation, as appropriate. Such statements commonly relate to features such as the expiry date, the storage conditions, the name and amount of any excipients and the directions for making the final preparations. Additional advisory labelling statements may relate to the directions for using the preparation or the precautions relating to the handling and use of the preparation.

H. Biological Assays and Tests

This section provides information and guidance concerning the biological assays and tests of the Pharmacopoeia and the standard preparations required for them.

1. Biological, including biochemical or immunochemical, methods are described for the determination of potency or other specific properties of certain substances and preparations where these properties cannot be adequately determined by chemical or physical means. The principle applied wherever possible throughout these methods is that of comparison with a standard preparation so as to determine how much of a sample being examined produces the same effect as a given quantity, the Unit, defined by the standard preparation. It is an essential condition of such methods that the tests on the standard preparation and on the sample, the potency or other property of which is being determined, shall be carried out at the same time and, in all other respects, under strictly comparable conditions.

2. Standard Preparations, as defined in the biological assays and tests of the Pharmacopoeia, are of two kinds: primary standards which are established, held and distributed by the appropriate international or national organisation and secondary (working) standards which are preparations the potencies of which have been determined by an adequate number of comparative tests in relation to the relevant primary standard.

3. A primary standard is a selected representative sample of the substance for which it is to serve as a basis of measurement. It is essential that primary standards shall be of uniform quality and as stable as possible. These conditions are usually ensured by providing the preparations in the dry state, dispensing them in sealed containers free from moisture and oxygen and storing them continuously at a low temperature and in the absence of light.

For the majority of biological assays of the Pharmacopoeia, the primary standards are the International Standards and Reference Preparations, established by the World Health Organization.

Laboratories in the United Kingdom may obtain these for the purposes of the biological assays described in the Pharmacopoeia from the National Institute for Biological Standards and Control, Blanche Lane, South Mimms, Potters Bar, Hertfordshire EN6 3QG, England.

In October 2005 the European Directorate for the Quality of Medicines & HealthCare (EDQM) was designated as the International Collaborative Centre for the establishment and distribution of WHO International Standards for Antibiotics (ISA) , replacing the National Institute for Biological Standards and Control (NIBSC) in this role. These standards may be obtained from the Sales Section, EDQM - Council of Europe, 7 allée Kastner, CS 30026, 67081 Strasbourg, France. Further information can be found on the EDQM website (www.edqm.eu).

For the assay of certain enzymes in the Pharmacopoeia the Standard Preparations, as defined in the appropriate method, are the primary standards established by the International Commission on Pharmaceutical Enzymes of the International Pharmaceutical Federation (FIP). These standards may be obtained from the Centre for Standards, Wolterslaan 12, B–9000, Ghent, Belgium or, where these standards have been established in co-operation with, and adopted as official preparations by, the European Pharmacopoeia Commission, they may be obtained from the EDQM address shown above.

4. As a measure of economy in the use of the primary standards it is recommended that working standards should be prepared and used in those biological methods of the Pharmacopoeia where the definition of the Standard Preparation is so worded as to permit this. However, in some instances the complexity or lack of precision of the method or difficulties associated with the preparation of a secondary standard render such a practice inadvisable and in any country in which a particular assay is controlled by law it may be necessary to obtain the approval of the appropriate authority for the use of working standards. The biological properties of the samples selected as working standards should conform as closely as possible to those of the primary standard and an assurance that these conditions have been fulfilled is usually obtained by comparing the behaviour of the two samples under varying conditions of comparative testing. In such tests a detailed study of the dose-response curves may indicate whether the sample selected to serve as a working standard is a suitable preparation. For the assay of certain biological materials in the Pharmacopoeia, for example oxytocin, European Pharmacopoeia Biological Reference Preparations have been established and are recommended for use as the working standards. Such preparations may be obtained from the European Pharmacopoeia Commission (address as above).

5. Wherever possible the primary standard is the International Standard, and the biological activity is expressed in International Units (IU). In other cases, where Units are referred to in the official assays and tests, the Unit for a particular substance is, for the United Kingdom, the specific biological activity contained in such an amount of the respective primary standard as the appropriate international or national organisation indicates. The necessary information is provided with the samples of the primary standard.

6. For enzymes in the Pharmacopoeia, where the assay is such that the stoichiometry of the reaction is known, for example, where the substrate is a synthetic ester, the activity is measured in microkatals or nanokatals. A microkatal is defined as the enzyme activity that under defined conditions, produces one micromole of the reaction product per second or alternatively consumes one micromole of the reaction substrate per second. For other enzymes in the Pharmacopoeia, where the reaction involved in the assay is more complex, for example, where the substrate is a naturally occurring macromolecule such as a protein, the activity is measured in Units as previously defined.

7. The methods of biological assay described in the Pharmacopoeia have been found satisfactory but will not necessarily be the best methods for use in all circumstances. In most instances they may be replaced by other methods if

it can be shown that such methods are at least equally accurate and precise and provide a measurement of the same active principles.

8. Any estimate of potency derived from a biological assay is subject to random error due to the inherent variability of biological responses and calculations of error should be made, if possible, from the results of each assay and recorded with the potency estimate even when the official method of assay is used. Guidance on the design of assays, the statistical analysis of results and the calculation of potency is provided in general text 5.3 of the European Pharmacopoeia (Supplementary Chapter IV G). The methods described therein take account of the inherent random error but assume that systematic errors, for example, errors in weighing or dilution, will not represent a major source of variation in the potency estimates. Alternative assay designs and methods of calculation may be used provided that they are not less reliable.

9. Where an immunoassay, that is an assay procedure based on the reversible and non-covalent binding of an antigen by antibody, is described in the Pharmacopoeia for detecting or quantifying either an antigen or an antibody, the considerations described in Appendix XIV B apply in addition to the general points referred to above.

J. Efficacy of Antimicrobial Preservation

This section provides background information on the purpose and scope of the test for efficacy of antimicrobial preservation. The Annex to this section provides guidance on some practical aspects of testing.

1. The test for efficacy of antimicrobial preservation, included in Appendix XVI C, has been accorded non-mandatory status in the Pharmacopoeia. This status is reflected in the test's inclusion as a general text in section 5 of the European Pharmacopoeia and in the form of reference to the test within Production sections of relevant general monographs of the European Pharmacopoeia. Non-mandatory status provides a degree of flexibility consistent with the intended purpose of the pharmacopoeial test.

2. The pharmacopoeial test is intended to serve as a model offering a manufacturer guidance concerning this aspect of quality and a foundation on which he can build to meet his own particular needs. The testing procedure is intended to serve as a means whereby, during product development, a manufacturer can assess the efficacy of any antimicrobial preservative included in the product. Within the context of the British Pharmacopoeia, Appendix XVI C serves to define what is meant by the term 'suitable antimicrobial preservative' in accordance with the relevant Notice in Part 2 of the General Notices.

3. If during development a fully quantitative, comparative evaluation of different preservative systems would be useful, the procedure described in the Appendix could be extended. For example, the incorporation of additional sample times would allow an estimation of microbial death rates.

4. The test is intended for application to those products that may support the growth or viability of microbial contaminants. It is based on the premise that preservatives are used primarily to protect products from in-use microbial contamination but also recognises their role in reducing the bioburden of 'non-sterile' products. In-use contamination is of relevance to multidose products, both sterile and non-sterile. Bioburden is of relevance to non-sterile products, both multidose and single dose.

5. The present European Pharmacopoeia text provides criteria for parenteral, ophthalmic, topical and oral preparations. The criteria for ear preparations are equivalent to those specified for topical preparations thus maintaining parity between these two types of product.

6. An unusual feature of the European Pharmacopoeia text is the inclusion of two sets of criteria (A and B) for parenteral and ophthalmic preparations and for topical preparations. The A criteria express the recommended efficacy to be achieved, that is, they represent generally applicable 'target' criteria.

7. It is recognised that for certain products, for example, some antacids and certain biological products, these target criteria are unlikely to be achieved except at the expense of some other property of equal or greater importance. The alternative criteria to be met in these circumstances are a matter for agreement between the manufacturer and the competent authority and should take account of any special considerations relevant to the specific product. The B criteria for parenteral and ophthalmic products and for topical products were adopted by the European Pharmacopoeia Commission in deference to those member states that wanted published guidance on the minimum values below which any alternative criteria should not fall.

8. As noted in paragraph 2 above, the pharmacopoeial test is intended to provide a framework within which to develop a test suitable for the particular product being examined. Detailed descriptions of all aspects of testing are therefore inappropriate within the pharmacopoeial test itself. The following Annex draws attention to some key features of the test and offers additional guidance on certain practical aspects of testing.

Annex

9. Several features have been identified as sources of procedural variation in the test that may contribute to variation in the outcome of the test. A clearly written and sufficiently detailed test protocol is therefore essential. Some of these features are discussed in this Annex.

10. **Test organisms; culture maintenance** Potential sources of variation to which attention should be given include the source, age and storage of the microbial culture and the number of passages between the freeze-dried strain collection culture (repository culture) and the suspension of organisms used to inoculate the product (the test suspension). It is recommended that the repository culture is cultured and aliquots freeze-dried or stored under liquid nitrogen. It is also recommended that the sub-culture used as the test suspension should be no more than 5 passages from the repository culture.

11. **Product inoculation** Potential sources of variation to which attention should be given include the culture media and method used to prepare the inoculum, the inoculum: product ratio and the homogeneity of the inoculum–product mixture. Appendix XVI C states that the test suspension shall be used immediately. It is recommended that this is interpreted to mean that, once prepared, the test suspension should not be stored but may be used over a period of 8 hours provided that it is kept at a temperature of 2° to 8°.

12. **Test container** It is emphasised that, as indicated in the Appendix, wherever possible, the product should be inoculated and incubated in its original container (market pack). Where, however, it is necessary to transfer the product to some other container, for example, in order to achieve

homogeneous distribution of the inoculum, careful consideration needs to be given to the choice of test container. Features of such a container that will influence its suitability as a test container include the material from which it is made, its shape and size (volume) and the type of closure. The material from which the container is made should not affect the product, for example, by leaching or by sorption of ingredients. Particular attention should be paid to possible changes in product pH since these can markedly affect preservative activity. A container with smooth surfaces and a wide neck that will allow ease of access and mixing is recommended for creams and other viscous preparations.

13. **Test procedure** Test conditions such as storage temperatures and sample times are given in Appendix XVI C. Working at a temperature within the stated range of 20° to 25°, it is advisable, wherever possible, to minimise the temperature difference from test to test.

14. **Counting survivors** The Appendix specifies that the count obtained for the inoculum is used as the baseline for calculating the reduction in viable micro-organisms.

15. **Preservative inactivation** When residual antimicrobial activity is removed either by the use of an inactivating agent or by dilution, it is necessary to confirm the ability of the system to support the growth of the test organisms by the use of appropriate controls. These controls should simulate test conditions and allow validation of the counts. In designing such controls an appropriate target recovery efficiency, for example 70% recovery, should be set.

16. **Interpretation of results** Where a test criterion is given as 'no increase' this is intended to be interpreted as no increase above the counts obtained at the previous specified sample time. It is expected that, having achieved the required reduction in counts at the shorter time interval specified, the preservative will maintain the microbial population at or below this lower level.

K. Stereochemistry

This section describes the way in which the stereochemistry of a substance is indicated in the chemical definitions and graphic formulae of the British Pharmacopoeia and the way it may be identified and/or controlled within the tests in a monograph.

1. Many medicinal substances that contain one or more chiral centres and that are already on the market have been made available for pharmaceutical use as racemic mixtures with little known about the biological activities of the separate isomers. This has been reflected in the monograph in the Pharmacopoeia and a test to show that the substance is the racemic mixture has not usually been included unless it was known that at least one of the separate enantiomers was also available commercially. Nevertheless, with increasing concern by regulatory authorities for substances to be made available as single isomers, tests for enantiomeric composition will become more common (see section on tests below).

Chemical definition

(monographs other than those of the European Pharmacopoeia)

2. In the case of substances containing a single chiral centre, the descriptor '(*RS*)-' is included at the appropriate position in the chemical definition of the substance to indicate a racemic mixture.

3. For substances containing multiple chiral centres and comprising a mixture of all possible stereoisomers the term '*all-rac*-' has been used, for example Isoaminile. In those few substances existing as diastereoisomeric mixtures, that is where in one or more centres the stereochemistry is explicit but in other centres it is not, each centre is defined either as the specific (*R*)- or (*S*)- configuration, or as racemic (*RS*)-, respectively.

Graphic formulae

4. When a medicinal substance is a racemate, an indication is given by means of the graphic formula.

5. Because in graphic formulae there is no generally accepted convention for depicting a racemate, each racemic substance with one chiral centre is shown in the (*R*)- form with the appended text 'and enantiomer' for example, Carteolol Hydrochloride. For the *all-rac*- mixtures, such as Docusate Sodium and Alpha Tocopheryl Acetate, non-stereospecific graphic formulae are drawn and the legend 'mixture of *n* stereoisomers' added beneath (where *n* is the number of possible stereoisomers); the stereogenic carbon atoms concerned are identified by means of asterisks.

6. In diastereoisomeric mixtures, the unique configuration centres are drawn as such, while each racemic centre (with equal amounts of the (*R*) and (*S*) configuration) are indictated by an asterisk and the legend 'racemic at C*' appended. For example, Carbenicillin Sodium is drawn in this way; the chiral atoms in the penicillanic acid ring each in their single specific configuration and the phenylmalonyl side-chain chiral atom marked with an asterisk.

Tests

7. In future, when a monograph describes an enantiomer, it will include both a test for specific optical rotation under Identification and a test, using methods such as chiral chromatography, to control enantiomeric purity.

8. When both the racemic mixture and the enantiomer are available, the monograph for the racemic mixture will, where appropriate, specify a test for angle of rotation together with a cross reference under Identification. The test for angle of rotation will normally specify low, symmetrical limits about zero where this has been shown to limit the presence of optically active impurities and demonstrate approximately equal proportions of the enantiomers.

9. When only the racemic mixture is available, the monograph for the racemic mixture may, where appropriate, specify a test for angle of rotation.

L. Microbiological Assay of Antibiotics

This section provides guidance on interpretation of statements in the Pharmacopoeia concerning content limits for those antibiotics and their preparations for which the monograph specifies a microbiological assay.

1. The statements in the Pharmacopoeia concerning the potency of antibiotics are framed to provide a control analyst with the means whereby he can ascertain whether or not the material or preparation in question is satisfactory, that is, they provide criteria appropriate to a 'check assay'.

2. In order to be confident that, when assayed by a control analyst, a material or preparation would meet the pharmacopoeial criteria a manufacturer would himself need to work to criteria appropriate to a 'release assay'.

3. The need for different criteria for check and release assay purposes arises from the inherent variability of biological systems which precludes assigning an exact value to the true potency. It is possible only to give a range of values within which the true potency can be expected to lie with a defined

degree of confidence (95%; $P = 0.95$ for pharmacopoeial purposes). In such circumstances the fiducial limits of error computed for the assay in question define the lower and upper limits within which the true potency of the sample lies, with a probability of 95%.

4. The required minimum precision for an acceptable assay of any particular antibiotic or preparation is defined in the appropriate monograph in the paragraph entitled Assay. This degree of precision is the minimum acceptable for determining that the final product complies with the official requirements. It may be inadequate for a decision about the potency that should be stated on the label or used as the basis for calculating the quantity of an antibiotic to be incorporated in a preparation. In such circumstances, assays of greater precision may be desirable with, for instance, fiducial limits of error of the order of 98 to 102%. With this degree of precision, the lower fiducial limit lies close to the estimated potency. By using this limit, instead of the estimated potency, to assign a potency to the antibiotic either for labelling or for calculating the quantity to be included in a preparation, there is less likelihood of the final preparation subsequently failing to comply with the official requirements for potency. Greater precision can be achieved by statistically combining the results of two or more independent assays.

Bulk antibiotic

5. In a monograph for a bulk antibiotic a minimum potency in IU is given under the heading Definition and this is to be interpreted in accordance with the second paragraph of the General Notice on Biological Assays and Tests. This states that 'The material is not of pharmacopoeial quality if the upper fiducial limit of error is less than the stated potency. For such antibiotics the required precision of the assay is stated in terms of the fiducial limits about the estimated potency.'

6. Taking Amphotericin, the monograph for which has a defined minimum potency of not less than 750 IU per mg, as an example of a bulk antibiotic: a control analyst would judge the material *unsatisfactory* with respect to potency only if the *upper* fiducial limit of error [UFLE] obtained in his assay was *less* than 750 IU per mg, calculated with reference to the dried substance. A manufacturer, on the other hand, would consider suitable for release only those batches for which the *lower* fiducial limit of error [LFLE] obtained in his assay was *greater* than 750 IU per mg, calculated with reference to the dried substance.

Bulk antibiotic (Fig. SC1L-1)

Probability = 0.95,

Precision = 95–105%

Let X be the monograph value for the minimum potency of the bulk antibiotic [750 IU per mg, for example, for Amphotericin].

Let R_1, R_2, R_3 be the estimated potency found by the manufacturer in the release assay.

To ensure (with 95% confidence) that the true potency of his bulk substance is not less than the desired minimum [*ie* that R not <X], the manufacturer releases only a batch such as R_1 for which the lower fiducial limit of error of the estimated potency is ≥X; he rejects batches such as R_2 and R_3 for which the lower fiducial limit of error is <X.

Let C_1, C_2, C_3, C_4 be the estimated potency found by the control analyst in the check assay.

Only when the upper fiducial limit of error of the estimated potency is <X can the control analyst conclude (with 95% confidence) that the true potency of the sample being examined is less than the desired minimum value. He would fail a batch with estimated potency C_4.

Formulated preparation

7. In a monograph for a formulated preparation the lower and upper limits of content are given under the heading Assay and are given as the values (stated in terms of percentage of labelled claim) within which the fiducial limits of error of the estimated potency obtained in the Assay must lie.

8. For a formulated preparation the results of the Assay and the content, or strength, stated on the label are given in terms of IU unless specific instruction to calculate a 'weight equivalent' is given as, for example, in the monograph for Streptomycin Injection. Such a 'weight equivalent' cannot be given for certain antibiotics, in particular those like Neomycin Sulfate which consist of a variable mixture of components.

9. Taking Neomycin Eye Ointment, the monograph for which requires the upper fiducial limit of error to be not less than 90% and the lower fiducial limit of error to be not more than 115% of the stated amount, as an example of a formulated preparation: a control analyst would judge a product *unsatisfactory* with respect to content only if the *upper* fiducial limit of error obtained in the check assay was *less* than 90% or if the *lower* fiducial limit of error was *greater* than 115% of the amount expected in accordance with the strength stated on the label. A manufacturer, on the other hand, would consider suitable for release only those batches of the product for which the *lower* fiducial limit of error obtained in his assay was *greater* than 90% and for which the *upper* fiducial limit of error was *less* than 115% of the amount expected in accordance with the strength stated on the label.

Formulated preparation (Fig. SC1L-2)

Probability = 0.95,

Precision = 95 – 105%

Let Y be the stated content/strength [labelled claim] of the preparation [3500 IU per g of ointment, for example, for Neomycin Eye Ointment].

Let R_1, R_2, R_3, R_4, R_5 be the estimated content/strength found by the manufacturer in the release assay.

To ensure (with 95% confidence) that the true content/strength is not less than the specified minimum [*ie* that R not <90% Y], the manufacturer releases only batches such as R_1 and R_2 for which the lower fiducial limit of error of the estimated potency is ≥90% Y; he rejects batches such as R_3 and R_4 for which the lower fiducial limit of error is <90% Y. To ensure (with 95% confidence) that the true content/strength is not greater than the specified maximum [*ie* that R not > 115% Y], the manufacturer also rejects batches such as R_5 for which the upper fiducial limit of error is > 115% Y. In order to avoid possible problems of compliance with monograph assay limits at the end of shelf-life for those antibiotics where stability is an issue, manufacturers are advised to exercise caution in releasing batches such as R_2 for which the lower fiducial limit of error is less than the value stated on the label. The advice given in paragraph 4 may be of assistance in these circumstances.

Let C_1, C_2, C_3, C_4, C_5, C_6 be the estimated content/strength found by the control analyst in the check assay.

Only when the lower fiducial limit of error of the estimated content/strength is > 115% Y or when the upper fiducial limit of error of the estimated content/strength is <90% Y can the control analyst conclude (with 95% confidence) that the true content/strength is outside the specified range; he would fail batches with estimated content/strength C_4 and C_6.

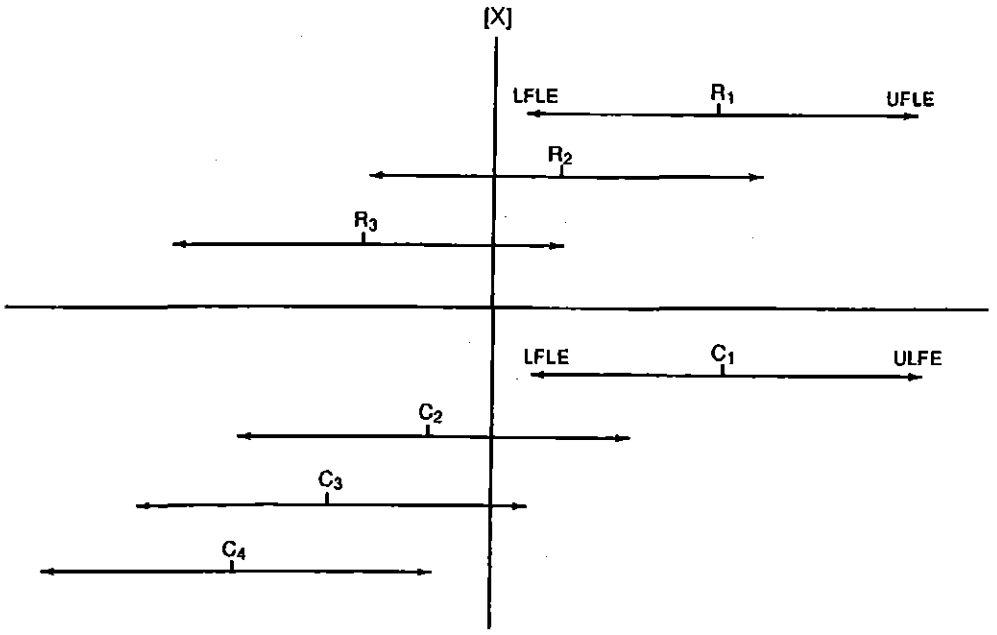


Fig. SC1L-1

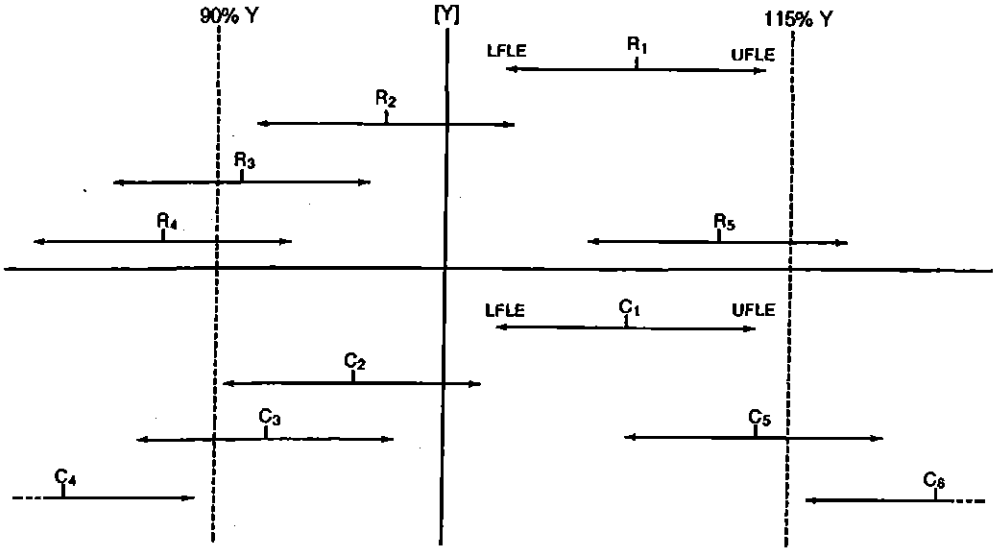


Fig. SC1L-2

It is important to recognise that, for the reasons explained in paragraph 3, a batch giving the estimate R_1 in a release assay may give the estimate C_4 in a check assay *without any change in the true potency*.

Definitions

Assay A single test carried out for the purpose of estimating the potency of a material/preparation or the pooled results of two or more such tests.

True potency The actual potency of the material/preparation at the time of assay. In practice this value can never be exactly evaluated.

Stated or labelled potency This is often a nominal value assigned to a formulated preparation from knowledge of the potency of the bulk material. In the case of bulk materials, it may be calculated from assay data.

Estimated potency The potency calculated from assay data. It is misleading to refer to a 'best estimate' [see under fiducial limits below].

Fiducial limits [confidence interval] These are limits to the true potency of the material/preparation and are calculated on the assumption that there is no bias in the assay system. They may be calculated for any desired level of probability; for pharmacopoeial purposes a level of 0.95 is applied. The concept is that, if the assay could be repeated many times, the true potency would lie within the fiducial limits in 95% of assays. Thus, in any particular assay, the true potency could be anywhere within the fiducial range (and occasionally outside it) and the estimated potency is no better measure of the true potency than any other value in the range.

Release and check assays A release assay is an assay carried out by those responsible for assigning a potency to a material or declaring a content or strength for a preparation [manufacturers]. A check assay is an assay carried out by those responsible for checking the potency of a material or the content or strength of a preparation [control authorities,

independent analysts and purchasers of bulk materials]. The use of these two terms does not imply a fundamental difference in the design or execution of an assay but rather in the interpretation of the results obtained. A check assay only enables a control analyst to say unequivocally when a material/preparation is unsatisfactory [see illustration above].

M. Microbial Contamination

This section of Supplementary Chapter I provides information on the status and application of the texts on microbial contamination included in Appendix XVI. These texts, which are taken from the European Pharmacopoeia (Ph Eur), are used both to satisfy mandatory requirements of the Pharmacopoeia and as guidelines for the purposes of microbiological monitoring. This chapter provides clarification on the application and interpretation of the test methods and criteria in the different situations.

1. Appendix XVI B and Appendix XVI F comprise several sub-sections describing tests for detecting different types of microbial contamination and Appendix XVI D and Appendix XVI G provide guidance on the microbial quality of pharmaceutical preparations and herbal medicinal products.

2. The tests for *specified micro-organisms*, included as Appendix XVI B1, are those included as method 2.6.13 of the Ph Eur. These tests describe the methods used to set mandatory requirements in monographs for certain bulk materials of natural origin. For example, the monograph for Dried Aluminium Hydroxide specifies the absence of *Escherichia coli* and from bile-tolerant gram-negative bacteria and that for Pancreatin specifies 1 g is free from *Escherichia coli* and 10 g is free from Salmonella. These methods are also used to support the relevant non-mandatory recommendations made within the guidelines on the microbiological quality of pharmaceutical preparations and herbal medicinal products (see paragraph 9 below).

3. The tests for *total viable aerobic count*, included as Appendix XVI B2, are those included as method 2.6.12 of the Ph Eur. These quantitative tests are used in two contexts within the Ph Eur. In addition to being invoked to set mandatory limits for a range of bulk materials of natural origin, the methods are also used to support the non-mandatory guidelines on the quality of pharmaceutical preparations and herbal medicinal products (see paragraph 9 below).

4. In Appendix XVI B2 of the British Pharmacopoeia (BP), an introductory paragraph states that the tests are designed primarily to determine whether a substance or preparation complies with an established specification for microbiological quality. For example, the monographs for Agar and for Dried Aluminium Hydroxide specify total aerobic microbial counts of not more than 10^3 micro-organisms per gram and total combined yeast/mould counts of not more than 10^2 micro-organisms per gram. The method indicates that, when used for such purposes, it is to be carried out in accordance with the instructions in the general text, including the number of samples to be taken, and the results are to be interpreted as stated.

5. When the tests are used by a manufacturer for monitoring raw materials and/or finished products or for process validation, the sampling plans for microbiological examination and the method of interpretation of the results are matters for agreement between the manufacturer and the competent authority. (See also Basis of Pharmacopoeial Requirements in the Introduction to Supplementary Chapter I.)

6. The *test for absence of mycoplasmas*, included as Appendix XVI B3, is method 2.6.7 of the Ph Eur as applied to vaccines for human use. This test is invoked as a mandatory requirement in the relevant parts of the Production section of certain monographs for viral vaccines produced in cell cultures or in eggs, for example, Inactivated Influenza Vaccine (Surface Antigen). (The test as applied to veterinary vaccines is reproduced in the BP (Veterinary).)

7. The *test for mycobacteria*, included as Appendix XVI B4, is method 2.6.2 of the Ph Eur. Reference to this method is made under the tests for extraneous agents in viral vaccines (see paragraph 8 below).

8. The *tests for extraneous agents in viral vaccines*, included as Appendix XVI B5, are those included as method 2.6.16 of the Ph Eur. These tests are invoked as mandatory requirements in the relevant parts of the Production section of certain monographs for viral vaccines.

9. As indicated in the general monograph for Pharmaceutical preparations, recommendations on the microbiological quality of non-sterile pharmaceutical preparations are provided in Appendix XVI D and Appendix XVI G of the BP.

The text on *Microbiological Quality of Non-sterile Pharmaceutical Preparations*, included as Appendix XVI D, is general text 5.1.4 of the Ph Eur. As described in the General Notices: 'General chapters become mandatory when referred to in a monograph, unless such reference is made in a way that indicates that it is not the intention to make the text referred to mandatory but rather to cite it for information'. Where general text 5.1.4 is referred to in a general monograph as giving recommendations on microbiological quality, different acceptance criteria may be justified.

The general monograph for Oral Liquids, for example, has a mandatory Production requirement that 'In the manufacture, packaging, storage and distribution of liquid preparations for oral use, suitable measures are taken to ensure their microbial quality' the following statement 'recommendations on this aspect are provided in the text on *Microbial Quality of Pharmaceutical Preparations (5.1.4)*' clearly constitutes a non-mandatory recommendation.

The text on *Microbiological Quality of Herbal Medicinal Products for Oral Use*, included as Appendix XVI G, is general text 5.1.8 of the Ph Eur. This text is described as 'recommended acceptance criteria for microbiological quality of herbal medicinal products'.

10. The texts of Appendix XVI D and Appendix XVI G are intended to provide guidance to those concerned with this important aspect of quality assurance of final dosage forms and herbal medicinal products for oral use. The advisory limits for products in both texts should be used by manufacturers for process validation and may be used as part of a general microbial monitoring programme to identify needs for corrective action. The availability of authoritative guidelines provides a useful yardstick for official control laboratories when conducting surveys of products on the market. It is emphasised that the inclusion of advisory limits in the Pharmacopoeia is not intended to imply the need for end-product microbiological testing on a batch-to-batch basis. Such testing would be inconsistent with the principles of good manufacturing practice and the analytical burden imposed by such testing could not be justified for the majority of products.

N. Particulate Contamination

This section of Supplementary Chapter I provides information on the status and application of the texts on particulate contamination included in Appendix XIII. Guidance is also offered on limits for visible particles in small-volume injections.

1. Appendix XIII comprises two methods for determining particulate contamination:- Sub-visible particles and Visible particles. Of these two methods, only that for sub-visible particles is currently invoked as a mandatory requirement within the monographs of the Pharmacopoeia.

2. The test for **sub-visible particles** included as Appendix XIII A is method text 2.9.19 of the European Pharmacopoeia. The criteria are invoked by means of the revised Ph Eur general monograph for Parenteral Preparations. The statements previously included in certain individual monographs of the British Pharmacopoeia such as Sodium Chloride Intravenous Infusion have therefore been deleted and reliance placed on the general monograph.

3. The test for **visible particles** included as Appendix XIII B is method text 2.9.20 of the European Pharmacopoeia. This text describes standardised viewing conditions but sets no criteria of acceptance. Contamination by visible particles is governed instead by the requirement of the Ph Eur general monograph for Parenteral Preparations that injections and intravenous infusions that are solutions are required to be 'clear and practically free from particles'. It is recognised that this latter requirement can give rise to problems of interpretation. These problems could, perhaps, be overcome by providing simple criteria for the test for visible particles suitable for application as a pharmacopoeial 'check-test', that is, for application by an independent analyst, for example, a hospital quality control pharmacist, as a means of judging the quality of a particular parenteral preparation.

4. The need to set limits is based on the premise that an expectation of total absence of visible particles from all containers from a batch of an injectable preparation is unreasonable and unrealistic. An attributes-based test using a small sample together with simple pass/fail criteria would be consistent with the needs and constraints of a check test. The criteria chosen would need to be capable of detecting a batch that was grossly contaminated while representing an acceptably low chance of condemning batches of satisfactory quality through a small sample being unrepresentative. It would, of course, be unlikely that, if a sample failed such a test, consequent action would be initiated by the competent authority on the result of a single test. In such circumstances the competent authority would investigate the occurrence further with the manufacturer.

5. The following criteria are offered as generally suitable for a check test for small volume injections that are solutions. For guidance, it is suggested that in total 20 containers are examined as described in Appendix XIII B and any particles recorded. It is suggested that the preparation being examined should be considered to have failed the test if one or more particles are found in more than one container.

6. It is emphasised that these criteria are not intended for use by a manufacturer for batch release purposes. It is expected that a manufacturer would obtain assurance of the quality of his product with respect to visible particulate matter by 100% inspection or by other appropriate means in accordance with good pharmaceutical manufacturing practice (GMP).

5.17.2. Recommendations on Testing of Particulate Contamination: Visible Particles

(Ph. Eur. general text 5.17.2)

This general chapter is non-mandatory; it provides information on visible particle testing of liquid preparations that refer to general chapter 2.9.20. Particulate contamination: visible particles in their monographs. This information represents considerations used in the field of visual inspection and in the control of visible particles in medicinal products. This chapter is not intended to elaborate on good manufacturing practice requirements, but should be read in conjunction with such requirements.

INTRODUCTION

Particulate contamination in liquid preparations consists of mobile, undissolved particles, other than gas bubbles, that are unintentionally present. Particulate contamination may originate from various sources and is to be minimised independent of its type. The level of particulate contamination in preparations must be controlled. Depending on the size of the particles, particulate contamination may be in the visible or the sub-visible size range. In products such as parenteral preparations, although there is little supporting evidence generated by clinical studies, visible particles are considered to be a potential safety concern, and their presence should therefore be minimised as far as possible in any product intended for parenteral administration to humans or animals. As visible particles can be assumed to be heterogeneous and non-uniformly distributed across a given product batch, it is important to use sufficiently large sample sizes. Whilst the presence of such particles in general is random, it can nonetheless be a sign of a systematic issue; the occurrence of particles must therefore be assessed and appropriate measures taken to eliminate any source of particulate contamination and/or improve the formulation, primary packaging or manufacturing process, as required.

Particles may be extrinsic (deriving from the environment, equipment, primary packaging or personnel) or intrinsic (related to the formulation, including active substances and excipients, process residuals or contaminants, or resulting from interactions between the formulation and primary packaging). Examples of extrinsic particles include cellulose fibres, glass, paint flakes, hair and insect parts. Examples of intrinsic particles include proteinaceous particles, silicone droplets, inorganic precipitates such as barium sulfate or aluminium sulfate, fatty acid particles from the degradation of polysorbates, and glass lamellae. It is sometimes only possible to detect particles during storage, i.e. during stability studies on the product, hence the importance of particulate contamination monitoring using appropriate test methods, such as that described in general chapter *2.9.20* for visible particles or those described in general chapter *2.9.19* for sub-visible particles. The assessment of particles should be based on the safety considerations related to the product and the route of administration.

In some cases, for example in cell therapy medicinal products or dispersions, particles may be the active substance itself and be intentionally present. However, with the exception of these cases, visible particles of any kind are unwanted in medicinal products, including biotechnology-derived products, and effort must be made to avoid or at least minimise their presence.

Additionally, it is recommended that end-users check for visible particles before administering the medicinal product.

VISUAL INSPECTION: GENERAL CONSIDERATIONS

The intrinsic characteristics of some medicinal products may render visual inspection for particles difficult. This is the case, for example, for opalescent (turbid) solutions or dispersions, coloured solutions or lyophilisates, opaque or coloured containers, and parenteral preparations such as suspensions and cell therapy products in which particles are naturally present. Measures must be taken to ensure that visual inspection operators are able to detect particulate matter during a 100 per cent inspection of a batch followed by an acceptable quality level (AQL) or equivalent sampling plan method and subsequent visual inspection within quality control testing. One such measure is the use of defect sets that are representative of the product and contain a variety of potential visible particle defects together with negative and positive controls. Operators must also receive in-depth training using performance limits for defect detection and unjustified rejects. Non-destructive measures to improve inspection techniques may also be required, for example the use of a light intensity higher than 3750 lux, inspection of each container for more than 5 s and against both black and white backgrounds. Destructive measures are used only for quality control purposes, for example dilution of the product with visible-particle-free water or another suitable diluent. In addition, when the container hampers inspection, it may be necessary to transfer the contents to a suitable clear container.

VISUAL INSPECTION DURING PRODUCTION

INSPECTION SCHEMES

Each container of a liquid preparation (e.g. vial, syringe, ampoule) is visually inspected after filling and closure. During this 100 per cent inspection, containers in which any particulates are observed are removed (culled) from the batch and rejected. An additional spot check is also often performed according to a sampling plan based on a pre-defined AQL; the sample size and the acceptance number can be deduced using the tables included in ISO 2859-1 or by other statistical means. The AQL confirms the suitability of the inspection to ensure an acceptable quality level.

Suitable AQL limits for defect categories (e.g. minor, major or critical defect) must be defined. The presence of visible particles in a container is classified as at least a 'major defect'. An investigation is usually triggered if the AQL limit is exceeded or if atypical particles are detected.

ASPECTS TO CONSIDER

During the 100 per cent inspection, visual inspection for particles is performed according to the method provided in general chapter *2.9.20.* This process can be manual or, if adequately validated against the manual inspection method, semi-automated or fully automated. Validation of automated or semi-automated methods against manual visual inspection typically comprises evaluation of rejected units against predefined acceptance criteria, including the false reject rate, using representative samples and defect sets. The defect set used for qualification of inspection operators should contain a range of defects, including units presenting typical particulate contamination, as mentioned above. The defect set itself should be appropriately stable over the intended period of use.

Typically, operators are unable, by visual inspection alone, to classify particulates into different categories and materials, and thus allow root-cause analysis.

The detection of visible particles is probabilistic by nature and depends on various factors such as: light intensity; inspection conditions; use and type of magnification; observation time; inspection background; distance from samples; type and speed of rotation; fill volume; primary packaging material, size and type; operator and equipment capability; and the size, number, type and refractive index of particles. Consequently, whilst the goal of a 100 per cent inspection followed by AQL testing is to eliminate all containers containing any visible particles, some of these may escape detection due to poor detectability and visibility. Every visual inspection system is therefore characterised by an accepted statistical probability to detect units with visible particles and an accepted statistical probability to miss units with visible particles. All containers found to contain visible particles or presenting any defects considered as unacceptable are excluded from the acceptable part of the batch, unless otherwise authorised or justified. In the case of freeze-dried products undergoing 100 per cent inspection followed by AQL testing, only the visible part of the cake and the primary packaging components can be examined. Particles originally present in the solution may become embedded in the cake during manufacture and remain undetected.

VISUAL INSPECTION FOR QUALITY CONTROL

Visible particle inspection for quality control is also conducted as described in general chapter *2.9.20.* This inspection is typically performed manually.

ASPECTS TO CONSIDER

Visual inspection for quality control is performed on either liquid products or reconstituted solutions of dried products such as lyophilisates. Inspection of visible particles in freeze-dried products is performed as part of quality control testing for batch release using destructive methods. Whereas the 100 per cent visual inspection and AQL testing are carried out on sample units without further preparation (e.g. reconstitution), samples of freeze-dried product for quality control testing are reconstituted under conditions simulating those of actual use by the patient or healthcare professional. In order to avoid potential contamination with particles, sample preparation for quality control testing should be conducted under appropriate conditions. Sample sizes should be determined based on statistical considerations, for example special sampling plans for destructive testing in ISO 2859-1.

Testing single units only is not recommended. However, the sample size available for quality control may be limited due to the size of the batches and other considerations, for instance in the case of biological products. In general practice, a sample size of 10-20 units is common.

Visible particle requirements are applicable until the product reaches the end of its shelf-life. Thus, quality control testing for visible particles should be performed regularly during long-term stability studies and preferably also during accelerated degradation studies. The emphasis is on stability-indicating attributes such as precipitation, agglomeration, discouloration and glass from delamination, and not on potential extrinsic particle contaminants. Any particles observed during stability testing that are considered indicative of a systematic issue, rather than single particles found in stability studies, should be identified to assess their possible root-cause and define suitable corrective actions.
If investigation confirms a systematic issue, such as protein particles due to the formulation or glass from delamination, the formulation, primary packaging or manufacturing process of the medicinal product should be addressed to mitigate the occurrence of particles, unless otherwise authorised or justified.

ACCEPTANCE CRITERIA

An additional quality control test for visible particles (e.g. for batch release testing) may be omitted for liquid preparations subjected to 100 per cent visual inspection followed by AQL testing. A successful AQL inspection indicates that the batch complies with the requirement 'practically free from visible particles'. However, destructive quality control testing cannot be avoided for lyophilisates, since it is performed on the reconstituted product, whereas both the 100 per cent visual examination and AQL testing are carried out on the dried material.

Quality control acceptance criteria often refer to the occurrence of visible particles per unit, across the number of samples analysed. For example, a few small particles in 1 unit out of a set of 10-20 samples analysed may not be of any significant concern. Significant amounts of particles in a single unit would, however, be a concern.

Establishing acceptance criteria for visible particles in single units is a difficult task and it is unreasonable to expect numerical values to be set. Whilst 'zero particles' remains the goal, it is not considered to be a realistic quality control acceptance criterion since the probabilistic nature and random occurrence of visible particles often leads to the discovery of single particles in single units. Appropriate approaches must be defined for each product. A universal recommendation is not suggested.

'Practically free from visible particles' reflects the capability of the manufacturing and testing process. The term is applicable at the batch level of a medicinal product, not for single units examined individually. However unrealistic, a 'zero particles' product is nonetheless a worthy goal.

Products administered using a filter

With some parenteral products, for example products for which there is insufficient product knowledge, filters may be used to reduce the risks related to particles that may form during storage. However, the use of such filters does not constitute acceptance of particles after manufacture or allow particulate contamination *per se*. If justified and authorised, products administered using a filter can be exempt from the 'practically free from particles' requirement, providing it has been demonstrated that the filter delivers a filtrate that complies.

O. Inhaled Products

Formulated Preparations: Specific Monographs

The following policy applies to BP monographs for inhaled products. BP monographs contribute to the safety and efficacy of inhaled products through the provision of quality specifications in line with current best practices and test methodology.

1. Alignment of BP terminology and test methodology with those used in the European Pharmacopoeia (Ph. Eur.)

a. Titles for BP monographs for inhaled products should use the relevant European Directorate for the Quality of Medicines and Healthcare (EDQM) full Standard Term for the dosage form, with the BP legacy title included as a subsidiary title for revised monographs.

b. The requirements should be consistent with those specified in the Ph. Eur.

2. Alignment of BP monographs for inhaled products with the Ph. Eur. general monographs for Preparations for Inhalation and Pharmaceutical Preparations.

a. Fine Particle Dose No tests for Fine Particle Dose tests will be included for specific BP finished inhaled product monographs under this revised policy, but a Production statement indicating that a test and limits should be agreed with the competent authority will be included in line with the Preparations for Inhalation General Monograph (with the exception of nebuliser preparations).

b. Uniformity of delivered dose The Uniformity of delivered dose test should be aligned with the Ph. Eur. General Monograph for Preparations for Inhalation and should be included in BP monographs for inhaled products with the exception of preparations intended for nebulisation.

c. Uniformity requirements for nebuliser preparations Appropriate Uniformity of content tests should be included in BP finished product monographs for preparations intended for nebulisation, to harmonise with the specifications of available products.

d. Assay An appropriate Assay (content of active requirement) for all BP monographs for inhaled product monographs is required. The type of Assay included in the monograph should follow the approach taken by the innovator product and the other established products in order to ensure a consistent approach. This is particularly important for preparations that are not intended for nebulisation. Where the average of the results of the Uniformity of delivered dose are used as an Assay, the content limits should be aligned with the Preparations for Inhalation general monograph, to harmonise with the specifications of licensed products.

3. Preparations for Inhalation of the British Pharmacopoeia This general monograph will be omitted for a future BP publication and suitable validated alternative methodology for the Content of active ingredient on actuation of the valve test will be established for existing product monographs when available. The Content of active ingredient on actuation of the valve test methodology from this general monograph will be moved to an Appendix and a cross-reference to this Appendix will be included in finished product monographs until alternative Assay methodology becomes available.

4. Water If control of water content has been shown to be important during product development, a requirement to determine water content should be included in a Production statement in BP monographs for inhaled products, which are not intended for nebulisation, to harmonise with the methodology and specifications used for established products.

5. Labelling With the exception of nebuliser preparations, where appropriate, Labelling statements should be added to BP monographs for finished inhaled products to reflect the label claim of the licensed products in terms of either the pre-metered dose, the metered dose or the delivered dose, as appropriate.

6. Inhalation Powder, Pre-Metered Where the expression *"the pre-metered unit is loaded into a dry-powder inhaler to generate an aerosol"* appears in individual BP monographs for Inhalation Powders, Pre-Metered, the term *"pre-metered unit"* refers to the single-dose pre-metered dosage unit, consisting of one or more powders of (a) solid active substance(s), presented in a suitable pharmaceutical form other than a hard capsule. The pre-metered unit is loaded into a dry powder inhaler to generate an aerosol.

Supplementary Chapter II

Names of Medicinal Substances and Preparations

This Supplementary Chapter provides information on Pharmacopoeial monograph titles, on the construction of titles for formulated preparations and on the structures and nomenclature of substances of natural or semi-synthetic origin.

Introduction

British Approved Names are devised or selected by the British Pharmacopoeia Commission and published by the Health Ministers on the recommendation of the Commission on Human Medicines. British Approved Names [BANs] are nonproprietary names established *inter alia* to provide suitable titles for monographs for medicinal substances in the Pharmacopoeia. The issue of a British Approved Name, however, does not imply that the substance will necessarily be included in the Pharmacopoeia. The guiding principles that are used in devising or selecting new British Approved Names are stated in the current edition of the British Approved Names book.

Where a Recommended International Nonproprietary Name [rINN] has been established by the World Health Organization, this name is now invariably adopted as the BAN and used as the title of the monograph, if any, in the Pharmacopoeia. In the past there were occasional differences between the rINN and the corresponding BAN. Many, but not all, of these differences were minor changes in spelling to accommodate normal English pronunciation and usage. Implementation of Directive 92/27/EEC, however, requires the use of rINN in the labelling of medicinal products throughout the member states of the European Community. In consequence, the relevant BANs have been modified to accord with the English version of the rINN. These changes were made by means of Supplement No. 4 to *British Approved Names 1997* and were reflected, where relevant, in the titles of the monographs in the 1998 edition and subsequent editions of the Pharmacopoeia as described in section A of this chapter.

The titles of monographs for medicinal preparations combine the British Approved Name with an appropriate term for the dosage form in question as described in section B of this chapter.

A. Changes in Monograph Titles

1. Certain changes in monograph titles were made in the British Pharmacopoeia 1998 in order to bring the titles in the British Pharmacopoeia in line with the names (recommended International Nonproprietary Names [rINNs]) that manufacturers were required to use on product labels and leaflets in accordance with EC Directive 92/27/EEC. The changed monograph titles remain in line with the rINNs manufacturers are required to use on product labels and leaflets in accordance with the Human Medicines Regulations 2012, as amended.

2. Normal practice within the British Pharmacopoeia when changing the title of a monograph is to retain the former title as a subsidiary title for a period of at least five years. This was not possible for these changes since use of the former name would no longer be permitted on product labels, *etc.* In order to provide continuity, a statement was therefore included in all affected monographs in the British Pharmacopoeia 1998 in the form 'When [former title] is prescribed or demanded, [current title] shall be dispensed or supplied.'. These statements were deleted from the British Pharmacopoeia 2003.

3. For substances for which there was a rINN, it was necessary to omit any subsidiary titles from the British Pharmacopoeia 1998. For example, the 'Vitamin C' subsidiary titles were omitted from the monographs for Ascorbic Acid, Ascorbic Acid Injection and Ascorbic Acid Tablets. Statements of the type described above (incorporating the name used in the former subsidiary title) were included and have been retained in subsequent editions, where appropriate, to provide continuity.

4. With the exception of Adrenaline and Noradrenaline, the practice of 'dual-labelling' (two names presented on separate lines) was discontinued in the British Pharmacopoeia 2003. In order to comply with EC Directive 92/27/EEC, the titles of all such affected monographs were replaced by the rINNs. A statement in the form '*The name [former title] was formerly used in the United Kingdom*' was included in all affected monographs in the British Pharmacopoeia 2003 and subsequent publications. These statements were deleted from the British Pharmacopoeia 2009.

5. Adrenaline and Noradrenaline are the terms used in the titles of monographs in the European Pharmacopoeia and are thus the official names in use in the Member States party to the Convention on the Elaboration of a European Pharmacopoeia.

B. Monograph Titles for Formulated Preparations

1. The titles of monographs for formulated preparations combine the appropriate drug substance name and pharmaceutical form.

2. The drug substance name is a British Approved Name [BAN] which in general is also the recommended International Nonproprietary Name [rINN]. In cases where the drug substance name is a British Approved Name (Modified), the modifying term is omitted from the title for the formulated preparation except where there exist two or more formulations containing different forms (salts, hydrates etc.) of the drug substance. In such cases the modifying term is retained in the monograph title. Thus, for example, the title of the monograph for tablets containing Clonidine Hydrochloride is Clonidine Tablets since tablets containing clonidine are formulated only with the hydrochloride. The title of the monograph for tablets containing Isosorbide Dinitrate, on the other hand, is Isosorbide Dinitrate Tablets, since tablets formulated with the mononitrate are also available.

3. The terms for the pharmaceutical form are either (i) derived from the relevant General Monograph; or (ii) terms in established use in the UK.

4. When the pharmaceutical form is described as the term in established use in the UK, reference to the pharmaceutical form from the relevant General Monograph will be provided as a Subsidiary Title and incorporated into the Definition

section of the monograph. For Codeine Linctus, for example, the title of the monograph and therefore the nonproprietary name of the preparation remains as Codeine Linctus, but the monograph contains the subsidiary title 'Codeine Oral Solution' and the Definition states that 'Codeine Linctus is an *oral solution* containing ...'.

5. Standard Terms, as published by the Council of Europe, are to be used for describing the pharmaceutical form in the Summary of Product Characteristics (SmPC), Patient Information Leaflet (PIL) and labelling of medicinal products receiving a European Community authorisation. Member States may use different product names for national authorisations.

C. Structures and Nomenclature of Substances of Natural or Semi-synthetic Origin

These notes provide a guide to the semi-systematic chemical nomenclature of certain groups of natural and semi-synthetic products. The following literature should be consulted for further information.

1. The definitive rules of the International Union of Pure and Applied Chemistry (IUPAC) for organic substances are contained in *Nomenclature of Organic Chemistry*, Section A to F and H, Pergamon Press, Oxford, 1979.

2. The IUPAC rules for inorganic substances are given in *Nomenclature of Inorganic Chemistry*, Blackwells, Oxford, 1990.

3. *Biochemical Nomenclature and Related Documents*, The Biochemical Society for the International Union of Biochemistry (IUB), 2nd edn. London, 1992. The following topics are included.

Stereochemistry (Nomenclature of Organic Chemistry, Section E)

Natural products and related compounds (Nomenclature of Organic Chemistry, Section F)

Abbreviations and Symbols

Amino acids, peptides, peptide hormones and immunoglobulins

Steroids

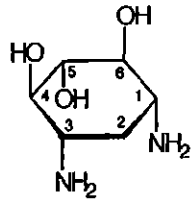
Carotenoids and tocopherols

Carbohydrates and cyclitols

Vitamins

A. Aminoglycoside Antibiotics

A.1 The aminoglycoside antibiotics are conveniently named by reference to 2-D-deoxystreptamine, **1**, in which name the configuration and numbering shown are implicit.



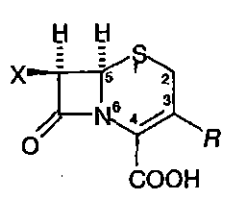
1

A.2 The aminoglycoside antibiotics commonly carry glycosyl radicals on the oxygen atoms attached to C-4 and C-6. The configuration and numbering shown in **1** should be strictly observed if confusion is to be avoided.

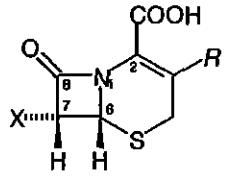
A.3 When one glycosyl radical is linked to another the names are separated by two locants which indicate the respective positions involved in this glycosidic union; these locants are enclosed in parentheses and separated by an arrow (pointing from the locant corresponding to the glycosyl carbon atom to the locant corresponding to the hydroxylic carbon atom involved).

B. Cephalosporin Antibiotics

B.1 The cephalosporin antibiotics are conveniently named by reference to either cephalosporanic acid (**2**, $R = CH_2OAc$, X = H) or cephem-4-carboxylic acid (**2**, R = X = H).



2



3

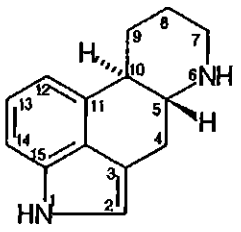
B.2 When names are based on cephalosporanic acid or cephem-carboxylic acid the traditional numbering shown in **2** is used.

B.3 Cephalosporanic acid is systematically named as (6*R*)-3-acetoxymethyl-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid. Compounds that are named systematically use the numbering and orientation shown in **3**.

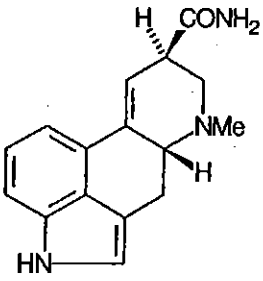
B.4 The cephalosporin antibiotics bear an acylamino group (X) at position 7 (under both numbering systems).

C. Ergot Alkaloids

C.1 Members of the ergoline group of substances are conveniently named by reference to either ergoline itself, **4**, or to D-lysergamide, **5**. When names are so based the traditional numbering shown in **4** is used. In 9,10-dihydro compounds the configuration at position-10 needs to be specified.



4



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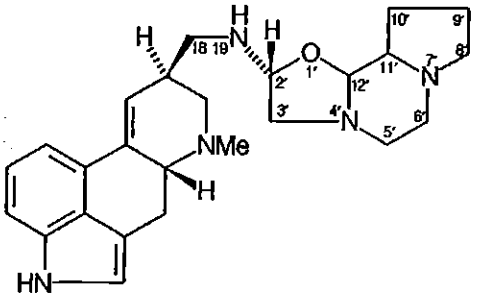
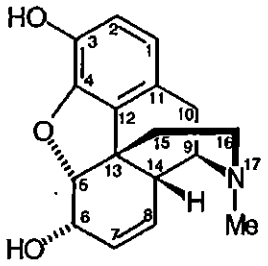


6

C.2 Members of the ergotamine group of substances are conveniently named by reference to ergotaman, **6**. Ergotamine itself is (5′*R*)-5′-benzyl-12′-hydroxy-2′-methyl-18-oxoergotaman-3′,6′-dione.

D. Morphines

D.1 Members of the morphine and codeine group of substances have traditionally been named with reference to morphine itself, **7**, using the numbering shown. However, names may be based more conveniently on either morphinan, **8**, or *ent*-morphinan, **9**.



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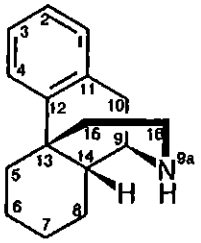

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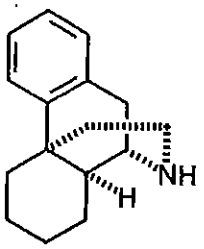


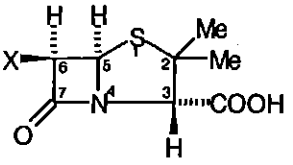
9

D.2 Morphine, **7**, is (5*R*, 6*S*)-4,5-epoxy-9a-methyl-7,8-didehydromorphinan-3,6-diol.

D.3 In certain morphine derivatives, an etheno or ethano bridge is present joining positions 6 and 14 and a hydroxyalkyl side chain is present at position 7.

E. Penicillin Antibiotics

E.1 The penicillin antibiotics are conveniently named by reference to penicillanic acid (**10**, X = H) when the classical numbering shown is used.



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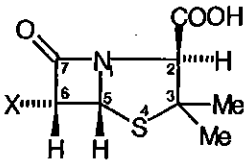


11

E.2 Penicillanic acid is systematically named as (2*S*, 5*R*)-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid. Compounds that are named systematically use the orientation and numbering shown in **11**.

E.3 The penicillin antibiotics are usually 6-acylamino penicillanic acid derivatives in which the configuration at position 6 is *R*.

F. Polypeptides

F.1 The following 3-letter and 1-letter symbols for amino acids, authorised by the IUPAC-IUB Joint Commission on Biochemical Nomenclature, are used for representing the sequences of polypeptides:

Alanine	Ala	A	*Leucine*	Leu	L
Arginine	Arg	R	*Lysine*	Lys	K
Asparagine	Asn	N	*Methionine*	Met	M
Aspartic Acid	Asp	D	*Phenylalanine*	Phe	F
Cysteine	Cys	C	*Proline*	Pro	P
Glutamine	Gln	Q	*Serine*	Ser	S
Glutamic acid	Glu	E	*Threonine*	Thr	T
Glycine	Gly	G	*Tryptophan*	Trp	W
Histidine	His	H	*Tyrosine*	Try	Y
Isoleucine	Ile	I	*Valine*	Val	V

F.2 The following symbols recommended by the Joint Commission are also used:

2-Aminohexanoic acid	Ahx
Sarcosine	Sar
Pyroglutamic acid	<Glu
tert-*Butoxycarbonyl*	Boc

F.3 When interpreting sequences of amino-acid residues, the hyphen should be considered as part of the symbol. Its use to separate the individual residues or radicals may be illustrated by the following example:

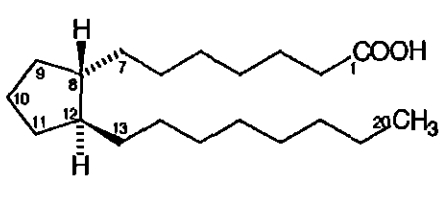
Gly	=	NH_2CH_2COOH
Gly-	=	$NH_2CH_2CO–$
-Gly	=	$–NHCH_2COOH$
-Gly-	=	$–NHCH_2CO–$
and thus,		
Gly-Gly-Gly	=	$NH_2CH_2CONHCH_2CONHCH_2COOH$

The residues are conventionally written with the amino group to the left and the carboxyl group to the right. This is implicit in the symbolism.

F.4 Where peptide sequences are shown using 3-letter symbols all amino acids except glycine have the 'L-' configuration unless otherwise indicated.

G. Prostaglandins

G.1 Members of the prostaglandin group of substances are conveniently named by reference to prostanoic acid, 12, using the numbering shown. Prostanoic acid may be systematically named as 7-[(1*S*, 2*S*)-2-octylcyclopentyl]heptanoic acid. *Dinoprost*, 13, may therefore be named as (5*Z*,12*E*)-(9*S*, 11*R*, 15*S*)-9, 11,15-trihydroxyprosta-5,13-dienoic acid.



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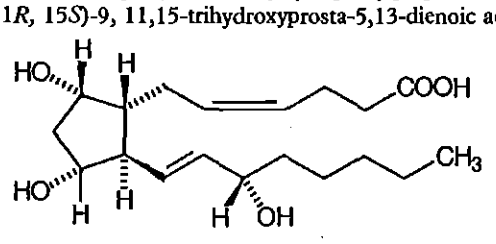
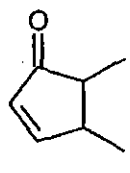
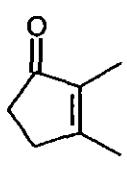


13

G.2 A convenient trivial nomenclature exists by which prostaglandins (PG) are classified into groups A to F according to the substitution pattern in the cyclopentane ring (shown below). The subscript numerals 1, 2 and 3 refer to the number of double bonds found in the side-chains and subscript a refers to the configuration of the C-9 hydroxy group. Thus, 13 is referred to as PGF_{2a}.



A



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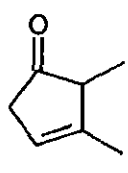

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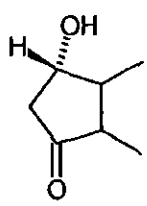

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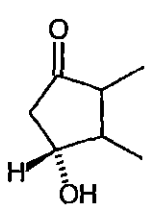

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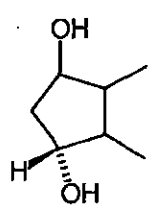
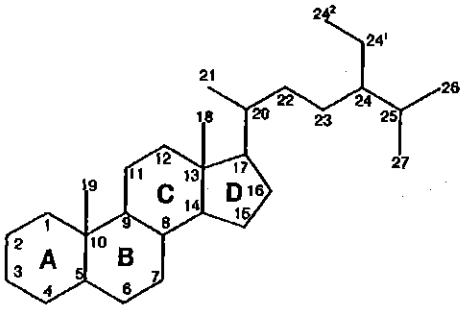


F

H. Steroids

H.1 Steroids are drawn and numbered, and the rings lettered, as shown in 14.



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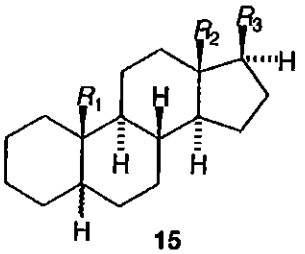
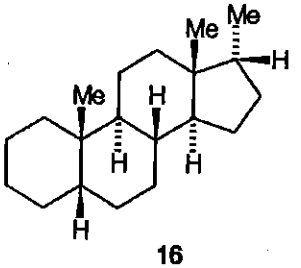


15

H.2 Steroids are named with reference to certain basic carbocycles some of which are defined in Table I. When so drawn, dotted bonds are regarded as lying below the plane of the paper and are designated a, thickened bonds are regarded as lying above the plane of the paper and are designated b and bonds of unknown configuration are shown by a wavy line and are designated x.

Table I

	R_1	R_2	R_3
Gonane	H	H	H
Estrane	H	Me	H
Androstane	Me	Me	H
Pregnane	Me	Me	Et



16

H.3 When the hydrogen atom at C-5 is present, its configuration is always specified, eg 5a-pregnane, 5b-androstane. The configuration at centres, 8,9,10,13,14 and 17 is assumed to be as shown in 15 unless otherwise specified.

H.4 When inversion of the normal configuration occurs, the positions concerned are specified; and thus 16 is named 5b,17a-pregnane.

H.5 When inversion occurs at all of the defined asymmetric centres, the original name is preceded by the italicised prefix *ent-*. Racemates are indicated by use of the italicised prefix *rac-*.

H.6 Further fundamental carbocycles are defined thus:

Table II

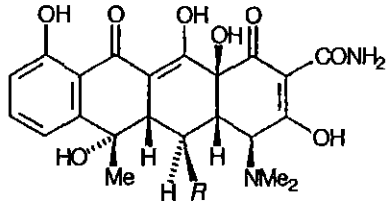
Side-chain	carbon positions present
Cholane	20 - 24
Cholestane	20 - 27
Ergostane	20 - 27, 24^1

In addition to retaining the configuration shown in 15, C-20 has an *R*-configuration in each carbocycle and C-24 in ergostane has an *S*-configuration. However, additional substituents at positions C-17, C-20, C-21 may alter the *R* and *S* propriety descriptions without any change at C-20.

H.7 A large number of therapeutically active steroids bear a carbonyl group at position 3 and unsaturation across positions 4 and 5. Ring A is often aromatic in estrogens.

J. Tetracyclines

J.1 The tetracycline antibiotics are conveniently named by reference to tetracycline itself, 17, (R = H), which may be defined as (4*S*,4a*S*,5a*S*,6*S*,12a*S*)-4-dimethylamino-1,4,4a,5a,6,11,12a-octahydro-3,6,10,12,12a-pentahydroxy-6-methyl-1,11-dioxonaphthacene-2-carboxamide.



17

J.2 When analogues of tetracycline are named the stereodescriptors R and S may be subject to change even though the steric configuration usually remains unchanged. For example, in oxytetracycline, the hydroxyl group at position 5 imposes assignment inversions at positions 4a and 5a from S to R although the steric configuration at these positions remains unchanged.

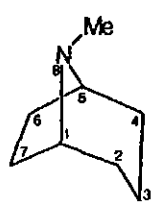
Table III

	Position					
	4	**4a**	**5**	**5a**	**6**	**12a**
Chlortetracycline	*S*	*S*	–	*S*	*S*	*S*
Clomocycline	*S*	*S*	–	*S*	*S*	*S*
Demeclocycline	*S*	*S*	–	*S*	*S*	*S*
Doxycycline	*S*	*R*	*S*	*R*	*R*	*S*
Meclocycline	*S*	*R*	*S*	*R*	–	*S*
Methacycline	*S*	*R*	*S*	*R*	–	*S*
Minocycline	*S*	*S*	–	*R*	–	*S*
Oxytetracycline	*S*	*R*	*S*	*R*	*S*	*S*
Tetracycline	*S*	*S*	–	*S*	*S*	*S*

J.3 When fully systematic names based on naphthacene-2-carboxamide are used, the stereodescriptors given in Table III should be used.

K. Tropanes

K.1 Members of the tropane group of substances are conveniently named by references to tropane itself, 18, when the numbering and orientation shown are used. Tropane is defined as (8*r*)-*N*-methyl-8-azabicyclo[3.2.1]octane.



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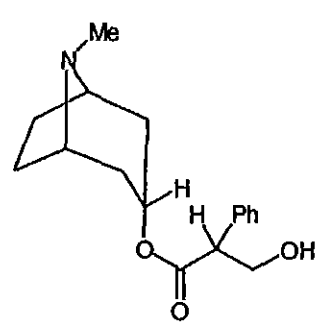


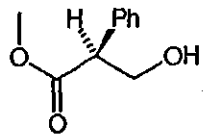
19

K.2 Atropine, **19**, is (1*R*,3*r*,5*S*,8*r*) tropan-3-yl (*RS*)-tropate where the term *tropate* represents 3-hydroxy-2-phenylpropionate.

K.3 In the (–)-series the tropoyl side-chain has the (*S*) configuration, **20**.

K.4 In y-tropane compounds, **21**, the configuration at position-3 is designated as '3s'.

K.5 In hyoscine and other atropine derivatives bearing a 6,7-epoxy bridge, the configuration is as shown in **22**.



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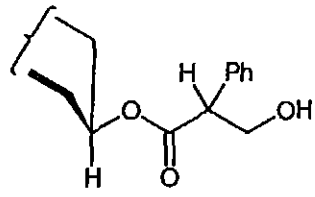

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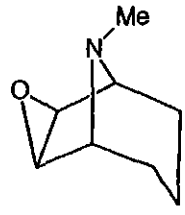
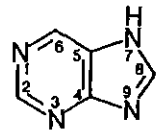


22

L. Xanthines

Members of the xanthine group of substances are conveniently named by reference to purine, **23**, when the non-systematic numbering shown is used.



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Supplementary Chapter III

Pharmacopoeial Organisation

This Supplementary Chapter provides information that may be helpful to those wishing to communicate with the British Pharmacopoeia Commission.

A1. Contact Points

The following table gives an indication of the member of the British Pharmacopoeia Secretariat responsible for particular areas of the Commission's work. The contact number is +44 (0)20 3080 followed by the extension listed in the table below.

E-mail (for reference substances)	bpcrs@mhra.gov.uk
E-mail (for general enquiries)	bpcom@mhra.gov.uk
E-mail (for invented names)	inventednames@mhra.gov.uk
Website	www.pharmacopoeia.com

Editor-in-Chief	Mr A Gibb	6430
Head of Analytical Science	Mr S Young	6790
British Pharmacopoeia Chemical Reference Substances (BPCRS)		
BPCRS sales	Ms F Chughtai	6745
	Mr B Delahunty	6144
	Ms J Paine	6470
	Ms N Begum	6415
BPCRS technical queries	Mr S Young	6790
Monographs		
General enquiries	Mr B Delahunty	6144
Antibiotics	Mr P Crowley	6126
	Ms A Thomson	6000
Analytical methods (general)	Mr S Young	6790
	Mr M Whaley	6224
Biologicals	Mr A Gibb	6185
	Dr G Kemp	7259
	Mr R Smith	7118
European Pharmacopoeia (general matters)[1]	Ms H Ashraf	7020
	Mr A Evans	6565
	Ms A Thomson	6000
General monographs	Mr S Maddocks	6678
Herbal medicines	Mr S Young	6790
	Ms H Ashraf	7020
Inorganic chemicals	Dr F J Swanson	6567
Microbiological aspects	Mr R Smith	7118
New monographs (general matters)[1]	Ms H Corns	6642
Nomenclature	Mr A Evans	6565
	Dr F J Swanson	6567
Organic Chemicals[2]	Ms H Corns	6642
	Mr P Crowley	6126
	Mr A Evans	6565
	Mr L Elanganathan	6879
	Mr S Maddocks	6678
	Ms H Ashraf	7020
	Dr H Bowden	7057
	Ms A Thomson	6000
Pharmaceutical aspects	Mr S Young	6790
	Mr S Maddocks	6678
Radioactive materials	Mr A Evans	6565
Reagents	Mr A Evans	6565
Surgical materials	Mr S Maddocks	6678
Unlicensed Medicines	Dr F J Swanson	6567
	Mr P Crowley	6126
Website		
www.pharmacopoeia.com	Dr G Kemp	7259
	Mr L Elanganathan	6879

A2. Expert Advisory Groups

The following table gives an indication of the members of the British Pharmacopoeia Secretariat responsible for particular areas within the remit of the Expert Advisory Groups of the British Pharmacopoeia Commission. Contact telephone numbers are provided in Supplementary Chapter III A1.

Expert Advisory Group	Class	Secretariat
MC1: Medicinal Chemicals	Aromatic Synthetics Heterocyclic synthetics Alkaloids and synthetic analogues	Ms H Corns Mr L Elanganathan
MC2: Medicinal Chemicals	Aromatic Synthetics Sulfonamides Phenothiazines	Ms H Corns Dr H Bowden Ms A Thomson
MC3: Medicinal Chemicals	Steroids and pseudosteroids Barbiturates Carbohydrates Tricyclics; Benzodiazepines Quaternary ammonium compounds Vitamins	Mr A Evans Ms H Ashraf
ABS: Antibiotics	Antibacterials Antibiotics	Mr P Crowley Ms A Thomson
BIO: Biological and Biotechnological Products	Biological Products	Mr A Gibb Mr R Smith Dr G Kemp
HCM: Herbal and Complementary Medicines	Crude Drugs Homoeopathic preparations Traditional Herbal Materials	Mr S Young Ms H Ashraf

[1] *For specific matters see under relevant subject entry*

[2] *See table in Supplementary Chapter III A2*

PCN: Pharmacy and Nomenclature	Pharmaceutical aspects and techniques General and dosage form monographs Nomenclature Invented names Pharmacological aspects	Mr S Maddocks Mr A Evans Dr F J Swanson
Unlicensed Medicines	General monographs Formulated Preparations	Dr F J Swanson Mr P Crowley

B. Monograph Development: Mechanism

The following Supplementary Chapter provides an outline of the mechanism by which monographs are selected and developed for inclusion in the British Pharmacopoeia.

The British Pharmacopoeia Commission will not usually develop monographs for drug substances or excipients. These will usually be elaborated by the European Pharmacopoeia Commission.

The British Pharmacopoeia Commission will consider a monograph for inclusion in the British Pharmacopoeia in the following circumstances:

1. The formulation is widely used (for example: products in the top 500 list of prescribed items; products in the top 100 list of items used in hospitals; Pharmacy (P) and General Sale List (GSL) products that are widely used, taking into account seasonal fluctuations; veterinary products that are widely used or where a need is identified).

2. The innovator product is approaching or past its patent expiry date (monographs will usually only be prepared in the two years preceding patent expiry. However there may be circumstances where it is justified to prepare a monograph at an earlier stage. These will be considered by the British Pharmacopoeia Commission individually).

3. There is a particular need based on the therapeutic category and/or the importance of the material concerned; the latter being particularly relevant to small patient populations.

4. Unlicensed products that are produced to meet particular patient needs (generally "specials" manufacture or extemporaneous preparation) (see also Supplementary Chapter V A).

5. The product/preparation is a widely used traditional herbal medicine.

6. The legal classification of the product has been changed from POM (prescription only medicine) to P or from P to GSL.

7. The product falls within a "family" of product presentations, of which there are already published monographs and/or monographs on the work programme.

8. Drug substances or excipients which are not on the European Pharmacopoeia work programme, but for which there is a specific UK need.

9. A request is received from the Competent Authority [Medicines and Healthcare Products Regulatory Agency (MHRA)].

10. A request is received from a manufacturer for one of their own products.

11. Support for relevant EC directives.

12. A request is received from official bodies [such as the World Health Organization (WHO)].

13. Other circumstances considered on a case-by-case basis.

It should be noted that compliance with any of the above criteria will not necessarily mean that a monograph will be included in the British Pharmacopoeia. The British Pharmacopoeia Commission may decide not to elaborate a monograph for a number of reasons, including a lack of interest from stakeholders, resource limitations or other circumstances, decided on a case-by-case basis.

The diagram provides a simplified, schematic representation of the development of a monograph for a medicinal substance or an associated formulated preparation.

C. Monograph Development: Guidance to Manufacturers

Bulk drug substances

Each monograph, taken as a whole, should provide a reliable basis for making an independent judgement as to the quality of the substance in the interests of the protection of the public. General guidance as to the types of test required and the level of control considered appropriate can be obtained by reference to current BP monographs for similar chemical entities where such are available. In general any monograph for a bulk drug substance should include the features listed below.

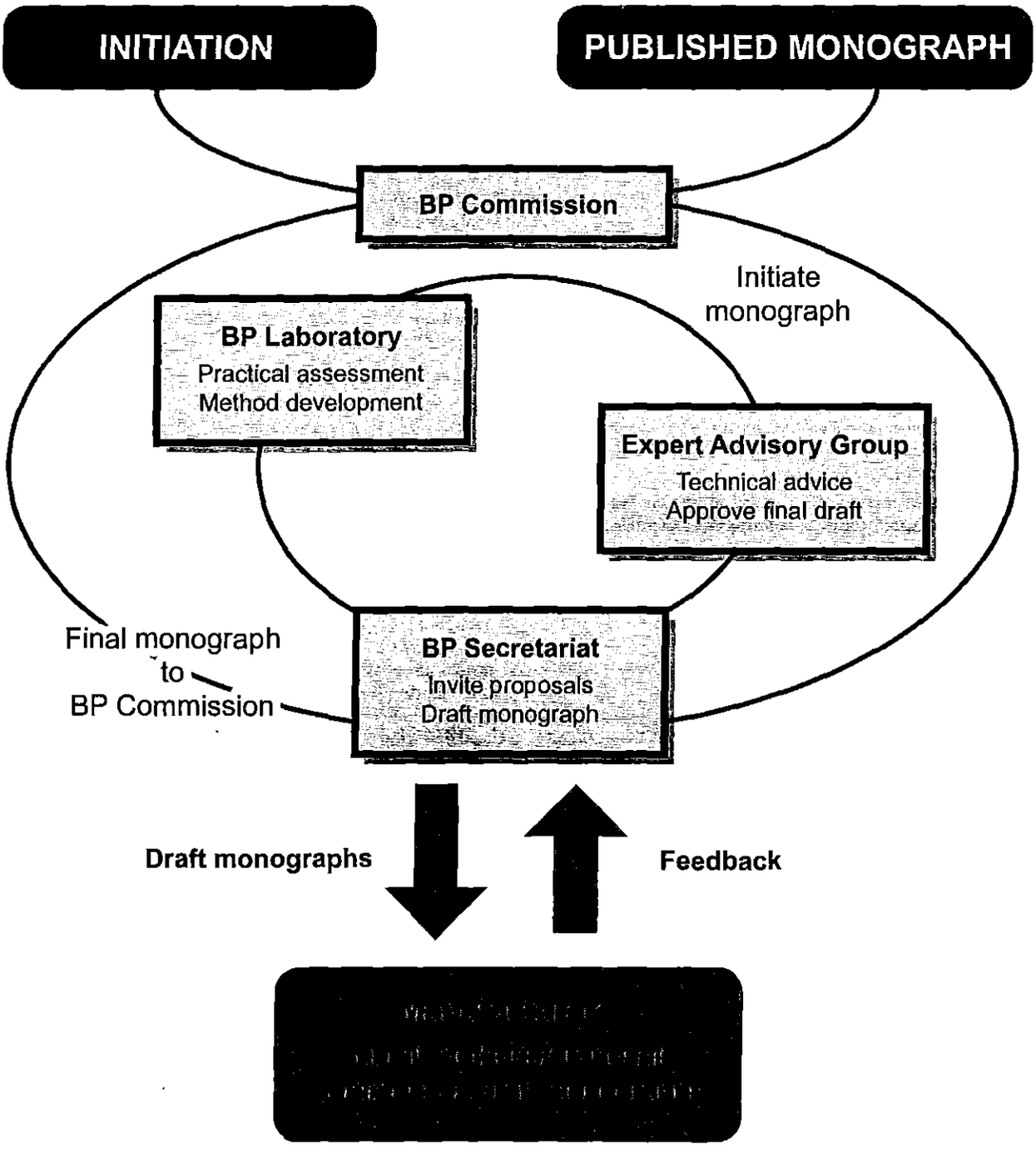
Attention is drawn to the General Notices of the Pharmacopoeia, to the general methods described in the Appendices, to the basis of pharmacopoeial requirements as described in Supplementary Chapter I and to other information provided in the Supplementary Chapters of the Pharmacopoeia.

1. **Definition/Characteristics** Brief description of physical form of material; whether hygroscopic. It would be helpful if information concerning polymorphism could be provided (see Supplementary Chapter I B).

2. **Solubility** Solubility in water, alcohol and at least one common organic solvent, expressed quantitatively or using defined BP terms.

3. **Identification** Qualitative tests capable of unequivocally establishing the identity of the substance. Infrared spectrometry is preferred, or if this is not possible, 2 or 3 identification tests to identify different characteristics of the substance may be used. A test for the counter ion should be included where appropriate.

4. **Impurities** (see also Supplementary Chapter I A) Both related substances and any other impurities that may be present in the substance as a result of the method of manufacture or from degradation on storage. It would be helpful to know the nature of such impurities, the reason for their presence (for example, by-product of synthesis, hydrolysis product, etc.), the amounts that may be encountered in material prepared under conditions of Good Manufacturing Practice and the manner in which the proportions may vary on storage. An indication of the toxicity of any impurities in relation to that of the substance itself and methods for their detection and control would enable the Committee preparing the monograph to decide whether control tests are necessary and, if so, the methods and limits to be applied. In many cases, it is possible to limit impurities



in chromatographic tests using a dilute solution of the substance being examined, and this is the preferred approach, wherever possible. In other cases, for example, where the response factor of an impurity is significantly different from that of the substance (outside the range 0.8 to 1.2), or where a stringent limit is necessary for toxicity reasons, a reference material is required (see paragraph 11 below and Supplementary Chapter III E).

Impurities other than related substances that might require control include inorganic impurities and residues of solvents and reagents used during synthesis and purification. Non-specific purity tests such as light absorption, specific optical rotation and sulfated ash should also be considered.

5. **Transparency** In keeping with BP policy on monograph transparency a suitable statement will be added to appropriate new monographs for medicinal substances giving the identities of impurities known to be limited by the specifications. It is to be emphasised that such statements are not intended to be exclusive and other, unnamed impurities may also be limited. Manufacturers are requested to provide information for such statements (see paragraph 4 above).

6. **Assay** Method and proposed limits calculated with reference to the anhydrous, dried or solvent-free material as appropriate. For bulk drug substances, it has been BP policy *generally* to use a robust and precise method of assay (such as titration) rather than a specific, but sometimes less precise, stability-indicating method (such as liquid chromatography). Wherever possible, control of potential impurities is provided separately by means of specific impurity tests (see paragraph 4 above). It is appreciated, however, that a manufacturer may use, and therefore propose, a chromatographic method for both related substances and assay. In such circumstances,

each case is judged on its merits on the basis of the data provided, which must relate to validated methods. Such methods normally require the establishment of a reference material of the substance with a declared content (see paragraph 11 below and Supplementary Chapter III E). Adequate means of demonstrating system suitability will need to be included in the monograph so that the analyst has an assurance that the results are accurate. As stated above, each monograph, taken as a whole, should provide a reliable basis for making an independent judgement as to the quality of the substance.

7. **Other tests** A test for water or for loss on drying is usually required.

8. **Storage** Any special storage conditions such as protection from light.

9. **Labelling** Any special labelling statements (see Supplementary Chapter I G).

10. **Preparations** Pharmaceutical dosage forms normally available and information on dose.

11. **Samples** A quantity of the material sufficient to carry out in duplicate all the tests and the assay in the proposed specification should be supplied (10 g is usually suitable). This sample should be taken from a typical production batch, that is, it should not be specially purified. In addition appropriate amounts of possible impurities should be supplied (0.1 to 0.5 g is usually suitable).

Many monographs require the use of one or more reference substances (BPCRS); these are established by the Laboratory before publication of the monograph (see Supplementary Chapter III E). If a reference substance for assay purposes is required (see paragraph 6 above) or for impurity control (see paragraph 4 above), please indicate if adequate supplies will be available. About 50 g of material is required to establish a reference material for an assay standard and about 10 g of a named impurity (see also Supplementary Chapter III E). If it is perceived that an ongoing supply of sufficient quantities of material(s) will not be possible, this should be drawn to the attention of the BP Secretariat so that an alternative approach may be considered.

When sending samples, Material Safety Data Sheets that comply with COSHH Regulations should be supplied for all materials including impurities, so that Pharmacopoeia staff are aware of possible hazards when handling these materials.

12. **Supporting data** Appropriate and relevant validation data relating to the proposed analytical procedures and methodology should be provided (see Supplementary Chapter III F). In particular, data to demonstrate the stability indicating nature of methods (i.e. forced degradation studies) and information identifying both synthetic impurities and degradation products should be provided. Additionally, appropriate and relevant batch and stability data to support the proposed specifications should be provided. This information will be kept confidential to the BP Secretariat and Laboratory and to the relevant Expert Advisory Group(s) of the British Pharmacopoeia Commission.

13. **Advice** The BP Secretariat is pleased to provide advice to manufacturers on the nature and extent of data required. It is appreciated that for some older products information on the identity of impurities and/or validation data for methods is not as substantial as that which is required for new chemical entities and advice in these cases is available. A list of contact points in the Secretariat is to be found in Supplementary Chapter III A.

Formulated preparations

Each monograph, taken as a whole, should provide a reliable basis for making an independent judgement as to the quality of the preparation in the interests of the protection of the public. General guidance as to the types of test required and the level of control considered appropriate can be obtained by reference to current BP monographs for the same dosage form of similar chemical entities where such are available. Reference should also be made to any general monograph for the dosage form in question for general requirements and any exceptions to, or modifications of, those requirements noted. In general any monograph for a formulated preparation should include the features listed below.

Attention is drawn to the General Notices of the Pharmacopoeia, to the general methods described in the Appendices, to the basis of pharmacopoeial requirements as described in the Introduction and Supplementary Chapter I and to other information provided in the Supplementary Chapters of the Pharmacopoeia.

1. **Definition/Description** A definition of the preparation in terms of the active ingredient(s) together with information on its presentation.

For sterile preparations (parenteral, ophthalmic and others) this should include information on the nature of the vehicle; the nature of any additives (eg antimicrobial preservatives, buffers) present; the method of sterilisation. In addition, for parenteral preparations information should be provided on whether it is a solution, a suspension, a dry powder or a concentrate for dilution.

For topical semi-solid preparations this should include information on the type of basis (water-in-oil, oil-in-water, etc) and the particle size of the active ingredient, if significant.

For tablets this should include whether or not they are coated. Where tablets are coated, the reason for coating should be provided.

Information concerning polymorphism should be included where relevant (see Supplementary Chapter I B).

2. **Content statement** Proposed limits as a percentage of the stated content of the active ingredient in the terms declared on the label (see Supplementary Chapter I F).

The purpose of the assay in preparation monographs is to determine whether the content of the active ingredient is within acceptable limits of the labelled claim and the limits are therefore of necessity stated in terms of *the moiety declared on the label*. That is, the same method of expression is used in the content statement as under the headings Assay and Labelling. The preferred means of expression is in terms of the therapeutically active part of the molecule. It should be noted that the mode of expressions chosen for the assay limits in the monograph for the bulk drug substance in no way circumscribes that which may be used in the monograph for the formulation.

3. **Identification** Identification tests should be based on those for the parent drug substance where applicable, with details of any necessary preliminary treatment such as extraction. Infrared spectrometry is preferred. A single liquid-chromatographic method for Identification, control of impurities, and Assay should be supplemented by an additional test for identification.

4. **Impurities** [As under Bulk drug substances] Additional information on any impurities arising on manufacture or storage of the dosage form.

The tests applied to the bulk drug substance, including those for impurities arising in manufacture of the bulk drug substance, should be applied, wherever possible - with any necessary modification - in order to demonstrate that material of pharmacopoeial quality has been used in making the formulation.

5. **Transparency** In keeping with BP policy on monograph transparency a suitable statement will be added to appropriate new monographs for formulated preparations giving the identities of impurities known to be limited by the specifications. It is to be emphasised that such statements are not intended to be exclusive and other, unnamed impurities may also be limited. Manufacturers are requested to provide information for such statements (see paragraph 4 above).

6. **Assay** The method of assay will not necessarily be that used for the bulk drug substance. For formulated preparations a specific, stability-indicating method is preferred. Such methods normally require the establishment of a reference material of the substance with a declared content (see paragraph 11 below and Supplementary Chapter III E).

7. **Other tests** Tests such as pH and clarity and colour of solution may be necessary depending on the type of dosage form. In addition, for single-dose preparations a test for uniformity of content and, in the case of solid dosage forms, a dissolution test may be required (see Supplementary Chapter I E).

8. **Storage** Any special storage conditions/containers.

9. **Labelling** Any special labelling statements (see Supplementary Chapter I G).

Please provide sample labels, outer packages, leaflets and a copy of the relevant Summary of Product Characteristics (SmPC) or data sheet.

10. **Strengths available/Dose** While no longer included in the published monograph, this information is of assistance during monograph development.

11. **Samples** A quantity of the formulation sufficient to carry out in duplicate all the tests (including those under paragraph 7) and the assay in the proposed specification should be supplied. Samples should be taken from a typical production batch.

The following suggested quantities are provided as a rough guide of the order of sample size for each strength of different dosage forms:

Solid single dose formulations (Tablets, Capsules, etc)	100 units

Liquid formulations

i)	Topical and Oral formulations	100 mL
ii)	Parenteral formulations	50 mL
	Semi-solid topical formulations	50 to 100 g

In addition appropriate amounts of possible impurities (arising from synthesis or degradation) should be supplied (approximately 200 to 500 mg, unless the impurity is required for use as a reference standard, see below)

Many monographs require the use of one or more reference substances (BPCRS); these are established by the Laboratory before publication of the monograph (see Supplementary Chapter III E). If a reference substance is required for assay purposes (see paragraph 6 above), or for impurity control (see paragraph 4 under bulk drug substances), please indicate if adequate supplies will be available. About 50 g of material is required to establish a reference material for an assay standard and about 10 g of a named impurity (see also Supplementary Chapter III E).

When sending samples, Material Safety Data Sheets that comply with COSHH Regulations should be supplied for all materials including impurities, so that Pharmacopoeia staff are aware of possible hazards when handling these materials.

12. **Supporting data** Appropriate and relevant validation data relating to the proposed analytical procedures and methodology should be provided (see Supplementary Chapter III F). In particular, data to demonstrate the stability indicating nature of methods (i.e. forced degradation studies) and information identifying both synthetic impurities and degradation products should be provided. Additionally, appropriate and relevant batch and stability data to support the proposed specifications should be provided. This information will be kept confidential to the BP Secretariat and Laboratory and to the relevant Expert Advisory Group(s) of the British Pharmacopoeia Commission.

13. **Advice** The BP Secretariat is pleased to provide advice to manufacturers on the nature and extent of data required. It is appreciated that for some older products information on the identity of impurities and/or validation data for methods is not as substantial as that which is required for new chemical entities and advice in these cases is available. A list of contact points in the Secretariat is to be found in Supplementary Chapter III A.

D. Monograph Development: Methods of Analysis

1. This Supplementary Chapter concerns the methods described in the Pharmacopoeia for the analysis of medicinal and pharmaceutical substances, pharmaceutical dosage forms and other articles. It provides information on the development, validation and use of pharmacopoeial methods so that users of the Pharmacopoeia may understand the purpose and limitations of a monograph and to guide manufacturers and other users when participating in the development of new monographs and the revision of existing monographs.

2. For further information regarding the elaboration of European Pharmacopoeia monographs, users may also consult the "Technical guide for the elaboration of monographs", which is published by the European Pharmacopoeia.

3. The Chapter does not set out the nature and extent of validation required in particular instances. Guidelines on such matters with respect to product registration are available from other sources such as the International Conference on Harmonisation (ICH) and are published as guideline Q2 (R1), "Validation of Analytical Procedures: Text and Methodology". The terms used, and illustrations of the data requirements to demonstrate that a method satisfies the definitions, are provided in Supplementary Chapter III F.

Method origin

3. Proposals for new or revised methods for inclusion are often provided by manufacturers and other users of the Pharmacopoeia either in response to a request from the Secretariat or Laboratory or, for revised methods, when a published method has been found to be unsuitable for any reason. A method may become unsuitable when, for example, a reagent or piece of apparatus is no longer readily available, knowledge of the material has increased, regulatory

requirements have altered or a more specific or sensitive test has been developed.

4. Any method that is to be considered for inclusion in a monograph has to be suitable for pharmacopoeial purposes and, wherever possible, be accompanied by appropriate supporting data.

4.1 Guidance on the suitability of any method for inclusion in the Pharmacopoeia may be obtained by reference to the General Notices (in particular, those dealing with official standards, excipients, identification and assays and tests) and Supplementary Chapter I concerning the basis of pharmacopoeial requirements. Each monograph, taken as a whole, should provide a reliable basis for making an independent judgement as to the quality of an article in the interests of the protection of the public.

4.2 A pharmacopoeial monograph applies throughout the shelf-life of a formulated preparation or throughout the period of use of a medicinal or pharmaceutical substance and the monograph when taken as a whole must be stability indicating and must be able to assure the quality of the substance throughout its claimed shelf life. Methods for inclusion in the Pharmacopoeia should therefore have been shown to be stability indicating where appropriate by means of forced degradation studies.

4.3 Other than in exceptional circumstances, methods should not specify the use of apparatus that is not widely available in reasonably equipped laboratories nor should they require extensive additional training of laboratory staff. Reagents and reference materials required for a proposed pharmacopoeial method should be generally available from the common sources of supply in the United Kingdom or a manufacturer should be able to supply sufficient quantity for use as a British Pharmacopoeia Chemical Reference Substance (BPCRS). The method should be described in sufficient detail that a competent analyst is able to repeat it.

4.4 Methods proposed by manufacturers should have been adequately validated in accordance with appropriate guidelines (paragraph 2 refers). Exceptionally, full validation data may not be available in some circumstances. For example, not all contributors to the Pharmacopoeia are able to demonstrate the *specificity* of a test method for application to a monograph for a formulated preparation since the nature of the excipients may be unknown; in this case a note of the extent of any validation carried out, with its limitations, is helpful.

4.5 It is of particular importance that method proposals include a robust and reliable identity test(s) for the active component in a substance or formulated preparation. Infrared spectrophotometry is preferred.

4.6 It is also of particular importance that any pharmacopoeial method is *robust* and *reproducible*. Data that demonstrate the transferability of a method are, therefore, especially helpful.

Method elaboration

5. All proposals for new and revised methods for publication are carefully examined. The nature and extent of the evaluation is determined by a number of factors including the following:

— whether the proposal is for a new monograph or for revision of an existing monograph,
— extent of validation and batch data available,
— how many specifications and/or samples are available for examination,
— complexity of the proposed method.

6. The evaluation of methods is carried out by the Commission's Expert Advisory Groups and Panels of Experts, the Secretariat and the Laboratory; practical evaluation is included in many cases. If necessary, more extensive practical work is carried out in consultation with the proposer of the method. Such practical work may be necessary, for example, where a proposed method is not directly applicable to other sources of the material or preparation or is shown to be insufficiently *robust* for pharmacopoeial use.

7. After initial evaluation, the method is drafted in the style of the Pharmacopoeia and those known to have an interest in the material or preparation are invited to comment. If necessary, further modifications may be made to the method and the consultation process repeated before the method is published. A diagrammatic representation of the process of monograph elaboration is provided as Supplementary Chapter III B.

Published methods

8. The user can expect that published methods:

are suitable for the purpose for which they are described in the Pharmacopoeia, have been evaluated and, where necessary, modified as described in the preceding section,

have been shown to be adequately validated, as appropriate to the type of test, and include tests to demonstrate the continuing suitability of the method, where necessary,

are described in sufficient detail that a competent analyst can perform the test using readily available apparatus and reagents and that any necessary reference materials are available, will be reviewed and revised when experience shows this to be necessary.

9. The user cannot, however, assume that a method forming part of a pharmacopoeial monograph will have been applied to all sources of a raw material or to all formulations of a dosage form currently available. The user is responsible for confirming that the method is applicable to the particular material being examined. It is essential that a manufacturer carries out sufficient checks to demonstrate that, for example, impurities arising from a new route of synthesis are controlled by the methods described in the monograph or that the excipients in a formulated preparation do not interfere with any of the tests in a monograph. It should be noted that an article cannot claim to be of pharmacopoeial quality unless it can be shown to comply with all of the tests specified in a monograph (see General Notice on Official Standards).

Feedback

The British Pharmacopoeia Commission welcomes constructive comments from users on the tests of the Pharmacopoeia and it is through such feedback that revision of the tests is initiated. A list of contact points in the Secretariat is to be found in Supplementary Chapter III A.

E. British Pharmacopoeia Chemical Reference Substances (BPCRS)

1. The current list of BPCRS may be found on the BP's website at *www.pharmacopoeia.com*. The quantity of material supplied is sufficient to carry out in duplicate each of the tests in which it is used in any one monograph.

Requirement

2. The establishment of a new BPCRS is based on scientific necessity following advice from the British Pharmacopoeia Commission's Expert Advisory Groups. A reference substance for the medicinal substance is usually required for specific, stability-indicating assays and may also be required for identification purposes. A reference substance for an impurity is required when control using a dilute solution of the substance being examined is not possible or is undesirable. Such cases include those where the response factor of the impurity is significantly different from that of the substance, or where it is necessary to limit a named impurity (see Supplementary Chapter I A).

The following criteria are taken into account when determining whether a new BPCRS is required.

Availability of the material A commercially available reagent is specified wherever it is found to be suitable and justified.

Analytical convenience Wherever possible, either the same EPCRS or the same BPCRS is specified throughout any one monograph.

Establishment

3. Manufacturers who have contributed to the development of the monograph(s) are asked to supply material for use as reference substances. Normally, about 50 g is required for an Assay standard and about 10 g for an impurity. In some cases it is possible to use a quantity of the medicinal substance admixed with smaller quantities of the impurities as an impurity reference standard. It would be helpful if manufacturers could indicate the likely availability of reference materials at an early stage during monograph development.

4. The materials are tested by the Laboratory to confirm their suitability for the intended purpose and are made available not later than the effective date of the monograph in which they are used.

Monitoring and replacement

5. All substances used for quantitative analyses are re-tested every three years and materials used for qualitative analysis every five years unless experience has shown that more frequent testing is necessary.

6. When a BPCRS is due for replacement (because of a fall in quality or exhaustion), a review of the use of the material and the number of units supplied is carried out.

Continued availability

7. When a monograph is omitted from the Pharmacopoeia the last published monograph remains the legal standard. As the usual reason for omission is low and declining use or withdrawal of the material from the market, demand for any associated reference substances from within the United Kingdom is normally low.

8. It is not possible to maintain reference substances for omitted materials indefinitely and, for materials no longer marketed in the United Kingdom, it is difficult to obtain replacement stocks. As a service to analysts, reference substances for monographs omitted from the current edition of the Pharmacopoeia are normally retained for about five years from the date of publication of the current edition unless the material becomes unsatisfactory or the supply is exhausted before that date.

9. Demand is monitored and any significant increase in demand for a reference substance for an omitted monograph is noted. If such increased demand appears to stem from a renewed interest in the medicinal or pharmaceutical product, consideration may be given to reinstating the monograph in the Pharmacopoeia.

F. Validation of Analytical Procedures

Introduction

Validation of an analytical procedure is performed in order to demonstrate that the procedure is suitable for its intended use. Validation is performed in order to show that the result(s) generated by a particular analytical procedure are reliable and accurate.

The principles and practices of validation of analytical procedures are covered by the International Conference on Harmonisation (ICH), are published as guideline Q2(R1), "Validation of Analytical Procedures: Text and Methodology" and are available from www.ich.org. A full discussion of the terms and methodology applicable to validation of analytical procedures is provided in the ICH documents.

Types of procedures to be validated

The objective of validation of an analytical procedure is to demonstrate that it is suitable for its intended purpose. A tabular summation of the characteristics applicable to identification, control of impurities and assay procedures is included.

Specificity

Specificity is the ability to assess unequivocally the analyte in the presence of components that may be expected to be present. Typically these might include impurities, degradants, matrix, etc.

Specificity may often be expressed as the degree of bias of test results obtained by analysis of samples containing added impurities, degradation products, related chemical compounds, or placebo ingredients when compared to test results without added substances.

Specificity is usually demonstrated by measuring the response of the sample matrix and any expected or known species (for example excipients, impurities or degradation products). It would normally be expected that no significant response would be obtained that interferes with the measurement of the analyte(s). However it is not always possible that an analytical procedure is specific for a particular analyte. In this instance a combination of two or more analytical procedures may be necessary to achieve the required discrimination.

Linearity

The linearity of an analytical procedure is its ability (within a given range) to obtain test results that are directly proportional to the concentration (amount) of analyte in the sample.

Linearity is usually demonstrated by visual inspection of a plot of signals as a function of analyte concentration or content. If there is a linear relationship, test results should be evaluated by appropriate statistical methods, for example, by calculation of a regression line by the method of least squares. In some cases, to obtain linearity between assays and sample concentrations, the test data may need to be

Type of analytical procedure characteristics	IDENTIFICATION	TESTING FOR IMPURITIES quantitat.	 limit	ASSAY - dissolution (measurement only) - content/potency
Accuracy	-	+	-	+
Precision				
Repeatability	-	+	-	+
Interm. Precision	-	+ (1)	-	+ (1)
Specificity (2)	+	+	+	+
Detection Limit	-	- (3)	+	-
Quantitation Limit	-	+	-	-
Linearity	-	+	-	+
Range	-	+	-	+

- signifies that this characteristic is not normally evaluated
+ signifies that this characteristic is normally evaluated
(1) in cases where reproducibility has been performed, intermediate precision is not needed
(2) lack of specificity of one analytical procedure could be compensated by other supporting analytical procedure(s)
(3) may be needed in some cases

subjected to a mathematical transformation prior to the regression analysis. Data from the regression line itself may be helpful to provide mathematical estimates of the degree of linearity.

The correlation coefficient, y-intercept, slope of the regression line and residual sum of squares should be calculated. A plot of the data should be included.
In addition, an analysis of the deviation of the actual data points from the regression line may also be helpful for evaluating linearity.

A minimum of five concentrations is recommended. Other approaches should be justified.

Accuracy
The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found. This is sometimes termed trueness.

Accuracy should be established across the specified range of the analytical procedure.

Accuracy is usually demonstrated by adding known amounts of analyte(s) to the sample matrix and determining the measured result using the analytical procedure. The recovery of measured against actual amounts is then calculated.
Usually a minimum of three determinations at each of three concentrations across the intended range is recommended.

Accuracy may also be demonstrated by the method of standard additions, or by cross-correlation of results with a second, independent, procedure. Accuracy may be inferred once precision, linearity and specificity have been established.

Precision
The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Precision may be considered at three levels: repeatability, intermediate precision and reproducibility.

Precision should be investigated using homogeneous, authentic samples. However, if it is not possible to obtain a homogeneous sample it may be investigated using artificially prepared samples or a sample solution.

The precision of an analytical procedure is usually expressed as the variance, standard deviation or coefficient of variation of a series of measurements.

Repeatability (intra-assay precision)
Repeatability expresses the precision under the same operating conditions over a short interval of time. Repeatability is also termed intra-assay precision.

Repeatability is usually demonstrated by repeated measurements of a single sample (e.g. use of the analytical procedure within a laboratory over a short period of time using the same analyst with the same equipment).
A minimum of three determinations at each of three concentrations across the intended range, or a minimum of six determinations at the test concentration is recommended.

Intermediate Precision
Intermediate precision expresses within-laboratory variations: different days, different analysts or equipment, etc.

Intermediate precision is usually demonstrated by repeated measurements of the sample used in the repeatability

experiment within the same laboratory. Usually the repeatability experiment is repeated on the same sample by a different analyst, on a different day, using different equipment if possible.

Reproducibility

Reproducibility expresses the precision between laboratories (collaborative studies, usually applied to standardisation of methodology).

Reproducibility is usually demonstrated by means of an inter-laboratory trial.

Detection Limit

The detection limit of an analytical procedure is the lowest concentration of analyte in a sample that can be detected but not necessarily quantitated as an exact value.

The detection limit is usually expressed as the concentration of analyte (e.g., percentage, parts per billion) in the sample.

The detection limit is usually demonstrated by measuring low concentrations of the analyte and showing that a response is obtained.

Quantitation Limit

The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. The quantitation limit is a parameter of quantitative assays for low levels of compounds in sample matrices, and is used particularly for the determination of impurities and/or degradation products.

It is usually expressed as the concentration of analyte (e.g. percentage, parts per billion) in the sample.

The quantitation limit is usually demonstrated by measuring low concentrations of the analyte and showing that a repeatable response is obtained.

Robustness

The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small but deliberate variations in method parameters and provides an indication of its reliability during normal usage.

Robustness is usually demonstrated by making small deliberate changes to one of the operating parameters of the method, analysing samples and comparing the results to those obtained using the prescribed method.

Range

The range of an analytical method is the interval between the upper and lower concentration (amounts) of analyte (including these concentrations) for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity.

The specified range is normally derived from linearity studies and depends on the intended application of the procedure. It is established by confirming that the analytical procedure provides an acceptable degree of linearity, accuracy and precision when applied to samples containing amounts of analyte within or at the extremes of the specified range of the analytical procedure.

For assays the range is usually not less than 80 to 120% of the test concentration.

For determination of content uniformity the range is usually not less than 70 to 130% of the test concentration.

For determination of impurities the range is usually not less than the reporting limit of the impurity to 120% of the specification.

For dissolution testing the range is usually +/- 20% over the expected concentrations.

Range is usually demonstrated by confirming that the analytical procedure provides an acceptable degree of linearity, accuracy and precision when applied to samples containing amounts of analyte within or at the extremes of the specified range.

System Suitability Testing

System suitability testing is an integral part of many analytical procedures. The tests are based on the concept that the equipment, electronics, analytical operations and samples to be analyzed constitute an integral system that can be evaluated as such. System suitability test parameters to be established for a particular procedure depend on the type of procedure being validated.

Criteria for assessing the suitability of chromatographic systems are described in the chapter on Chromatographic separation techniques (Appendix III (*Ph. Eur. method 2.2.46*)). The extent to which adjustments of parameters of the chromatographic system can be made to satisfy the criteria of system suitability are also given in this chapter.

Supplementary Chapter IV

European Pharmacopoeia

This Supplementary Chapter provides information concerning the European Pharmacopoeia.

A. Membership of the European Pharmacopoeia Commission

In addition to the United Kingdom, the Member States party to the Convention on the Elaboration of a European Pharmacopoeia are as follows: Albania, Austria, Belgium, Bosnia and Herzegovina, Bulgaria, Croatia, Cyprus, The Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Iceland, Ireland, Italy, Latvia, Lithuania, Luxembourg, Malta, Montenegro, The Netherlands, Norway, Poland, Portugal, Republic of Moldova, Romania, Serbia, Slovak Republic, Slovenia, Spain, Sweden, Switzerland, The Former Yugoslav Republic of Macedonia, Turkey and Ukraine. The European Union is also party to the Convention.

Observers to the European Pharmacopoeia Commission at the time of publication are: Algeria, Argentina, Armenia, Australia, Azerbaijan, Belarus, Brazil, Canada, China, Georgia, Guinea, India, Israel, Japan, Kazakhstan, Republic of Korea, Madagascar, Malaysia, Mexico, Morocco, The Russian Federation, Senegal, Singapore, South Africa, Syria, Tunisia, the United States of America, Uzbekistan, the Taiwan Food and Drug Administration (TFDA) and the World Health Organization (WHO).

The United Kingdom delegation to the European Pharmacopoeia Commission up to 31 December 2021 was as follows:

Professor A Davidson

Mr J Pound

Professor K Taylor

Alternate members of the United Kingdom delegation up to 31 December 2021 were as follows:

Mr A Gibb

Dr R Horder

Mr S Young

UK Membership of Groups of Experts of the European Pharmacopoeia Commission

The United Kingdom is represented in all of the major Groups of Experts of the European Pharmacopoeia Commission. The UK membership of the Groups on 1 January 2021 is as listed in the table below.

1	Microbiology	Mr M Whaley
6	Biological Substances	Dr B Cowper
6B	Human Blood and Blood Products	Dr C Thelwell
7	Antibiotics	Mr J Sumal
9G	Medicinal Gases	Mr P Henrys *(Chairman)*
10A	Organic Chemistry (Synthetic Products)	Mr D Malpas *(Specialist)*
10B	Organic Chemistry (Synthetic Products)	Dr E Bush
10C	Organic Chemistry (Synthetic Products)	Dr J Mckendrick
10D	Organic Chemistry (Synthetic Products)	Mr C T Goddard
11	Organic Chemistry (Natural Products)	Ms H Corns
12	Dosage Forms and Methods	Dr R Horder *(Chairman)* Dr E Gray
13B	Phytochemistry B	Mr P Anderson
14	Radioactive Compounds	Dr R D Pickett
15	Sera and Vaccines	Dr P Stickings Dr S Schepelmann *(Specialist)*
15V	Veterinary Sera and Vaccines	Dr A-M Brady *(Specialist)* Dr R Cooney
17	Medicinal Products containing Chemically Defined Active Substances	Mr S Young
P4	Procedure 4	Mr A Evans

UK Membership of Working Parties of the European Pharmacopoeia Commission

The UK membership of the Working Parties on 1 January 2021 is as follows.

Alkyl Mesilates	*Vacant*
Bacterial Endotoxins Test	Dr Karin Nordgren
Cell Therapy Products	Ms K Cornish
Chromatographic Separation Techniques	Mr S Young
Excipient Performance	*Vacant*
Extracts	Dr M Pires
General Methods	Dr E Gray
Gene Therapy	Dr Y Zhao
Host-cell Proteins	Dr A Kippen
Inhalanda	Professor K Taylor
Monoclonal Antibodies	Dr S Prior
	Dr P Varley
	Dr M Wadhwa
Mycoplasma	Mr R Hawkins
Paediatric Formulary	Dr K Boon
Pyrrolizidine Alkaloids	Ms S Macdonald
P4 Procedure 4 for Biologicals	Dr L Both
	Dr M Wadhwa
Raw Materials for the Production of Cellular and Gene Therapy Products	Dr L Bisset
Rules of Procedure	Mr A Gibb
Special Revision Programme	Mr A Evans
Standard Terms	Dr M Ahmed
Statistics	Dr R Gaines Das

B. Dates of Implementation

Under the 1964 Convention[1] on the Elaboration of a European Pharmacopoeia the standards of the European Pharmacopoeia are required to take precedence over the standards of the national pharmacopoeias of the contracting parties, thus ensuring a common standard.

In addition to the United Kingdom the countries party to the Convention are: Austria, Belgium, Bosnia and Herzegovina, Bulgaria, Croatia, Cyprus, The Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Iceland, Ireland, Italy, Latvia, Lithuania, Luxembourg, Malta, Montenegro, The Netherlands, Norway, Poland, Portugal, Romania, Serbia, Slovakia, Slovenia, Spain, Sweden, Switzerland, The Former Yugoslav Republic of Macedonia, Turkey and Ukraine. The European Union is also party to the Convention.

The 10th Edition, published in July 2019, came into force on 1 January 2020 and will be amended by Supplements 10.1 to 10.8. The publication remains in force until 31 December 2022 and the supplements are non-cumulative.

The date of entry into force of each publication is agreed by the members of the Convention. For new texts and monographs the agreed date is the latest date by which all member states must have implemented the standard but for replacement texts and monographs the standard enters into force on the same day in all states party to the Convention.

The dates of entry into force of the monographs in the 10th Edition and Supplements are given in the Table below.

Publication	Implementation Date
10th Edition	1 January 2020
Supplement 10.1	1 April 2020
Supplement 10.2	1 July 2020
Supplement 10.3	1 January 2021
Supplement 10.4	1 April 2021
Supplement 10.5	1 July 2021
Supplement 10.6	1 January 2022
Supplement 10.7	1 April 2022
Supplement 10.8	1 July 2022

To provide the user of the British Pharmacopoeia with a comprehensive reference to pharmacopoeial standards applicable in the United Kingdom, monographs of the European Pharmacopoeia, as amended by the Supplements, are included in the British Pharmacopoeia or the British Pharmacopoeia (Veterinary), as appropriate (see the General Notice on the European Pharmacopoeia).

Where the title of the monograph entry included in the British Pharmacopoeia is different from the English title of the European Pharmacopoeia monograph, an approved synonym has been created by the British Pharmacopoeia Commission (see Appendix XXI B).

[1] *The Convention on the Elaboration of a European Pharmacopoeia (European Treaty Series No. 50; UK Treaty Series No.32 (1974) CMND 5763) as amended by the Protocol (European Treaty Series No. 134; UK Treaty Series No. MISC16 (1990) CMND 1133).*

C. Certification Scheme

1. The scheme is operated in accordance with the procedures described in Council of Europe Resolution AP-CSP(07) 1. Additional information, including a list of certificates granted, is published regularly in Pharmeuropa.

2. The scheme has been extended by means of Resolution AP-CSP(07)1 and the Human Medicines Regulations 2012, as amended, to cover active substances or excipients (organic or inorganic, obtained by synthesis, extraction or fermentation), any product with transmissible spongiform encephalopathy (TSE) risk, or herbal products used in the production or preparation of pharmaceutical products.

3. For the established process, the certificate is a '*Certificate of Suitability of a Monograph of the European Pharmacopoeia*'. It certifies that the relevant Ph. Eur. monograph adequately controls the substance as manufactured by the company concerned at the time that the certificate is granted (mainly that the impurity tests are adequate to control the impurity profile associated with the particular source/route of synthesis). It is *not* a *certificate of compliance*. It does not certify that the substance, as manufactured by the company concerned at the time that the certificate is granted, complies with the requirements of the relevant Ph. Eur. monograph.

4. The Certification Scheme is recognised by all signatory states of the European Pharmacopoeia Convention. Canada, Australia, New Zealand, Tunisia and Morocco also recognise the scheme.

5. The certificate is intended to facilitate the licensing process. It can be used to simplify the data required within the Control of starting materials section of an application as stated in the Human Medicines Regulations 2012, as amended. The Certification Scheme is intended to operate in an analogous fashion to that for European Drug Master Files (EDMFs) for which it is the preferred alternative. Its general purpose is to avoid duplication of work by marketing authorisation applicants in preparing dossiers and by the authorities in the assessment process. The scheme also assists in eliminating differences in the interpretation of Ph. Eur. monographs by various competent authorities. The operation of the scheme provides the European Pharmacopoeia Commission with a mechanism for updating monographs, for example, to cover substances made by different manufacturing methods.

6. The scope of the procedure was extended by means of Resolution AP-CSP(07)1. It now covers substances produced by fermentation as indirect gene products, which are metabolites of microorganisms, irrespective of whether or not the microorganisms have been modified by traditional procedures or r-DNA technology and products with risk of transmitting agents of animal spongiform encephalopathies (TSE).

Substances which are direct gene products, such as proteins, and substances obtained from human tissues, vaccines and blood products and preparations remain excluded from the Certification procedure.

The final decision on eligibility of an application for a certificate of suitability for a material of animal origin is taken by the relevant board of the procedure if necessary.

D. Residual Solvents

(Ph. Eur. general texts 5.4)

LIMITING RESIDUAL SOLVENT LEVELS IN ACTIVE SUBSTANCES, EXCIPIENTS AND MEDICINAL PRODUCTS

The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) has adopted Impurities Guidelines for Residual Solvents which prescribes limits for the content of solvents which may remain in active substances, excipients and medicinal products after processing. This guideline, the text of which is reproduced below, excludes existing marketed products. The European Pharmacopoeia is, however, applying the same principles enshrined in the guideline to existing active substances, excipients and medicinal products whether or not they are the subject of a monograph of the Pharmacopoeia. All substances and products are to be tested for the content of solvents likely to be present in a substance or product.

Where the limits to be applied comply with those given below, tests for residual solvents are not generally mentioned in specific monographs since the solvents employed may vary from one manufacturer to another and the requirements of this general chapter are applied via the general monograph on *Substances for Pharmaceutical Use (2034)*. The competent authority is to be informed of the solvents employed during the production process. This information is also given in the dossier submitted for a certificate of suitability of the monographs of the European Pharmacopoeia and is mentioned on the certificate.

Where only Class 3 solvents are used, a test for loss on drying may be applied or a specific determination of the solvent may be made. If for a Class 3 solvent a justified and authorised limit higher than 0.5 per cent is applied, a specific determination of the solvent is required.

When Class 1 residual solvents or Class 2 residual solvents (or Class 3 residual solvents which exceed the 0.5 per cent) are used, the methodology described in the general method *(2.4.24)* is to be applied wherever possible. Otherwise an appropriate validated method is to be employed.

When a quantitative determination of a residual solvent is carried out, the result is taken into account for the calculation of the content of the substance except where a test for drying is carried out.

IMPURITIES: GUIDELINES FOR RESIDUAL SOLVENTS (CHMP/ICH/82260/2006)

1 INTRODUCTION
2 SCOPE OF THE GUIDELINE
3 GENERAL PRINCIPLES
3.1 CLASSIFICATION OF RESIDUAL SOLVENTS BY RISK ASSESSMENT
3.2 METHODS FOR ESTABLISHING EXPOSURE LIMITS
3.3 OPTIONS FOR DESCRIBING LIMITS OF CLASS 2 SOLVENTS
3.4 ANALYTICAL PROCEDURES
3.5 REPORTING LEVELS OF RESIDUAL SOLVENTS

4 LIMITS OF RESIDUAL SOLVENTS
4.1 SOLVENTS TO BE AVOIDED
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APPENDIX 1. LIST OF SOLVENTS INCLUDED IN THE GUIDELINE
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1 INTRODUCTION

The objective of this guideline is to recommend acceptable amounts of residual solvents in pharmaceuticals for the safety of the patient. The guideline recommends the use of less toxic solvents and describes levels considered to be toxicologically acceptable for some residual solvents.

Residual solvents in pharmaceuticals are defined here as organic volatile chemicals that are used or produced in the manufacture of active substances or excipients, or in the preparation of medicinal products. The solvents are not completely removed by practical manufacturing techniques. Appropriate selection of the solvent for the synthesis of active substance may enhance the yield, or determine characteristics such as crystal form, purity, and solubility. Therefore, the solvent may sometimes be a critical parameter in the synthetic process. This guideline does not address solvents deliberately used as excipients nor does it address solvates. However, the content of solvents in such products should be evaluated and justified.

Since there is no therapeutic benefit from residual solvents, all residual solvents should be removed to the extent possible to meet product specifications, good manufacturing practices, or other quality-based requirements. Medicinal products should contain no higher levels of residual solvents than can be supported by safety data. Some solvents that are known to cause unacceptable toxicities (Class 1, Table 1) should be avoided in the production of active substances, excipients, or medicinal products unless their use can be strongly justified in a risk-benefit assessment. Some solvents associated with less severe toxicity (Class 2, Table 2) should be limited in order to protect patients from potential adverse effects. Ideally, less toxic solvents (Class 3, Table 3) should be used where practical. The complete list of solvents included in this guideline is given in Appendix 1.

The lists are not exhaustive and other solvents can be used and later added to the lists. Recommended limits of Class 1 and 2 solvents or classification of solvents may change as new safety data becomes available. Supporting safety data in a marketing application for a new medicinal product containing a new solvent may be based on concepts in this guideline or the concept of qualification of impurities as expressed in the guideline for active substances (*Q3A*, Impurities in New Active Substances) or medicinal products (*Q3B*, Impurities in New Medicinal Products), or all three guidelines.

2 SCOPE OF THE GUIDELINE

Residual solvents in active substances, excipients, and in medicinal products are within the scope of this guideline. Therefore, testing should be performed for residual solvents when production or purification processes are known to result in the presence of such solvents. It is only necessary to test for solvents that are used or produced in the manufacture or purification of active substances, excipients, or medicinal product. Although manufacturers may choose to test the medicinal product, a cumulative method may be used to calculate the residual solvent levels in the medicinal product from the levels in the ingredients used to produce the medicinal product. If the calculation results in a level equal to or below that recommended in this guideline, no testing of the medicinal product for residual solvents need be considered. If however, the calculated level is above the recommended level, the medicinal product should be tested to ascertain whether the formulation process has reduced the relevant solvent level to within the acceptable amount. Medicinal product should also be tested if a solvent is used during its manufacture.

This guideline does not apply to potential new active substances, excipients, or medicinal products used during the clinical research stages of development, nor does it apply to existing marketed medicinal products.

The guideline applies to all dosage forms and routes of administration. Higher levels of residual solvents may be acceptable in certain cases such as short term (30 days or less) or topical application. Justification for these levels should be made on a case by case basis.

See Appendix 2 for additional background information related to residual solvents.

3 GENERAL PRINCIPLES

3.1 CLASSIFICATION OF RESIDUAL SOLVENTS BY RISK ASSESSMENT

The term "tolerable daily intake" (TDI) is used by the International Program on Chemical Safety (IPCS) to describe exposure limits of toxic chemicals and "acceptable daily intake" (ADI) is used by the World Health Organization (WHO) and other national and international health authorities and institutes. The new term "permitted daily exposure" (PDE) is defined in the present guideline as a pharmaceutically acceptable intake of residual solvents to avoid confusion of differing values for ADI's of the same substance.

Residual solvents assessed in this guideline are listed in Appendix 1 by common names and structures. They were evaluated for their possible risk to human health and placed into one of three classes as follows:

Class 1 solvents: solvents to be avoided
Known human carcinogens, strongly suspected human carcinogens, and environmental hazards.

Class 2 solvents: solvents to be limited
Non-genotoxic animal carcinogens or possible causative agents of other irreversible toxicity such as neurotoxicity or teratogenicity.

Solvents suspected of other significant but reversible toxicities.

Class 3 solvents: solvents with low toxic potential
Solvents with low toxic potential to man; no health-based exposure limit is needed. Class 3 solvents have PDEs of 50 mg or more per day.

3.2 METHODS FOR ESTABLISHING EXPOSURE LIMITS

The method used to establish permitted daily exposures for residual solvents is presented in Appendix 3. Summaries of the toxicity data that were used to establish limits are published in *Pharmeuropa*, Vol. 9, No. 1, Supplement April 1997.

3.3 OPTIONS FOR DESCRIBING LIMITS OF CLASS 2 SOLVENTS

Two options are available when setting limits for Class 2 solvents.

Option 1

The concentration limits in parts per million stated in Table 2 can be used. They were calculated using equation (1) below by assuming a product mass of 10 g administered daily.

$$\text{Concentration (ppm)} = \frac{1000 \times \text{PDE}}{\text{dose}} \quad (1)$$

Here, PDE is given in terms of mg/day and dose is given in g/day.

These limits are considered acceptable for all substances, excipients, or products. Therefore this option may be applied if the daily dose is not known or fixed. If all excipients and active substances in a formulation meet the limits given in Option 1, then these components may be used in any proportion. No further calculation is necessary provided the daily dose does not exceed 10 g. Products that are administered in doses greater than 10 g per day should be considered under Option 2.

Option 2

It is not considered necessary for each component of the medicinal product to comply with the limits given in Option 1. The PDE in terms of mg/day as stated in Table 2 can be used with the known maximum daily dose and equation (1) above to determine the concentration of residual solvent allowed in a medicinal product. Such limits are considered acceptable provided that is has been demonstrated that the residual solvent has been reduced to the practical minimum. The limits should be realistic in relation to analytical precision, manufacturing capability, reasonable variation in the manufacturing process, and the limits should reflect contemporary manufacturing standards.

Option 2 may be applied by adding the amounts of a residual solvent present in each of the components of the medicinal product. The sum of the amounts of solvent per day should be less than that given by the PDE.

Consider an example of the use of Option 1 and Option 2 applied to acetonitrile in a medicinal product. The permitted daily exposure to acetonitrile is 4.1 mg per day; thus, the Option 1 limit is 410 ppm. The maximum administered daily mass of a medicinal product is 5.0 g, and the medicinal product contains two excipients. The composition of the medicinal product and the calculated maximum content of residual acetonitrile are given in the following table.

Component	Amount in formulation	Acetonitrile content	Daily exposure
Active substance	0.3 g	800 ppm	0.24 mg
Excipient 1	0.9 g	400 ppm	0.36 mg
Excipient 2	3.8 g	800 ppm	3.04 mg
Medicinal product	5.0 g	728 ppm	3.64 mg

Excipient 1 meets the Option 1 limit, but the active substance, excipient 2, and medicinal product do not meet the Option 1 limit. Nevertheless, the product meets the Option 2 limit of 4.1 mg per day and thus conforms to the recommendations in this guideline.

Consider another example using acetonitrile as residual solvent. The maximum administered daily mass of a medicinal product is 5.0 g, and the medicinal product contains two excipients. The composition of the medicinal product and the calculated maximum content of residual acetonitrile is given in the following table.

Component	Amount in formulation	Acetonitrile content	Daily exposure
Active substance	0.3 g	800 ppm	0.24 mg
Excipient 1	0.9 g	2000 ppm	1.80 mg
Excipient 2	3.8 g	800 ppm	3.04 mg
Medicinal product	5.0 g	1016 ppm	5.08 mg

In this example, the product meets neither the Option 1 nor the Option 2 limit according to this summation.

The manufacturer could test the medicinal product to determine if the formulation process reduced the level of acetonitrile. If the level of acetonitrile was not reduced during formulation to the allowed limit, then the manufacturer of the medicinal product should take other steps to reduce the amount of acetonitrile in the medicinal product. If all of these steps fail to reduce the level of residual solvent, in exceptional cases the manufacturer could provide a summary of efforts made to reduce the solvent level to meet the guideline value, and provide a risk-benefit analysis to support allowing the product to be utilised containing residual solvent at a higher level.

3.4 ANALYTICAL PROCEDURES

Residual solvents are typically determined using chromatographic techniques such as gas chromatography. Any harmonised procedures for determining levels of residual solvents as described in the pharmacopoeias should be used, if feasible. Otherwise, manufacturers would be free to select the most appropriate validated analytical procedure for a particular application. If only Class 3 solvents are present, a non-specific method such as loss on drying may be used.

Validation of methods for residual solvents should conform to ICH guidelines "Text on Validation of Analytical Procedures" and "Extension of the ICH Text on Validation of Analytical Procedures".

3.5 REPORTING LEVELS OF RESIDUAL SOLVENTS

Manufacturers of pharmaceutical products need certain information about the content of residual solvents in excipients or active substances in order to meet the criteria of this guideline. The following statements are given as acceptable examples of the information that could be provided from a supplier of excipients or active substances to a pharmaceutical manufacturer. The supplier might choose one of the following as appropriate:

— only Class 3 solvents are likely to be present. Loss on drying is less than 0.5 per cent;
— only Class 2 solvents X, Y, ... are likely to be present. All are below the Option 1 limit;

(Here the supplier would name the Class 2 solvents represented by X, Y, ...)

— only Class 2 solvents X, Y, ... and Class 3 solvents are likely to be present. Residual Class 2 solvents are below the Option 1 limit and residual Class 3 solvents are below 0.5 per cent.

If Class 1 solvents are likely to be present, they should be identified and quantified. "Likely to be present" refers to the solvent used in the final manufacturing step and to solvents that are used in earlier manufacturing steps and not removed consistently by a validated process.

If solvents of Class 2 or Class 3 are present at greater than their Option 1 limits or 0.5 per cent, respectively, they should be identified and quantified.

4 LIMITS OF RESIDUAL SOLVENTS

4.1 SOLVENTS TO BE AVOIDED

Solvents in Class 1 should not be employed in the manufacture of active substances, excipients, and medicinal products because of their unacceptable toxicity or their deleterious environmental effect. However, if their use is unavoidable in order to produce a medicinal product with a significant therapeutic advance, then their levels should be restricted as shown in Table 1, unless otherwise justified. 1,1,1-Trichloroethane is included in Table 1 because it is an environmental hazard. The stated limit of 1500 ppm is based on a review of the safety data.

Table 1. – *Class 1 solvents in pharmaceutical products (solvents that should be avoided)*

Solvent	Concentration limit (ppm)	Concern
Benzene	2	Carcinogen
Carbon tetrachloride	4	Toxic and environmental hazard
1,2-Dichloroethane	5	Toxic
1,1-Dichloroethene	8	Toxic
1,1,1-Trichloroethane	1500	Environmental hazard

4.2 SOLVENTS TO BE LIMITED

Solvents in Table 2 should be limited in pharmaceutical products because of their inherent toxicity. PDEs are given to the nearest 0.1 mg/day, and concentrations are given to the nearest 10 ppm. The stated values do not reflect the necessary analytical precision of determination. Precision should be determined as part of the validation of the method.

Table 2. – *Class 2 solvents in pharmaceutical products*

Solvent	PDE (mg/day)	Concentration limit (ppm)
Acetonitrile	4.1	410
Chlorobenzene	3.6	360
Chloroform	0.6	60
Cumene	0.7	70
Cyclohexane	38.8	3880
1,2-Dichloroethene	18.7	1870
Dichloromethane	6.0	600
1,2-Dimethoxyethane	1.0	100
N,N-Dimethylacetamide	10.9	1090
N,N-Dimethylformamide	8.8	880
1,4-Dioxane	3.8	380
2-Ethoxyethanol	1.6	160
Ethyleneglycol	6.2	620
Formamide	2.2	220
Hexane	2.9	290
Methanol	30.0	3000
2-Methoxyethanol	0.5	50
Methylbutylketone	0.5	50
Methylcyclohexane	11.8	1180
Methylisobutylketone	45.0	4500
N-Methylpyrrolidone	5.3	530
Nitromethane	0.5	50
Pyridine	2.0	200
Sulfolane	1.6	160
Tetrahydrofuran	7.2	720
Tetralin	1.0	100
Toluene	8.9	890
1,1,2-Trichloroethene	0.8	80
Xylene[a]	21.7	2170

[a]usually 60 per cent *m*-xylene, 14 per cent *p*-xylene, 9 per cent *o*-xylene with 17 per cent ethyl benzene.

4.3 SOLVENTS WITH LOW TOXIC POTENTIAL

Solvents in Class 3 (shown in Table 3) may be regarded as less toxic and of lower risk to human health. Class 3 includes no solvent known as a human health hazard at levels normally accepted in pharmaceuticals. However, there are no long-term toxicity or carcinogenicity studies for many of the solvents in Class 3. Available data indicate that they are less toxic in acute or short-term studies and negative in genotoxicity studies. It is considered that amounts of these residual solvents of 50 mg per day or less (corresponding to 5000 ppm or 0.5 per cent under Option 1) would be acceptable without justification. Higher amounts may also be acceptable provided they are realistic in relation to manufacturing capability and good manufacturing practice.

Table 3. – *Class 3 solvents which should be limited by GMP or other quality-based requirements*

Acetic acid	Heptane
Acetone	Isobutyl acetate
Anisole	Isopropyl acetate
1-Butanol	Methyl acetate
2-Butanol	3-Methyl-1-butanol
Butyl acetate	Methylethylketone
tert-Butylmethyl ether	2-Methyl-1-propanol
Dimethyl sulfoxide	Pentane
Ethanol	1-Pentanol
Ethyl acetate	1-Propanol
Ethyl ether	2-Propanol
Ethyl formate	Propyl acetate
Formic acid	Triethylamine

4.4 SOLVENTS FOR WHICH NO ADEQUATE TOXICOLOGICAL DATA WAS FOUND

The following solvents (Table 4) may also be of interest to manufacturers of excipients, active substances, or medicinal products. However, no adequate toxicological data on which to base a PDE was found. Manufacturers should supply justification for residual levels of these solvents in pharmaceutical products.

Table 4. – *Solvents for which no adequate toxicological data was found*

1,1-Diethoxypropane	Methylisopropylketone
1,1-Dimethoxymethane	Methyltetrahydrofuran
2,2-Dimethoxypropane	Petroleum ether
Isooctane	Trichloroacetic acid
Isopropyl ether	Trifluoroacetic acid

GLOSSARY

Genotoxic carcinogens Carcinogens which produce cancer by affecting genes or chromosomes.

LOEL Abbreviation for *lowest-observed effect level.*

Lowest-observed effect level The lowest dose of substance in a study or group of studies that produces biologically significant increases in frequency or severity of any effects in the exposed humans or animals.

Modifying factor A factor determined by professional judgement of a toxicologist and applied to bioassay data to relate that data safely to humans.

Neurotoxicity The ability of a substance to cause adverse effects on the nervous system.

NOEL Abbreviation for *no-observed-effect level.*

No-observed-effect level The highest dose of substance at which there are no biologically significant increases in

frequency or severity of any effects in the exposed humans or animals.

PDE Abbreviation for *permitted daily exposure.*

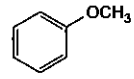
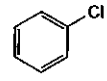
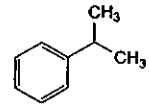
Permitted daily exposure The maximum acceptable intake per day of residual solvent in pharmaceutical products.

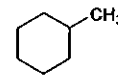
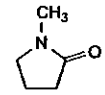
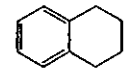
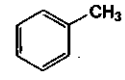
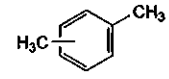
Reversible toxicity The occurrence of harmful effects that are caused by a substance and which disappear after exposure to the substance ends.

Strongly suspected human carcinogen A substance for which there is no epidemiological evidence of carcinogenesis but there are positive genotoxicity data and clear evidence of carcinogenesis in rodents.

Teratogenicity The occurrence of structural malformations in a developing foetus when a substance is administered during pregnancy.

APPENDIX 1. LIST OF SOLVENTS INCLUDED IN THE GUIDELINE

Solvent	Other Names	Structure	Class
Acetic acid	Ethanoic acid	CH_3COOH	Class 3
Acetone	2-Propanone Propan-2-one	CH_3COCH_3	Class 3
Acetonitrile		CH_3CN	Class 2
Anisole	Methoxybenzene		Class 3
Benzene	Benzol		Class 1
1-Butanol	*n*-Butyl alcohol Butan-1-ol	$CH_3[CH_2]_3OH$	Class 3
2-Butanol	*sec*-Butyl alcohol Butan-2-ol	$CH_3CH_2CH(OH)CH_3$	Class 3
Butyl acetate	Acetic acid butyl ester	$CH_3COO[CH_2]_3CH_3$	Class 3
tert-Butylmethyl ether	2-Methoxy-2-methylpropane	$(CH_3)_3COCH_3$	Class 3
Carbon tetrachloride	Tetrachloromethane	CCl_4	Class 1
Chlorobenzene			Class 2
Chloroform	Trichloromethane	$CHCl_3$	Class 2
Cumene	Isopropylbenzene (1-Methylethyl)benzene		Class 2
Cyclohexane	Hexamethylene		Class 2
1,2-Dichloroethane	*sym*-Dichloroethane Ethylene dichloride Ethylene chloride	CH_2ClCH_2Cl	Class 1
1,1-Dichloroethene	1,1-Dichloroethylene Vinylidene chloride	$H_2C=CCl_2$	Class 1
1,2-Dichloroethene	1,2-Dichloroethylene Acetylene dichloride	$ClHC=CHCl$	Class 2
Dichloromethane	Methylene chloride	CH_2Cl_2	Class 2
1,2-Dimethoxyethane	Ethyleneglycol dimethyl ether Monoglyme Dimethyl cellosolve	$H_3COCH_2CH_2OCH_3$	Class 2
N,*N*-Dimethylacetamide	DMA	$CH_3CON(CH_3)_2$	Class 2
N,*N*-Dimethylformamide	DMF	$HCON(CH_3)_2$	Class 2
Dimethyl sulfoxide	Methylsulfinylmethane Methyl sulfoxide DMSO	$(CH_3)_2SO$	Class 3
1,4-Dioxane	*p*-Dioxane [1,4]Dioxane		Class 2
Ethanol	Ethyl alcohol	CH_3CH_2OH	Class 3
2-Ethoxyethanol	Cellosolve	$CH_3CH_2OCH_2CH_2OH$	Class 2
Ethyl acetate	Acetic acid ethyl ester	$CH_3COOCH_2CH_3$	Class 3
Ethyleneglycol	1,2-Dihydroxyethane 1,2-Ethanediol	$HOCH_2CH_2OH$	Class 2
Ethyl ether	Diethyl ether Ethoxyethane 1,1'-Oxybisethane	$CH_3CH_2OCH_2CH_3$	Class 3
Ethyl formate	Formic acid ethyl ester	$HCOOCH_2CH_3$	Class 3
Formamide	Methanamide	$HCONH_2$	Class 2
Formic acid		$HCOOH$	Class 3
Heptane	*n*-Heptane	$CH_3[CH_2]_5CH_3$	Class 3
Hexane	*n*-Hexane	$CH_3[CH_2]_4CH_3$	Class 2
Isobutyl acetate	Acetic acid isobutyl ester	$CH_3COOCH_2CH(CH_3)_2$	Class 3
Isopropyl acetate	Acetic acid isopropyl ester	$CH_3COOCH(CH_3)_2$	Class 3
Methanol	Methyl alcohol	CH_3OH	Class 2
2-Methoxyethanol	Methyl cellosolve	$CH_3OCH_2CH_2OH$	Class 2
Methyl acetate	Acetic acid methyl ester	CH_3COOCH_3	Class 3

Solvent	Other Names	Structure	Class
3-Methyl-1-butanol	Isoamyl alcohol Isopentyl alcohol 3-Methylbutan-1-ol	$(CH_3)_2CHCH_2CH_2OH$	Class 3
Methylbutylketone	2-Hexanone Hexan-2-one	$CH_3[CH_2]_3COCH_3$	Class 2
Methylcyclohexane	Cyclohexylmethane		Class 2
Methylethylketone	2-Butanone MEK Butan-2-one	$CH_3CH_2COCH_3$	Class 3
Methylisobutylketone	4-Methylpentan-2-one 4-Methyl-2-pentanone MIBK	$CH_3COCH_2CH(CH_3)_2$	Class 2
2-Methyl-1-propanol	Isobutyl alcohol 2-Methylpropan-1-ol	$(CH_3)_2CHCH_2OH$	Class 3
N-Methylpyrrolidone	1-Methylpyrrolidin-2-one 1-Methyl-2-pyrrolidinone		Class 2
Nitromethane		CH_3NO_2	Class 2
Pentane	*n*-Pentane	$CH_3[CH_2]_3CH_3$	Class 3
1-Pentanol	Amyl alcohol Pentan-1-ol Pentyl alcohol	$CH_3[CH_2]_3CH_2OH$	Class 3
1-Propanol	Propan-1-ol Propyl alcohol	$CH_3CH_2CH_2OH$	Class 3
2-Propanol	Propan-2-ol Isopropyl alcohol	$(CH_3)_2CHOH$	Class 3
Propyl acetate	Acetic acid propyl ester	$CH_3COOCH_2CH_2CH_3$	Class 3
Pyridine			Class 2
Sulfonane	Tetrahydrothiophene 1,1-dioxide		Class 2
Tetrahydrofuran	Tetramethylene oxide Oxacyclopentane		Class 2
Tetralin	1,2,3,4-Tetrahydronaphthalene		Class 2
Toluene	Methylbenzene		Class 2
1,1,1-Trichloroethane	Methylchloroform	CH_3CCl_3	Class 1
1,1,2-Trichloroethene	Trichloroethene	$HClC{=}CCl_2$	Class 2
Triethylamine	*N*,*N*-Diethylethanamine	$N(CH_2CH_3)_3$	Class 3
Xylene*	Dimethybenzene Xylol		Class 2

*usually 60 per cent *m*-xylene, 14 per cent *p*-xylene, 9 per cent *o*-xylene with 17 per cent ethyl benzene.

APPENDIX 2. ADDITIONAL BACKGROUND

A2.1 ENVIRONMENTAL REGULATION OF ORGANIC VOLATILE SOLVENTS

Several of the residual solvents frequently used in the production of pharmaceuticals are listed as toxic chemicals in Environmental Health Criteria (EHC) monographs and the Integrated Risk Information System (IRIS). The objectives of such groups as the International Programme on Chemical Safety (IPCS), the United States Environmental Protection Agency (USEPA) and the United States Food and Drug Administration (USFDA) include the determination of acceptable exposure levels. The goal is protection of human health and maintenance of environmental integrity against the possible deleterious effects of chemicals resulting from long-term environmental exposure. The methods involved in the estimation of maximum safe exposure limits are usually based on long-term studies. When long-term study data are unavailable, shorter term study data can be used with modification of the approach such as use of larger safety factors. The approach described therein relates primarily to long-term or life-time exposure of the general population in the ambient environment, i.e. ambient air, food, drinking water and other media.

A2.2 RESIDUAL SOLVENTS IN PHARMACEUTICALS

Exposure limits in this guideline are established by referring to methodologies and toxicity data described in EHC and IRIS monographs. However, some specific assumptions about residual solvents to be used in the synthesis and

formulation of pharmaceutical products should be taken into account in establishing exposure limits. They are:

1) Patients (not the general population) use pharmaceuticals to treat their diseases or for prophylaxis to prevent infection or disease.

2) The assumption of life-time patient exposure is not necessary for most pharmaceutical products but may be appropriate as a working hypothesis to reduce risk to human health.

3) Residual solvents are unavoidable components in pharmaceutical production and will often be a part of medicinal products.

4) Residual solvents should not exceed recommended levels except in exceptional circumstances.

5) Data from toxicological studies that are used to determine acceptable levels for residual solvents should have been generated using appropriate protocols such as those described for example, by OECD, EPA, and the FDA Red Book.

APPENDIX 3. METHODS FOR ESTABLISHING EXPOSURE LIMITS

The Gaylor-Kodell method of risk assessment (Gaylor, D. W. and Kodell, R. L. Linear Interpolation algorithm for low dose assessment of toxic substance. J. Environ. Pathology, 4, 305, 1980) is appropriate for Class 1 carcinogenic solvents. Only in cases where reliable carcinogenicity data are available should extrapolation by the use of mathematical models be applied to setting exposure limits. Exposure limits for Class 1 solvents could be determined with the use of a large safety factor (i.e., 10 000 to 100 000) with respect to the no-observed-effect level (NOEL). Detection and quantification of these solvents should be by state-of-the-art analytical techniques.

Acceptable exposure levels in this guideline for Class 2 solvents were established by calculation of PDE values according to the procedures for setting exposure limits in pharmaceuticals (*Pharmacopeial Forum*, Nov-Dec 1989), and the method adopted by IPCS for Assessing Human Health Risk of Chemicals (*Environmental Health Criteria 170*, WHO, 1994). These methods are similar to those used by the USEPA (IRIS) and the USFDA (*Red Book*) and others. The method is outlined here to give a better understanding of the origin of the PDE values. It is not necessary to perform these calculations in order to use the PDE values tabulated in Section 4 of this document.

PDE is derived from the no-observed-effect level (NOEL), or the lowest-observed effect level (LOEL), in the most relevant animal study as follows:

$$\text{PDE} = \frac{\text{NOEL} \times \text{Weight Adjustment}}{\text{F1} \times \text{F2} \times \text{F3} \times \text{F4} \times \text{F5}}$$

The PDE is derived preferably from a NOEL. If no NOEL is obtained, the LOEL may be used. Modifying factors proposed here, for relating the data to humans, are the same kind of "uncertainty factors" used in Environmental Health Criteria (*Environmental Health Criteria* 170, World Health Organization, Geneva, 1994), and "modifying factors" or "safety factors" in *Pharmacopoeial Forum*. The assumption of 100 per cent systemic exposure is used in all calculations regardless of route of administration.

The modifying factors are as follows:

F1 = a factor to account for extrapolation between species:

- F1 = 2 for extrapolation from dogs to humans;
- F1 = 2.5 for extrapolation from rabbits to humans;
- F1 = 3 for extrapolation from monkeys to humans;
- F1 = 5 for extrapolation from rats to humans;
- F1 = 10 for extrapolation from other animals to humans;
- F1 = 12 for extrapolation from mice to humans.

F1 takes into account the comparative surface area: body weight ratios for the species concerned and for man. Surface area (S) is calculated as:

$$S = km^{0.67}$$

in which m = body mass, and the constant k has been taken to be 10. The body weight used in the equation are those shown below in Table A3.-1.

Table A3.-1. – *Values used in the calculations in this document*

Rat body weight	425 g
Pregnant rat body weight	330 g
Mouse body weight	28 g
Pregnant mouse body weight	30 g
Guinea-pig body weight	500 g
Rhesus monkey body weight	2.5 kg
Rabbit body weight (pregnant or not)	4 kg
Beagle dog body weight	11.5 kg
Rat respiratory volume	290 L/day
Mouse respiratory volume	43 L/day
Rabbit respiratory volume	1440 L/day
Guinea-pig respiratory volume	430 L/day
Human respiratory volume	28800 L/day
Dog respiratory volume	9000 L/day
Monkey respiratory volume	1150 L/day
Mouse water consumption	5 mL/day
Rat water consumption	30 mL/day
Rat food consumption	30 g/day

F2 = a factor of 10 to account for variability between individuals. A factor of 10 is generally given for all organic solvents, and 10 is used consistently in this guideline.

F3 = a variable factor to account for toxicity studies of short-term exposure:

- F3 = 1 for studies that last at least one half-lifetime (1 year for rodents or rabbits; 7 years for cats, dogs and monkeys);
- F3 = 1 for reproductive studies in which the whole period of organogenesis is covered;
- F3 = 2 for a 6 month study in rodents, or a 3.5 year study in non-rodents;
- F3 = 5 for a 3 month study in rodents, or a 2 year study in non-rodents;
- F3 = 10 for studies of a shorter duration.

In all cases, the higher factor has been used for study durations between the time points, e.g. a factor of 2 for a 9 month rodent study.

F4 = a factor that may be applied in cases of severe toxicity, e.g. non-genotoxic carcinogenicity, neurotoxicity or teratogenicity. In studies of reproductive toxicity, the following factors are used:

- F4 = 1 for foetal toxicity associated with maternal toxicity;
- F4 = 5 for foetal toxicity without maternal toxicity;
- F4 = 5 for a teratogenic effect with maternal toxicity;
- F4 = 10 for a teratogenic effect without maternal toxicity.

F5 = a variable factor that may be applied if the no-effect level was not established.

When only a LOEL is available, a factor of up to 10 can be used depending on the severity of the toxicity.

The weight adjustment assumes an arbitrary adult human body weight for either sex of 50 kg. This relatively low weight provides an additional safety factor against the standard weights of 60 kg or 70 kg that are often used in this type of calculation. It is recognised that some adult patients weigh less than 50 kg; these patients are considered to be accommodated by the built-in safety factors used to determine a PDE. If the solvent was present in a formulation specifically intended for paediatric use, an adjustment for a lower body weight would be appropriate.

As an example of the application of this equation, consider the toxicity study of acetonitrile in mice that is summarised in *Pharmeuropa*, Vol. 9. No. 1, Supplement, April 1997, page S24. The NOEL is calculated to be 50.7 mg kg^{-1} day^{-1}. The PDE for acetonitrile in this study is calculated as follows:

$$\text{PDE} = \frac{50.7\text{mg kg}^{-1}\ \text{day}^{-1} \times 50\ \text{kg}}{12 \times 10 \times 5 \times 1 \times 1} = 4.22\ \text{mg day}^{-1}$$

In this example,

F1	=	12 to account for the extrapolation from mice to humans;
F2	=	10 to account for differences between individual humans;
F3	=	5 because the duration of the study was only 13 weeks;
F4	=	1 because no severe toxicity was encountered;
F5	=	1 because the no-effect level was determined.

The equation for an ideal gas, $PV = nRT$, is used to convert concentrations of gases used in inhalation studies from units of ppm to units of mg/L or mg/m^3. Consider as an example the rat reproductive toxicity study by inhalation of carbon tetrachloride (molecular weight 153.84) summarised in *Pharmeuropa*, Vol. 9, No. 1, Supplement, April 1997, page S9.

$$\frac{n}{V} = \frac{P}{RT} = \frac{300 \times 10^{-6}\ \text{atm} \times 153\ 840\ \text{mg mol}^{-1}}{0.082\ \text{L atm K}^{-1}\text{mol}^{-1} \times 298\ \text{K}} = \frac{46.15\ \text{mg}}{24.45\ \text{L}}$$

$$= 1.89\ \text{mg/L}$$

The relationship 1000 L = 1 m^3 is used to convert to mg/m^3.

E. Alcoholimetric Tables

(Ph. Eur. general texts 5.5)

The general formula agreed by the Council of the European Communities in its Directive of 27 July 1976 on alcoholimetry served as the basis for establishing the following tables.

% *V/V*	% *m/m*	ρ_{20} (kg/m^3)
0.0	0.0	998.20
0.1	0.08	998.05
0.2	0.16	997.90
0.3	0.24	997.75
0.4	0.32	997.59
0.5	0.40	997.44
0.6	0.47	997.29
0.7	0.55	997.14
0.8	0.63	996.99
0.9	0.71	996.85
1.0	0.79	996.70
1.1	0.87	996.55
1.2	0.95	996.40
1.3	1.03	996.25
1.4	1.11	996.11
1.5	1.19	995.96
1.6	1.27	995.81
1.7	1.35	995.67
1.8	1.43	995.52
1.9	1.51	995.38
2.0	1.59	995.23
2.1	1.67	995.09
2.2	1.75	994.94
2.3	1.82	994.80
2.4	1.90	994.66
2.5	1.98	994.51
2.6	2.06	994.37
2.7	2.14	994.23
2.8	2.22	994.09
2.9	2.30	993.95
3.0	2.38	993.81
3.1	2.46	993.66
3.2	2.54	993.52
3.3	2.62	993.38
3.4	2.70	993.24
3.5	2.78	993.11
3.6	2.86	992.97
3.7	2.94	992.83
3.8	3.02	992.69
3.9	3.10	992.55
4.0	3.18	992.41
4.1	3.26	992.28
4.2	3.34	992.14
4.3	3.42	992.00
4.4	3.50	991.87
4.5	3.58	991.73
4.6	3.66	991.59
4.7	3.74	991.46
4.8	3.82	991.32
4.9	3.90	991.19

% *V/V*	% *m/m*	ρ_{20} (kg/m^3)
5.0	3.98	991.06
5.1	4.06	990.92
5.2	4.14	990.79
5.3	4.22	990.65
5.4	4.30	990.52
5.5	4.38	990.39
5.6	4.46	990.26
5.7	4.54	990.12
5.8	4.62	989.99
5.9	4.70	989.86
6.0	4.78	989.73
6.1	4.86	989.60
6.2	4.95	989.47
6.3	5.03	989.34
6.4	5.11	989.21
6.5	5.19	989.08
6.6	5.27	988.95
6.7	5.35	988.82
6.8	5.43	988.69
6.9	5.51	988.56
7.0	5.59	988.43
7.1	5.67	988.30
7.2	5.75	988.18
7.3	5.83	988.05
7.4	5.91	987.92
7.5	5.99	987.79
7.6	6.07	987.67
7.7	6.15	987.54
7.8	6.23	987.42
7.9	6.32	987.29
8.0	6.40	987.16
8.1	6.48	987.04
8.2	6.56	986.91
8.3	6.64	986.79
8.4	6.72	986.66
8.5	6.80	986.54
8.6	6.88	986.42
8.7	6.96	986.29
8.8	7.04	986.17
8.9	7.12	986.05
9.0	7.20	985.92
9.1	7.29	985.80
9.2	7.37	985.68
9.3	7.45	985.56
9.4	7.53	985.44
9.5	7.61	985.31
9.6	7.69	985.19
9.7	7.77	985.07
9.8	7.85	984.95
9.9	7.93	984.83
10.0	8.01	984.71
10.1	8.10	984.59
10.2	8.18	984.47
10.3	8.26	984.35
10.4	8.34	984.23
10.5	8.42	984.11
10.6	8.50	983.99
10.7	8.58	983.88
10.8	8.66	983.76
10.9	8.75	983.64

% *V/V*	% *m/m*	ρ_{20} (kg/m^3)
11.0	8.83	983.52
11.1	8.91	983.40
11.2	8.99	983.29
11.3	9.07	983.17
11.4	9.15	983.05
11.5	9.23	982.94
11.6	9.32	982.82
11.7	9.40	982.70
11.8	9.48	982.59
11.9	9.56	982.47
12.0	9.64	982.35
12.1	9.72	982.24
12.2	9.80	982.12
12.3	9.89	982.01
12.4	9.97	981.89
12.5	10.05	981.78
12.6	10.13	981.67
12.7	10.21	981.55
12.8	10.29	981.44
12.9	10.37	981.32
13.0	10.46	981.21
13.1	10.54	981.10
13.2	10.62	980.98
13.3	10.70	980.87
13.4	10.78	980.76
13.5	10.87	980.64
13.6	10.95	980.53
13.7	11.03	980.42
13.8	11.11	980.31
13.9	11.19	980.19
14.0	11.27	980.08
14.1	11.36	979.97
14.2	11.44	979.86
14.3	11.52	979.75
14.4	11.60	979.64
14.5	11.68	979.52
14.6	11.77	979.41
14.7	11.85	979.30
14.8	11.93	979.19
14.9	12.01	979.08
15.0	12.09	978.97
15.1	12.17	978.86
15.2	12.26	978.75
15.3	12.34	978.64
15.4	12.42	978.53
15.5	12.50	978.42
15.6	12.59	978.31
15.7	12.67	978.20
15.8	12.75	978.09
15.9	12.83	977.98
16.0	12.91	977.87
16.1	13.00	977.76
16.2	13.08	977.65
16.3	13.16	977.55
16.4	13.24	977.44
16.5	13.32	977.33
16.6	13.41	977.22
16.7	13.49	977.11
16.8	13.57	977.00
16.9	13.65	976.89

% *V/V*	% *m/m*	ρ_{20} (kg/m^3)
17.0	13.74	976.79
17.1	13.82	976.68
17.2	13.90	976.57
17.3	13.98	976.46
17.4	14.07	976.35
17.5	14.15	976.25
17.6	14.23	976.14
17.7	14.31	976.03
17.8	14.40	975.92
17.9	14.48	975.81
18.0	14.56	975.71
18.1	14.64	975.60
18.2	14.73	975.49
18.3	14.81	975.38
18.4	14.89	975.28
18.5	14.97	975.17
18.6	15.06	975.06
18.7	15.14	974.95
18.8	15.22	974.85
18.9	15.30	974.74
19.0	15.39	974.63
19.1	15.47	974.52
19.2	15.55	974.42
19.3	15.63	974.31
19.4	15.72	974.20
19.5	15.80	974.09
19.6	15.88	973.99
19.7	15.97	973.88
19.8	16.05	973.77
19.9	16.13	973.66
20.0	16.21	973.56
20.1	16.30	973.45
20.2	16.38	973.34
20.3	16.46	973.24
20.4	16.55	973.13
20.5	16.63	973.02
20.6	16.71	972.91
20.7	16.79	972.80
20.8	16.88	972.70
20.9	16.96	972.59
21.0	17.04	972.48
21.1	17.13	972.37
21.2	17.21	972.27
21.3	17.29	972.16
21.4	17.38	972.05
21.5	17.46	971.94
21.6	17.54	971.83
21.7	17.62	971.73
21.8	17.71	971.62
21.9	17.79	971.51
22.0	17.87	971.40
22.1	17.96	971.29
22.2	18.04	971.18
22.3	18.12	971.08
22.4	18.21	970.97
22.5	18.29	970.86
22.6	18.37	970.75
22.7	18.46	970.64
22.8	18.54	970.53
22.9	18.62	970.42

% *V/V*	% *m/m*	ρ_{20} (kg/m^3)
23.0	18.71	970.31
23.1	18.79	970.20
23.2	18.87	970.09
23.3	18.96	969.98
23.4	19.04	969.87
23.5	19.13	969.76
23.6	19.21	969.65
23.7	19.29	969.54
23.8	19.38	969.43
23.9	19.46	969.32
24.0	19.54	969.21
24.1	19.63	969.10
24.2	19.71	968.99
24.3	19.79	968.88
24.4	19.88	968.77
24.5	19.96	968.66
24.6	20.05	968.55
24.7	20.13	968.43
24.8	20.21	968.32
24.9	20.30	968.21
25.0	20.38	968.10
25.1	20.47	967.99
25.2	20.55	967.87
25.3	20.63	967.76
25.4	20.72	967.65
25.5	20.80	967.53
25.6	20.88	967.42
25.7	20.97	967.31
25.8	21.05	967.19
25.9	21.14	967.08
26.0	21.22	966.97
26.1	21.31	966.85
26.2	21.39	966.74
26.3	21.47	966.62
26.4	21.56	966.51
26.5	21.64	966.39
26.6	21.73	966.28
26.7	21.81	966.16
26.8	21.90	966.05
26.9	21.98	965.93
27.0	22.06	965.81
27.1	22.15	965.70
27.2	22.23	965.58
27.3	22.32	965.46
27.4	22.40	965.35
27.5	22.49	965.23
27.6	22.57	965.11
27.7	22.65	964.99
27.8	22.74	964.88
27.9	22.82	964.76
28.0	22.91	964.64
28.1	22.99	964.52
28.2	23.08	964.40
28.3	23.16	964.28
28.4	23.25	964.16
28.5	23.33	964.04
28.6	23.42	963.92
28.7	23.50	963.80
28.8	23.59	963.68
28.9	23.67	963.56

% *V/V*	% *m/m*	ρ_{20} (kg/m^3)
29.0	23.76	963.44
29.1	23.84	963.32
29.2	23.93	963.20
29.3	24.01	963.07
29.4	24.10	962.95
29.5	24.18	962.83
29.6	24.27	962.71
29.7	24.35	962.58
29.8	24.44	962.46
29.9	24.52	962.33
30.0	24.61	962.21
30.1	24.69	962.09
30.2	24.78	961.96
30.3	24.86	961.84
30.4	24.95	961.71
30.5	25.03	961.59
30.6	25.12	961.46
30.7	25.20	961.33
30.8	25.29	961.21
30.9	25.38	961.08
31.0	25.46	960.95
31.1	25.55	960.82
31.2	25.63	960.70
31.3	25.72	960.57
31.4	25.80	960.44
31.5	25.89	960.31
31.6	25.97	960.18
31.7	26.06	960.05
31.8	26.15	959.92
31.9	26.23	959.79
32.0	26.32	959.66
32.1	26.40	959.53
32.2	26.49	959.40
32.3	26.57	959.27
32.4	26.66	959.14
32.5	26.75	959.01
32.6	26.83	958.87
32.7	26.92	958.74
32.8	27.00	958.61
32.9	27.09	958.47
33.0	27.18	958.34
33.1	27.26	958.20
33.2	27.35	958.07
33.3	27.44	957.94
33.4	27.52	957.80
33.5	27.61	957.66
33.6	27.69	957.53
33.7	27.78	957.39
33.8	27.87	957.26
33.9	27.95	957.12
34.0	28.04	956.98
34.1	28.13	956.84
34.2	28.21	956.70
34.3	28.30	956.57
34.4	28.39	956.43
34.5	28.47	956.29
34.6	28.56	956.15
34.7	28.65	956.01
34.8	28.73	955.87
34.9	28.82	955.73

% *V/V*	% *m/m*	ρ_{20} (kg/m^3)
35.0	28.91	955.59
35.1	28.99	955.45
35.2	29.08	955.30
35.3	29.17	955.16
35.4	29.26	955.02
35.5	29.34	954.88
35.6	29.43	954.73
35.7	29.52	954.59
35.8	29.60	954.44
35.9	29.69	954.30
36.0	29.78	954.15
36.1	29.87	954.01
36.2	29.95	953.86
36.3	30.04	953.72
36.4	30.13	953.57
36.5	30.21	953.42
36.6	30.30	953.28
36.7	30.39	953.13
36.8	30.48	952.98
36.9	30.56	952.83
37.0	30.65	952.69
37.1	30.74	952.54
37.2	30.83	952.39
37.3	30.92	952.24
37.4	31.00	952.09
37.5	31.09	951.94
37.6	31.18	951.79
37.7	31.27	951.63
37.8	31.35	951.48
37.9	31.44	951.33
38.0	31.53	951.18
38.1	31.62	951.02
38.2	31.71	950.87
38.3	31.79	950.72
38.4	31.88	950.56
38.5	31.97	950.41
38.6	32.06	950.25
38.7	32.15	950.10
38.8	32.24	949.94
38.9	32.32	949.79
39.0	32.41	949.63
39.1	32.50	949.47
39.2	32.59	949.32
39.3	32.68	949.16
39.4	32.77	949.00
39.5	32.86	948.84
39.6	32.94	948.68
39.7	33.03	948.52
39.8	33.12	948.37
39.9	33.21	948.21
40.0	33.30	948.05
40.1	33.39	947.88
40.2	33.48	947.72
40.3	33.57	947.56
40.4	33.66	947.40
40.5	33.74	947.24
40.6	33.83	947.08
40.7	33.92	946.91
40.8	34.01	946.75
40.9	34.10	946.58

% *V/V*	% *m/m*	ρ_{20} (kg/m^3)
41.0	34.19	946.42
41.1	34.28	946.26
41.2	34.37	946.09
41.3	34.46	945.93
41.4	34.55	945.76
41.5	34.64	945.59
41.6	34.73	945.43
41.7	34.82	945.26
41.8	34.91	945.09
41.9	35.00	944.93
42.0	35.09	944.76
42.1	35.18	944.59
42.2	35.27	944.42
42.3	35.36	944.25
42.4	35.45	944.08
42.5	35.54	943.91
42.6	35.63	943.74
42.7	35.72	943.57
42.8	35.81	943.40
42.9	35.90	943.23
43.0	35.99	943.06
43.1	36.08	942.88
43.2	36.17	942.71
43.3	36.26	942.54
43.4	36.35	942.37
43.5	36.44	942.19
43.6	36.53	942.02
43.7	36.62	941.84
43.8	36.71	941.67
43.9	36.80	941.49
44.0	36.89	941.32
44.1	36.98	941.14
44.2	37.07	940.97
44.3	37.16	940.79
44.4	37.25	940.61
44.5	37.35	940.43
44.6	37.44	940.26
44.7	37.53	940.08
44.8	37.62	939.90
44.9	37.71	939.72
45.0	37.80	939.54
45.1	37.89	939.36
45.2	37.98	939.18
45.3	38.08	939.00
45.4	38.17	938.82
45.5	38.26	938.64
45.6	38.35	938.46
45.7	38.44	938.28
45.8	38.53	938.10
45.9	38.62	937.91
46.0	38.72	937.73
46.1	38.81	937.55
46.2	38.90	937.36
46.3	38.99	937.18
46.4	39.08	937.00
46.5	39.18	936.81
46.6	39.27	936.63
46.7	39.36	936.44
46.8	39.45	936.26
46.9	39.54	936.07

% *V/V*	% *m/m*	ρ_{20} (kg/m^3)
47.0	39.64	935.88
47.1	39.73	935.70
47.2	39.82	935.51
47.3	39.91	935.32
47.4	40.00	935.14
47.5	40.10	934.95
47.6	40.19	934.76
47.7	40.28	934.57
47.8	40.37	934.38
47.9	40.47	934.19
48.0	40.56	934.00
48.1	40.65	933.81
48.2	40.75	933.62
48.3	40.84	933.43
48.4	40.93	933.24
48.5	41.02	933.05
48.6	41.12	932.86
48.7	41.21	932.67
48.8	41.30	932.47
48.9	41.40	932.28
49.0	41.49	932.09
49.1	41.58	931.90
49.2	41.68	931.70
49.3	41.77	931.51
49.4	41.86	931.31
49.5	41.96	931.12
49.6	42.05	930.92
49.7	42.14	930.73
49.8	42.24	930.53
49.9	42.33	930.34
50.0	42.43	930.14
50.1	42.52	929.95
50.2	42.61	929.75
50.3	42.71	929.55
50.4	42.80	929.35
50.5	42.90	929.16
50.6	42.99	928.96
50.7	43.08	928.76
50.8	43.18	928.56
50.9	43.27	928.36
51.0	43.37	928.16
51.1	43.46	927.96
51.2	43.56	927.77
51.3	43.65	927.57
51.4	43.74	927.36
51.5	43.84	927.16
51.6	43.93	926.96
51.7	44.03	926.76
51.8	44.12	926.56
51.9	44.22	926.36
52.0	44.31	926.16
52.1	44.41	925.95
52.2	44.50	925.75
52.3	44.60	925.55
52.4	44.69	925.35
52.5	44.79	925.14
52.6	44.88	924.94
52.7	44.98	924.73
52.8	45.07	924.53
52.9	45.17	924.32

% *V/V*	% *m/m*	ρ_{20} (kg/m^3)
53.0	45.26	924.12
53.1	45.36	923.91
53.2	45.46	923.71
53.3	45.55	923.50
53.4	45.65	923.30
53.5	45.74	923.09
53.6	45.84	922.88
53.7	45.93	922.68
53.8	46.03	922.47
53.9	46.13	922.26
54.0	46.22	922.06
54.1	46.32	921.85
54.2	46.41	921.64
54.3	46.51	921.43
54.4	46.61	921.22
54.5	46.70	921.01
54.6	46.80	920.80
54.7	46.90	920.59
54.8	46.99	920.38
54.9	47.09	920.17
55.0	47.18	919.96
55.1	47.28	919.75
55.2	47.38	919.54
55.3	47.47	919.33
55.4	47.57	919.12
55.5	47.67	918.91
55.6	47.77	918.69
55.7	47.86	918.48
55.8	47.96	918.27
55.9	48.06	918.06
56.0	48.15	917.84
56.1	48.25	917.63
56.2	48.35	917.42
56.3	48.45	917.20
56.4	48.54	916.99
56.5	48.64	916.77
56.6	48.74	916.56
56.7	48.84	916.35
56.8	48.93	916.13
56.9	49.03	915.91
57.0	49.13	915.70
57.1	49.23	915.48
57.2	49.32	915.27
57.3	49.42	915.05
57.4	49.52	914.83
57.5	49.62	914.62
57.6	49.72	914.40
57.7	49.81	914.18
57.8	49.91	913.97
57.9	50.01	913.75
58.0	50.11	913.53
58.1	50.21	913.31
58.2	50.31	913.09
58.3	50.40	912.87
58.4	50.50	912.65
58.5	50.60	912.43
58.6	50.70	912.22
58.7	50.80	912.00
58.8	50.90	911.78
58.9	51.00	911.55

% *V/V*	% *m/m*	ρ_{20} (kg/m^3)
59.0	51.10	911.33
59.1	51.19	911.11
59.2	51.29	910.89
59.3	51.39	910.67
59.4	51.49	910.45
59.5	51.59	910.23
59.6	51.69	910.01
59.7	51.79	909.78
59.8	51.89	909.56
59.9	51.99	909.34
60.0	52.09	909.11
60.1	52.19	908.89
60.2	52.29	908.67
60.3	52.39	908.44
60.4	52.49	908.22
60.5	52.59	908.00
60.6	52.69	907.77
60.7	52.79	907.55
60.8	52.89	907.32
60.9	52.99	907.10
61.0	53.09	906.87
61.1	53.19	906.64
61.2	53.29	906.42
61.3	53.39	906.19
61.4	53.49	905.97
61.5	53.59	905.74
61.6	53.69	905.51
61.7	53.79	905.29
61.8	53.89	905.06
61.9	53.99	904.83
62.0	54.09	904.60
62.1	54.19	904.37
62.2	54.30	904.15
62.3	54.40	903.92
62.4	54.50	903.69
62.5	54.60	903.46
62.6	54.70	903.23
62.7	54.80	903.00
62.8	54.90	902.77
62.9	55.00	902.54
63.0	55.11	902.31
63.1	55.21	902.08
63.2	55.31	901.85
63.3	55.41	901.62
63.4	55.51	901.39
63.5	55.61	901.15
63.6	55.72	900.92
63.7	55.82	900.69
63.8	55.92	900.46
63.9	56.02	900.23
64.0	56.12	899.99
64.1	56.23	899.76
64.2	56.33	899.53
64.3	56.43	899.29
64.4	56.53	899.06
64.5	56.64	898.83
64.6	56.74	898.59
64.7	56.84	898.36
64.8	56.94	898.12
64.9	57.05	897.89

% *V/V*	% *m/m*	ρ_{20} (kg/m^3)	% *V/V*	% *m/m*	ρ_{20} (kg/m^3)
65.0	57.15	897.65	71.0	63.46	883.06
65.1	57.25	897.42	71.1	63.56	882.81
65.2	57.36	897.18	71.2	63.67	882.56
65.3	57.46	896.94	71.3	63.78	882.31
65.4	57.56	896.71	71.4	63.89	882.06
65.5	57.67	896.47	71.5	63.99	881.81
65.6	57.77	896.23	71.6	64.10	881.55
65.7	57.87	896.00	71.7	64.21	881.30
65.8	57.98	895.76	71.8	64.32	881.05
65.9	58.08	895.52	71.9	64.43	880.79
66.0	58.18	895.28	72.0	64.53	880.54
66.1	58.29	895.05	72.1	64.64	880.29
66.2	58.39	894.81	72.2	64.75	880.03
66.3	58.49	894.57	72.3	64.86	879.78
66.4	58.60	894.33	72.4	64.97	879.52
66.5	58.70	894.09	72.5	65.08	879.27
66.6	58.81	893.85	72.6	65.19	879.01
66.7	58.91	893.61	72.7	65.29	878.75
66.8	59.01	893.37	72.8	65.40	878.50
66.9	59.12	893.13	72.9	65.51	878.24
67.0	59.22	892.89	73.0	65.62	877.99
67.1	59.33	892.65	73.1	65.73	877.73
67.2	59.43	892.41	73.2	65.84	877.47
67.3	59.54	892.17	73.3	65.95	877.21
67.4	59.64	891.93	73.4	66.06	876.96
67.5	59.74	891.69	73.5	66.17	876.70
67.6	59.85	891.45	73.6	66.28	876.44
67.7	59.95	891.20	73.7	66.39	876.18
67.8	60.06	890.96	73.8	66.50	875.92
67.9	60.16	890.72	73.9	66.61	875.66
68.0	60.27	890.48	74.0	66.72	875.40
68.1	60.37	890.23	74.1	66.83	875.14
68.2	60.48	889.99	74.2	66.94	874.88
68.3	60.58	889.75	74.3	67.05	874.62
68.4	60.69	889.50	74.4	67.16	874.36
68.5	60.80	889.26	74.5	67.27	874.10
68.6	60.90	889.01	74.6	67.38	873.84
68.7	61.01	888.77	74.7	67.49	873.58
68.8	61.11	888.52	74.8	67.60	873.32
68.9	61.22	888.28	74.9	67.71	873.06
69.0	61.32	888.03	75.0	67.82	872.79
69.1	61.43	887.79	75.1	67.93	872.53
69.2	61.54	887.54	75.2	68.04	872.27
69.3	61.64	887.29	75.3	68.15	872.00
69.4	61.75	887.05	75.4	68.26	871.74
69.5	61.85	886.80	75.5	68.38	871.48
69.6	61.96	886.55	75.6	68.49	871.21
69.7	62.07	886.31	75.7	68.60	870.95
69.8	62.17	886.06	75.8	68.71	870.68
69.9	62.28	885.81	75.9	68.82	870.42
70.0	62.39	885.56	76.0	68.93	870.15
70.1	62.49	885.31	76.1	69.04	869.89
70.2	62.60	885.06	76.2	69.16	869.62
70.3	62.71	884.82	76.3	69.27	869.35
70.4	62.81	884.57	76.4	69.38	869.09
70.5	62.92	884.32	76.5	69.49	868.82
70.6	63.03	884.07	76.6	69.61	868.55
70.7	63.13	883.82	76.7	69.72	868.28
70.8	63.24	883.57	76.8	69.83	868.02
70.9	63.35	883.32	76.9	69.94	867.75

% *V/V*	% *m/m*	ρ_{20} (kg/m^3)
77.0	70.06	867.48
77.1	70.17	867.21
77.2	70.28	866.94
77.3	70.39	866.67
77.4	70.51	866.40
77.5	70.62	866.13
77.6	70.73	865.86
77.7	70.85	865.59
77.8	70.96	865.32
77.9	71.07	865.05
78.0	71.19	864.78
78.1	71.30	864.50
78.2	71.41	864.23
78.3	71.53	863.96
78.4	71.64	863.69
78.5	71.76	863.41
78.6	71.87	863.14
78.7	71.98	862.86
78.8	72.10	862.59
78.9	72.21	862.31
79.0	72.33	862.04
79.1	72.44	861.76
79.2	72.56	861.49
79.3	72.67	861.21
79.4	72.79	860.94
79.5	72.90	860.66
79.6	73.02	860.38
79.7	73.13	860.10
79.8	73.25	859.83
79.9	73.36	859.55
80.0	73.48	859.27
80.1	73.60	858.99
80.2	73.71	858.71
80.3	73.83	858.43
80.4	73.94	858.15
80.5	74.06	857.87
80.6	74.18	857.59
80.7	74.29	857.31
80.8	74.41	857.03
80.9	74.53	856.75
81.0	74.64	856.46
81.1	74.76	856.18
81.2	74.88	855.90
81.3	74.99	855.62
81.4	75.11	855.33
81.5	75.23	855.05
81.6	75.34	854.76
81.7	75.46	854.48
81.8	75.58	854.19
81.9	75.70	853.91
82.0	75.82	853.62
82.1	75.93	853.34
82.2	76.05	853.05
82.3	76.17	852.76
82.4	76.29	852.48
82.5	76.41	852.19
82.6	76.52	851.90
82.7	76.64	851.61
82.8	76.76	851.32
82.9	76.88	851.03

% *V/V*	% *m/m*	ρ_{20} (kg/m^3)
83.0	77.00	850.74
83.1	77.12	850.45
83.2	77.24	850.16
83.3	77.36	849.87
83.4	77.48	849.58
83.5	77.60	849.29
83.6	77.72	848.99
83.7	77.84	848.70
83.8	77.96	848.41
83.9	78.08	848.11
84.0	78.20	847.82
84.1	78.32	847.53
84.2	78.44	847.23
84.3	78.56	846.93
84.4	78.68	846.64
84.5	78.80	846.34
84.6	78.92	846.05
84.7	79.04	845.75
84.8	79.16	845.45
84.9	79.28	845.15
85.0	79.40	844.85
85.1	79.53	844.55
85.2	79.65	844.25
85.3	79.77	843.95
85.4	79.89	843.65
85.5	80.01	843.35
85.6	80.14	843.05
85.7	80.26	842.75
85.8	80.38	842.44
85.9	80.50	842.14
86.0	80.63	841.84
86.1	80.75	841.53
86.2	80.87	841.23
86.3	81.00	840.92
86.4	81.12	840.62
86.5	81.24	840.31
86.6	81.37	840.00
86.7	81.49	839.70
86.8	81.61	839.39
86.9	81.74	839.08
87.0	81.86	838.77
87.1	81.99	838.46
87.2	82.11	838.15
87.3	82.24	837.84
87.4	82.36	837.52
87.5	82.49	837.21
87.6	82.61	836.90
87.7	82.74	836.59
87.8	82.86	836.27
87.9	82.99	835.96
88.0	83.11	835.64
88.1	83.24	835.32
88.2	83.37	835.01
88.3	83.49	834.69
88.4	83.62	834.37
88.5	83.74	834.05
88.6	83.87	833.73
88.7	84.00	833.41
88.8	84.13	833.09
88.9	84.25	832.77

% *V/V*	% *m/m*	ρ_{20} (kg/m^3)
89.0	84.38	832.45
89.1	84.51	832.12
89.2	84.64	831.80
89.3	84.76	831.48
89.4	84.89	831.15
89.5	85.02	830.82
89.6	85.15	830.50
89.7	85.28	830.17
89.8	85.41	829.84
89.9	85.54	829.51
90.0	85.66	829.18
90.1	85.79	828.85
90.2	85.92	828.52
90.3	86.05	828.19
90.4	86.18	827.85
90.5	86.31	827.52
90.6	86.44	827.18
90.7	86.57	826.85
90.8	86.71	826.51
90.9	86.84	826.17
91.0	86.97	825.83
91.1	87.10	825.49
91.2	87.23	825.15
91.3	87.36	824.81
91.4	87.49	824.47
91.5	87.63	824.13
91.6	87.76	823.78
91.7	87.89	823.44
91.8	88.02	823.09
91.9	88.16	822.74
92.0	88.29	822.39
92.1	88.42	822.04
92.2	88.56	821.69
92.3	88.69	821.34
92.4	88.83	820.99
92.5	88.96	820.63
92.6	89.10	820.28
92.7	89.23	819.92
92.8	89.37	819.57
92.9	89.50	819.21
93.0	89.64	818.85
93.1	89.77	818.49
93.2	89.91	818.12
93.3	90.05	817.76
93.4	90.18	817.40
93.5	90.32	817.03
93.6	90.46	816.66
93.7	90.59	816.30
93.8	90.73	815.93
93.9	90.87	815.55
94.0	91.01	815.18
94.1	91.15	814.81
94.2	91.29	814.43
94.3	91.43	814.06
94.4	91.56	813.68
94.5	91.70	813.30
94.6	91.84	812.92
94.7	91.98	812.54
94.8	92.13	812.15
94.9	92.27	811.77
95.0	92.41	811.38
95.1	92.55	810.99
95.2	92.69	810.60
95.3	92.83	810.21
95.4	92.98	809.82
95.5	93.12	809.42
95.6	93.26	809.02
95.7	93.41	808.63
95.8	93.55	808.23
95.9	93.69	807.82
96.0	93.84	807.42
96.1	93.98	807.01
96.2	94.13	806.61
96.3	94.27	806.20
96.4	94.42	805.78
96.5	94.57	805.37
96.6	94.71	804.96
96.7	94.86	804.54
96.8	95.01	804.12
96.9	95.16	803.70
97.0	95.31	803.27
97.1	95.45	802.85
97.2	95.60	802.42
97.3	95.75	801.99
97.4	95.90	801.55
97.5	96.05	801.12
97.6	96.21	800.68
97.7	96.36	800.24
97.8	96.51	799.80
97.9	96.66	799.35
98.0	96.81	798.90
98.1	96.97	798.45
98.2	97.12	798.00
98.3	97.28	797.54
98.4	97.43	797.08
98.5	97.59	796.62
98.6	97.74	796.15
98.7	97.90	795.68
98.8	98.06	795.21
98.9	98.22	794.73
99.0	98.38	794.25
99.1	98.53	793.77
99.2	98.69	793.28
99.3	98.86	792.79
99.4	99.02	792.30
99.5	99.18	791.80
99.6	99.34	791.29
99.7	99.50	790.79
99.8	99.67	790.28
99.9	99.83	789.76
100.0	100.0	789.24

F. Pharmacopoeial Harmonisation

(Ph. Eur. general texts 5.8)

This general chapter is included for guidance of users.

The chapter does not affect in any way the status of the monographs and general chapters as the authoritative reference in case of doubt or dispute, where compliance with the European Pharmacopoeia is required.

The European Pharmacopoeia Commission recognises the utility of working with other pharmacopoeial bodies to develop harmonised monographs and general chapters. Such harmonisation is fully compatible with the declared aims of the Commission and has benefits of different kinds, notably the simplification and rationalisation of quality control methods and licensing procedures. Such harmonisation also enhances the benefits of the work of the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) and the International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products (VICH), since some of the guidelines developed depend on pharmacopoeial general chapters for their application.

Work on harmonisation is carried out by a well-defined but informal process in the Pharmacopoeial Discussion Group (PDG), in which the European Pharmacopoeia, the Japanese Pharmacopoeia and the United States Pharmacopeia are associated. Pharmacopoeial harmonisation has the following 2 aims:

- for general chapters, the aim is to arrive at interchangeable methods or requirements so that demonstration of compliance using a general chapter from one of the 3 pharmacopoeias implies that the same result would be obtained using the general chapter of either of the other pharmacopoeias; when a formal declaration of interchangeability has been recommended by ICH, the corresponding information is available on the ICH website;
- for monographs, the aim is to arrive at identical requirements for all attributes of a substance; for most substances it can be extremely difficult to achieve complete harmonisation, for example because of differences in legal status and interpretation; it has therefore appeared worthwhile to the PDG to approve and publish monographs in which as many attributes as possible are harmonised.

Any non-harmonised attributes/provisions and any local requirements (i.e. attributes/provisions that are present only in the Ph. Eur. text) are indicated in the relevant Ph. Eur. general chapters and monographs: the non-harmonised attributes/provisions are placed between black diamonds (♦♦), while the local requirements are placed between white diamonds (◊◊).

The non-mandatory Functionality-related characteristics section is specific to the Ph. Eur.; it is not subject to pharmacopoeial harmonisation and is therefore not placed between black or white diamonds.

Harmonisation is not achieved until the text becomes official in all 3 pharmacopoeias.

It remains the ultimate responsibility of the user to verify the current content of the texts in force in the respective pharmacopoeias.

The 3 pharmacopoeias have undertaken not to make unilateral changes to harmonised monographs and general chapters but rather to apply the agreed revision procedure whereby all partners adopt a revision simultaneously.

G. Statistical Analysis of Results of Biological Assays and Tests

(Ph. Eur. general text 5.3)

1. INTRODUCTION

This chapter provides guidance for the design of bioassays prescribed in the European Pharmacopoeia (Ph. Eur.) and for analysis of their results. It is intended for use by those whose primary training and responsibilities are not in statistics, but who have responsibility for analysis or interpretation of the results of these assays, often without the help and advice of a statistician. The methods of calculation described in this annex are not mandatory for the bioassays which themselves constitute a mandatory part of the Ph. Eur. Alternative methods can be used and may be accepted by the competent authorities, provided that they are supported by relevant data and justified during the assay validation process. A wide range of computer software is available and may be useful depending on the facilities available to, and the expertise of, the analyst.

Professional advice should be obtained in situations where: a comprehensive treatment of design and analysis suitable for research or development of new products is required; the restrictions imposed on the assay design by this chapter are not satisfied, for example particular laboratory constraints may require customized assay designs, or equal numbers of equally spaced doses may not be suitable; analysis is required for extended non-linear dose-response curves, for example as may be encountered in immunoassays. An outline of extended dose-response curve analysis for one widely used model is nevertheless included in Section 3.4 and a simple example is given in Section 5.4.

1.1. GENERAL DESIGN AND PRECISION

Biological methods are described for the assay of certain substances and preparations whose potency cannot be adequately assured by chemical or physical analysis.

The principle applied wherever possible throughout these assays is that of comparison with a standard preparation so as to determine how much of the substance to be examined produces the same biological effect as a given quantity, the *Unit*, of the standard preparation. It is an essential condition of such methods of biological assay that the tests on the standard preparation and on the substance to be examined be carried out at the same time and under identical conditions.

For certain assays (determination of virus titre for example) the potency of the test sample is not expressed relative to a standard. This type of assay is dealt with in Section 4.5.

Any estimate of potency derived from a biological assay is subject to random error due to the inherent variability of biological responses and calculations of error should be made, if possible, from the results of each assay, even when the official method of assay is used. Methods for the design of assays and the calculation of their errors are, therefore, described below. In every case, before a statistical method is adopted, a preliminary test is to be carried out with an appropriate number of assays, in order to ascertain the applicability of this method.

The confidence interval for the potency gives an indication of the precision with which the potency has been estimated in the assay. It is calculated with due regard to the experimental design and the sample size. The 95 per cent confidence interval is usually chosen in biological assays. Mathematical statistical methods are used to calculate these limits so as to

warrant the statement that there is a 95 per cent probability that these limits include the true potency. Whether this precision is acceptable to the European Pharmacopoeia depends on the requirements set in the monograph for the preparation concerned.

The terms "mean" and "standard deviation" are used here as defined in most current textbooks of biometry.

The terms "stated potency" or "labelled potency", "assigned potency", "assumed potency", "potency ratio" and "estimated potency" are used in this section to indicate the following concepts:

— "stated potency" or "labelled potency": in the case of a formulated product a nominal value assigned from knowledge of the potency of the bulk material; in the case of bulk material the potency estimated by the manufacturer;
— "assigned potency": the potency of the standard preparation;
— "assumed potency": the provisionally assigned potency of a preparation to be examined which forms the basis of calculating the doses that would be equipotent with the doses to be used of the standard preparation;
— "potency ratio" of an unknown preparation; the ratio of equipotent doses of the standard preparation and the unknown preparation under the conditions of the assay;
— "estimated potency": the potency calculated from assay data.

Section 9 (Glossary of symbols) is a tabulation of the more important uses of symbols throughout this annex. Where the text refers to a symbol not shown in this section or uses a symbol to denote a different concept, this is defined in that part of the text.

2. RANDOMISATION AND INDEPENDENCE OF INDIVIDUAL TREATMENTS

The allocation of the different treatments to different experimental units (animals, tubes, etc.) should be made by some strictly random process. Any other choice of experimental conditions that is not deliberately allowed for in the experimental design should also be made randomly. Examples are the choice of positions for cages in a laboratory and the order in which treatments are administered.

In particular, a group of animals receiving the same dose of any preparation should not be treated together (at the same time or in the same position) unless there is strong evidence that the relevant source of variation (for example, between times, or between positions) is negligible. Random allocations may be obtained from computers by using the built-in randomisation function. The analyst must check whether a different series of numbers is produced every time the function is started.

The preparations allocated to each experimental unit should be as independent as possible. Within each experimental group, the dilutions allocated to each treatment are not normally divisions of the same dose, but should be prepared individually. Without this precaution, the variability inherent in the preparation will not be fully represented in the experimental error variance. The result will be an under-estimation of the residual error leading to:

1) an unjustified increase in the stringency of the test for the analysis of variance (see Sections 3.2.3 and 3.2.4),

2) an under-estimation of the true confidence limits for the test, which, as shown in Section 3.2.5, are calculated from the estimate of s^2, the residual error mean square.

3. ASSAYS DEPENDING UPON QUANTITATIVE RESPONSES

3.1. STATISTICAL MODELS

3.1.1. GENERAL PRINCIPLES

The bioassays included in the Ph. Eur. have been conceived as "dilution assays", which means that the unknown preparation to be assayed is supposed to contain the same active principle as the standard preparation, but in a different ratio of active and inert components. In such a case the unknown preparation may in theory be derived from the standard preparation by dilution with inert components.

To check whether any particular assay may be regarded as a dilution assay, it is necessary to compare the dose-response relationships of the standard and unknown preparations. If these dose-response relationships differ significantly, then the theoretical dilution assay model is not valid. Significant differences in the dose-response relationships for the standard and unknown preparations may suggest that one of the preparations contains, in addition to the active principle, other components which are not inert but which influence the measured responses.

To make the effect of dilution in the theoretical model apparent, it is useful to transform the dose-response relationship to a linear function on the widest possible range of doses. 2 statistical models are of interest as models for the bioassays prescribed: the parallel-line model and the slope-ratio model.

The application of either is dependent on the fulfilment of the following conditions:

1) the different treatments have been randomly assigned to the experimental units,

2) the responses to each treatment are normally distributed,

3) the standard deviations of the responses within each treatment group of both standard and unknown preparations do not differ significantly from one another.

When an assay is being developed for use, the analyst has to determine that the data collected from many assays meet these theoretical conditions.

— Condition 1 can be fulfilled by an efficient use of Section 2.
— Condition 2 is an assumption which in practice is almost always fulfilled. Minor deviations from this assumption will in general not introduce serious flaws in the analysis as long as several replicates per treatment are included. In case of doubt, a test for deviations from normality (e.g. the Shapiro-Wilk[1] test) may be performed.
— Condition 3 can be checked with a test for homogeneity of variances (e.g. Bartlett's[2] test, Cochran's[3] test). Inspection of graphical representations of the data can also be very instructive for this purpose (see examples in Section 5).

When conditions 2 and/or 3 are not met, a transformation of the responses may bring a better fulfilment of these conditions. Examples are $\ln y$, $\sqrt{y}$, y^2.

— Logarithmic transformation of the responses y to $\ln y$ can be useful when the homogeneity of variances is not satisfactory. It can also improve the normality if the distribution is skewed to the right.

[1] *Wilk, M.B. and Shapiro, S.S. (1968). The joint assessment of normality of several independent samples, Technometrics 10, 825-839.*
[2] *Bartlett, M.S. (1937). Properties of sufficiency and statistical tests, Proc. Roy. Soc. London, Series A 160, 280-282.*
[3] *Cochran, W.G. (1951). Testing a linear relation among variances, Biometrics 7, 17-32.*

— The transformation of y to $\sqrt{y}$ is useful when the observations follow a Poisson distribution i.e. when they are obtained by counting.
— The square transformation of y to y^2 can be useful if, for example, the dose is more likely to be proportional to the area of an inhibition zone rather than the measured diameter of that zone.

For some assays depending on quantitative responses, such as immunoassays or cell-based *in vitro* assays, a large number of doses is used. These doses give responses that completely span the possible response range and produce an extended non-linear dose-response curve. Such curves are typical for all bioassays, but for many assays the use of a large number of doses is not ethical (for example, *in vivo* assays) or practical, and the aims of the assay may be achieved with a limited number of doses. It is therefore customary to restrict doses to that part of the dose-response range which is linear under suitable transformation, so that the methods of Sections 3.2 or 3.3 apply. However, in some cases analysis of extended dose-response curves may be desirable. An outline of one model which may be used for such analysis is given in Section 3.4 and a simple example is shown in Section 5.4.

There is another category of assays in which the response cannot be measured in each experimental unit, but in which only the fraction of units responding to each treatment can be counted. This category is dealt with in Section 4.

3.1.2. ROUTINE ASSAYS

When an assay is in routine use, it is seldom possible to check systematically for conditions 1 to 3, because the limited number of observations per assay is likely to influence the sensitivity of the statistical tests. Fortunately, statisticians have shown that, in symmetrical balanced assays, small deviations from homogeneity of variance and normality do not seriously affect the assay results. The applicability of the statistical model needs to be questioned only if a series of assays shows doubtful validity. It may then be necessary to perform a new series of preliminary investigations as discussed in Section 3.1.1.

Two other necessary conditions depend on the statistical model to be used:

— for the parallel-line model:

4A) the relationship between the logarithm of the dose and the response can be represented by a straight line over the range of doses used,

5A) for any unknown preparation in the assay the straight line is parallel to that for the standard.

— for the slope-ratio model:

4B) the relationship between the dose and the response can be represented by a straight line for each preparation in the assay over the range of doses used,

5B) for any unknown preparation in the assay the straight line intersects the y-axis (at zero dose) at the same point as the straight line of the standard preparation (i.e. the response functions of all preparations in the assay must have the same intercept as the response function of the standard).

Conditions 4A and 4B can be verified only in assays in which at least 3 dilutions of each preparation have been tested. The use of an assay with only 1 or 2 dilutions may be justified when experience has shown that linearity and parallelism or equal intercept are regularly fulfilled.

After having collected the results of an assay, and before calculating the relative potency of each test sample, an analysis of variance is performed, in order to check whether conditions 4A and 5A (or 4B and 5B) are fulfilled. For this, the total sum of squares is subdivided into a certain number of sum of squares corresponding to each condition which has to be fulfilled. The remaining sum of squares represents the residual experimental error to which the absence or existence of the relevant sources of variation can be compared by a series of F-ratios.

When validity is established, the potency of each unknown relative to the standard may be calculated and expressed as a potency ratio or converted to some unit relevant to the preparation under test e.g. an International Unit. Confidence limits may also be estimated from each set of assay data.

Assays based on the parallel-line model are discussed in Section 3.2 and those based on the slope-ratio model in Section 3.3.

If any of the 5 conditions (1, 2, 3, 4A, 5A or 1, 2, 3, 4B, 5B) are not fulfilled, the methods of calculation described here are invalid and an investigation of the assay technique should be made.

The analyst should not adopt another transformation unless it is shown that non-fulfilment of the requirements is not incidental but is due to a systematic change in the experimental conditions. In this case, testing as described in Section 3.1.1 should be repeated before a new transformation is adopted for the routine assays.

Excess numbers of invalid assays due to non-parallelism or non-linearity, in a routine assay carried out to compare similar materials, are likely to reflect assay designs with inadequate replication. This inadequacy commonly results from incomplete recognition of all sources of variability affecting the assay, which can result in underestimation of the residual error leading to large F-ratios.

It is not always feasible to take account of all possible sources of variation within one single assay (e.g. day-to-day variation). In such a case, the confidence intervals from repeated assays on the same sample may not satisfactorily overlap, and care should be exercised in the interpretation of the individual confidence intervals. In order to obtain a more reliable estimate of the confidence interval it may be necessary to perform several independent assays and to combine these into one single potency estimate and confidence interval (see Section 6).

For the purpose of quality control of routine assays it is recommended to keep record of the estimates of the slope of regression and of the estimate of the residual error in control charts.

— An exceptionally high residual error may indicate some technical problem. This should be investigated and, if it can be made evident that something went wrong during the assay procedure, the assay should be repeated.
  An unusually high residual error may also indicate the presence of an occasional outlying or aberrant observation. A response that is questionable because of failure to comply with the procedure during the course of an assay is rejected. If an aberrant value is discovered after the responses have been recorded, but can then be traced to assay irregularities, omission may be justified.
  The arbitrary rejection or retention of an apparently aberrant response can be a serious source of bias.
  In general, the rejection of observations solely because a test for outliers is significant, is discouraged.
— An exceptionally low residual error may once in a while occur and cause the F-ratios to exceed the critical values. In such a case it may be justified to replace the residual error estimated from the individual assay, by an average residual error based on historical data recorded in the control charts.

3.1.3. CALCULATIONS AND RESTRICTIONS

According to general principles of good design the following 3 restrictions are normally imposed on the assay design. They have advantages both for ease of computation and for precision.

a) Each preparation in the assay must be tested with the same number of dilutions.

b) In the parallel-line model, the ratio of adjacent doses must be constant for all treatments in the assay; in the slope-ratio model, the interval between adjacent doses must be constant for all treatments in the assay.

c) There must be an equal number of experimental units to each treatment.

If a design is used which meets these restrictions, the calculations are simple. The formulae are given in Sections 3.2 and 3.3. It is recommended to use software which has been developed for this special purpose. There are several programs in existence which can easily deal with all assay-designs described in the monographs. Not all programs may use the same formulae and algorithms, but they should all lead to the same results.

Assay designs not meeting the above mentioned restrictions may be both possible and correct, but the necessary formulae are too complicated to describe in this text. A brief description of methods for calculation is given in Section 7.1. These methods can also be used for the restricted designs, in which case they are equivalent with the simple formulae.

The formulae for the restricted designs given in this text may be used, for example, to create *ad hoc* programs in a spreadsheet. The examples in Section 5 can be used to clarify the statistics and to check whether such a program gives correct results.

3.2. THE PARALLEL-LINE MODEL

3.2.1. INTRODUCTION

The parallel-line model is illustrated in Figure 3.2.1.-I. The logarithm of the doses are represented on the horizontal axis with the lowest concentration on the left and the highest concentration on the right. The responses are indicated on the vertical axis. The individual responses to each treatment are indicated with black dots. The 2 lines are the calculated ln(dose)-response relationship for the standard and the unknown.

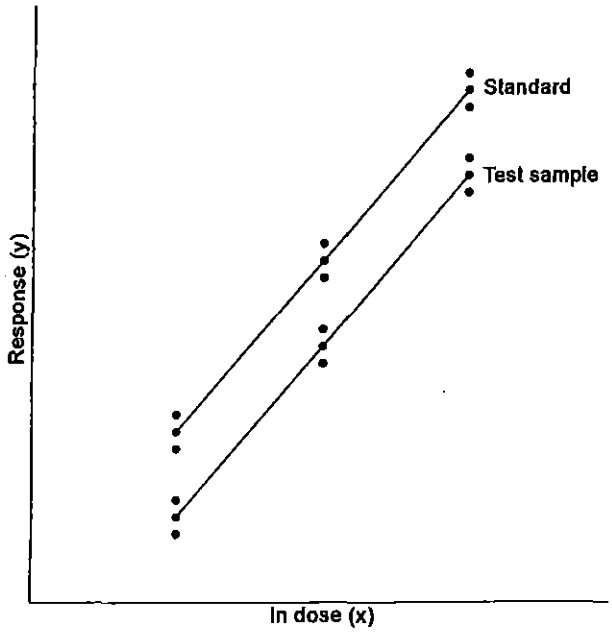


Figure 3.2.1.-I. – *The parallel-line model for a 3 + 3 assay*

Note The natural logarithm (ln or $\log_e$) is used throughout this text. Wherever the term "antilogarithm" is used, the quantity e^x is meant. However, the Briggs or "common" logarithm (log or $\log_{10}$) can equally well be used. In this case the corresponding antilogarithm is 10^x.

For a satisfactory assay the assumed potency of the test sample must be close to the true potency. On the basis of this assumed potency and the assigned potency of the standard, equipotent dilutions (if feasible) are prepared, i.e. corresponding doses of standard and unknown are expected to give the same response. If no information on the assumed potency is available, preliminary assays are carried out over a wide range of doses to determine the range where the curve is linear.

The more nearly correct the assumed potency of the unknown, the closer the 2 lines will be together, for they should give equal responses at equal doses. The horizontal distance between the lines represents the "true" potency of the unknown, relative to its assumed potency. The greater the distance between the 2 lines, the poorer the assumed potency of the unknown. If the line of the unknown is situated to the right of the standard, the assumed potency was overestimated, and the calculations will indicate an estimated potency lower than the assumed potency. Similarly, if the line of the unknown is situated to the left of the standard, the assumed potency was underestimated, and the calculations will indicate an estimated potency higher than the assumed potency.

3.2.2. ASSAY DESIGN

The following considerations will be useful in optimising the precision of the assay design:

1) the ratio between the slope and the residual error should be as large as possible,

2) the range of doses should be as large as possible,

3) the lines should be as close together as possible,

i.e. the assumed potency should be a good estimate of the true potency.

The allocation of experimental units (animals, tubes, etc.) to different treatments may be made in various ways.

3.2.2.1. Completely randomised design

If the totality of experimental units appears to be reasonably homogeneous with no indication that variability in response will be smaller within certain recognisable sub-groups, the allocation of the units to the different treatments should be made randomly.

If units in sub-groups such as physical positions or experimental days are likely to be more homogeneous than the totality of the units, the precision of the assay may be increased by introducing one or more restrictions into the design. A careful distribution of the units over these restrictions permits irrelevant sources of variation to be eliminated.

3.2.2.2. Randomised block design

In this design it is possible to segregate an identifiable source of variation, such as the sensitivity variation between litters of experimental animals or the variation between Petri dishes in a diffusion microbiological assay. The design requires that every treatment be applied an equal number of times in every block (litter or Petri dish) and is suitable only when the block is large enough to accommodate all treatments once. This is illustrated in Section 5.1.3. It is also possible to use a randomised design with repetitions. The treatments should be allocated randomly within each block. An algorithm to obtain random permutations is given in Section 8.5.

3.2.2.3. Latin square design

This design is appropriate when the response may be affected by two different sources of variation each of which can assume k different levels or positions. For example, in a plate assay of an antibiotic the treatments may be arranged in a $k \times k$ array on a large plate, each treatment occurring once in each row and each column. The design is suitable when the number of rows, the number of columns and the number of treatments are equal. Responses are recorded in a square format known as a Latin square. Variations due to differences in response among the k rows and among the k columns may be segregated, thus reducing the error. An example of a Latin square design is given in Section 5.1.2. An algorithm to obtain Latin squares is given in Section 8.6. More complex designs in which one or more treatments are replicated within the Latin square may be useful in some circumstances. The simplified formulae given in this Chapter are not appropriate for such designs, and professional advice should be obtained.

3.2.2.4. Cross-over design

This design is useful when the experiment can be sub-divided into blocks but it is possible to apply only 2 treatments to each block. For example, a block may be a single unit that can be tested on 2 occasions. The design is intended to increase precision by eliminating the effects of differences between units while balancing the effect of any difference between general levels of response at the 2 occasions. If 2 doses of a standard and of an unknown preparation are tested, this is known as a twin cross-over test.

The experiment is divided into 2 parts separated by a suitable time interval. Units are divided into 4 groups and each group receives 1 of the 4 treatments in the first part of the test. Units that received one preparation in the first part of the test receive the other preparation on the second occasion, and units receiving small doses in one part of the test receive large doses in the other. The arrangement of doses is shown in Table 3.2.2.-I. An example can be found in Section 5.1.5.

Table 3.2.2.-I. – *Arrangement of doses in cross-over design*

Group of units	Time I	Time II
1	S_1	T_2
2	S_2	T_1
3	T_1	S_2
4	T_2	S_1

3.2.3. ANALYSIS OF VARIANCE

This section gives formulae that are required to carry out the analysis of variance and will be more easily understood by reference to the worked examples in Section 5.1. Reference should also be made to the glossary of symbols (Section 9).

The formulae are appropriate for symmetrical assays where one or more preparations to be examined (T, U, etc.) are compared with a standard preparation (S). It is stressed that the formulae can only be used if the doses are equally spaced, if equal numbers of treatments per preparation are applied, and each treatment is applied an equal number of times. It should not be attempted to use the formulae in any other situation.

Apart from some adjustments to the error term, the basic analysis of data derived from an assay is the same for completely randomised, randomised block and Latin square designs. The formulae for cross-over tests do not entirely fit this scheme and these are incorporated into Example 5.1.5.

Having considered the points discussed in Section 3.1 and transformed the responses, if necessary, the values should be averaged over each treatment and each preparation, as shown in Table 3.2.3.-I. The linear contrasts, which relate to the slopes of the ln(dose)-response lines, should also be formed. 3 additional formulae, which are necessary for the construction of the analysis of variance, are shown in Table 3.2.3.-II.

The total variation in response caused by the different treatments is now partitioned as shown in Table 3.2.3.-III the sums of squares being derived from the values obtained in Tables 3.2.3.-I and 3.2.3.-II. The sum of squares due to non-linearity can only be calculated if at least 3 doses per preparation are included in the assay.

The residual error of the assay is obtained by subtracting the variations allowed for in the design from the total variation in response (Table 3.2.3.-IV). In this table $\bar{y}$ represents the mean of all responses recorded in the assay. It should be noted that for a Latin square the number of replicate responses (n) is equal to the number of rows, columns or treatments (dh).

The analysis of variance is now completed as follows. Each sum of squares is divided by the corresponding number of degrees of freedom to give mean squares. The mean square for each variable to be tested is now expressed as a ratio to the residual error (s^2) and the significance of these values (known as F-ratios) are assessed by use of Table 8.1 or a suitable sub-routine of a computer program.

3.2.4. TESTS OF VALIDITY

Assay results are said to be "statistically valid" if the outcome of the analysis of variance is as follows.

1) The linear regression term is significant, i.e. the calculated probability is less than 0.05. If this criterion is not met, it is not possible to calculate 95 per cent confidence limits.

2) The term for non-parallelism is not significant, i.e. the calculated probability is not less than 0.05. This indicates that condition 5A, Section 3.1, is satisfied.

3) The term for non-linearity is not significant, i.e. the calculated probability is not less than 0.05. This indicates that condition 4A, Section 3.1, is satisfied.

A significant deviation from parallelism in a multiple assay may be due to the inclusion in the assay-design of a preparation to be examined that gives an ln(dose)-response line with a slope different from those for the other preparations. Instead of declaring the whole assay invalid, it may then be decided to eliminate all data relating to that preparation and to restart the analysis from the beginning.

When statistical validity is established, potencies and confidence limits may be estimated by the methods described in the next section.

3.2.5. ESTIMATION OF POTENCY AND CONFIDENCE LIMITS

If I is the ln of the ratio between adjacent doses of any preparation, the common slope (b) for assays with d doses of each preparation is obtained from:

$$b = \frac{H_L(L_S + L_T + \ldots)}{Inh} \qquad (3.2.5.\text{-}1)$$

and the logarithm of the potency ratio of a test preparation, for example T, is:

$$M'_T = \frac{P_T - P_S}{db} \qquad (3.2.5.\text{-}2)$$

Table 3.2.3.-I. – *Formulae for parallel-line assays with d doses of each preparation*

	Standard (*S*)	1st Test sample (*T*)	2nd Test sample (*U*, etc.)
Mean response lowest dose	S_1	T_1	U_1
Mean response 2nd dose	S_2	T_2	U_2
...	...	...	...
Mean response highest dose	S_d	T_d	U_d
Total preparation	$P_S = S_1 + S_2 + ... + S_d$	$P_T = T_1 + T_2 + ... + T_d$	P_U = ...etc.
Linear contrast	$L_S = 1S_1 + 2S_2 + ... + dS_d - \frac{1}{2}(d+1)P_S$	$L_T = 1T_1 + 2T_2 + ... + dT_d - \frac{1}{2}(d+1)P_T$	L_U = ...etc.

Table 3.2.3.-II. – *Additional formulae for the construction of the analysis of variance*

$H_P = \frac{n}{d}$	$H_L = \frac{12n}{d^3 - d}$	$K = \frac{n(P_S + P_T + ...)^2}{hd}$

Table 3.2.3.-III. – *Formulae to calculate the sum of squares and degrees of freedom*

Source of variation	Degrees of freedom (*f*)	Sum of squares
Preparations	$h - 1$	$SS_{prep} = H_P(P_S^2 + P_T^2 + ...) - K$
Linear regression	1	$SS_{reg} = \frac{1}{h}H_L(L_S + L_T + ...)2$
Non-parallelism	$h - 1$	$SS_{par} = H_L(L_S^2 + L_T^2 + ...) - SS_{reg}$
Non-linearity*	$h(d-2)$	$SS_{lin} = SS_{treat} - SS_{prep} - SS_{reg} - SS_{par}$
Treatments	$hd - 1$	$SS_{treat} = n(S_1^2 + ... + S_d^2 + T_1^2 + ... + T_d^2 + ...) - K$
* Not calculated for 2-dose assays		

Table 3.2.3.-IV. – *Estimation of the residual error*

Source of variation		Degrees of freedom	Sum of squares
Blocks (rows)*		$n - 1$	$SS_{block} = hd(R_1^2 + ... + R_n^2) - K$
Columns**		$n - 1$	$SS_{col} = hd(C_1^2 + ... + C_n^2) - K$
Residual error***	Completely randomised	$hd(n-1)$	$SS_{res} = SS_{tot} - SS_{treat}$
	Randomised block	$(hd-1)(n-1)$	$SS_{res} = SS_{tot} - SS_{treat} - SS_{block}$
	Latin square	$(hd-2)(n-1)$	$SS_{res} = SS_{tot} - SS_{treat} - SS_{block} - SS_{col}$
Total		$nhd + n - 1$	$SS_{tot} = \sum(y - \bar{y})^2$

For Latin square designs, these formulae are only applicable if $n = hd$
* Not calculated for completely randomised designs
** Only calculated for Latin square designs
*** Depends on the type of design

The calculated potency is an estimate of the "true potency" of each unknown. Confidence limits may be calculated as the antilogarithms of:

$$CM'_T \pm \sqrt{(C-1)(CM'^2_T + 2V)} \qquad (3.2.5.-3)$$

where $C = \frac{SS_{reg}}{SS_{reg} - s^2t^2}$ and $V = \frac{SS_{reg}}{b^2dn}$

The value of t may be obtained from Table 8.2 for $p = 0.05$ and degrees of freedom equal to the number of the degrees

of freedom of the residual error. The estimated potency (R_T) and associated confidence limits are obtained by multiplying the values obtained by A_T after antilogarithms have been taken. If the stock solutions are not exactly equipotent on the basis of assigned and assumed potencies, a correction factor is necessary (See Examples 5.1.2 and 5.1.3).

3.2.6. MISSING VALUES

In a balanced assay, an accident totally unconnected with the applied treatments may lead to the loss of one or more responses, for example because an animal dies. If it is considered that the accident is in no way connected with the composition of the preparation administered, the exact calculations can still be performed but the formulae are necessarily more complicated and can only be given within the framework of general linear models (see Section 7.1). However, there exists an approximate method which keeps the simplicity of the balanced design by replacing the missing response by a calculated value. The loss of information is taken into account by diminishing the degrees of freedom for the total sum of squares and for the residual error by the number of missing values and using one of the formulae below for the missing values. It should be borne in mind that this is only an approximate method, and that the exact method is to be preferred.

If more than one observation is missing, the same formulae can be used. The procedure is to make a rough guess at all the missing values except one, and to use the proper formula for this one, using all the remaining values including the rough guesses. Fill in the calculated value. Continue by similarly calculating a value for the first rough guess. After calculating all the missing values in this way the whole cycle is repeated from the beginning, each calculation using the most recent guessed or calculated value for every response to which the formula is being applied. This continues until 2 consecutive cycles give the same values; convergence is usually rapid.

Provided that the number of values replaced is small relative to the total number of observations in the full experiment (say less than 5 per cent), the approximation implied in this replacement and reduction of degrees of freedom by the number of missing values so replaced is usually fairly satisfactory. The analysis should be interpreted with great care however, especially if there is a preponderance of missing values in one treatment or block, and a biometrician should be consulted if any unusual features are encountered. Replacing missing values in a test without replication is a particularly delicate operation.

Completely randomised design

In a completely randomised assay the missing value can be replaced by the arithmetic mean of the other responses to the same treatment.

Randomised block design

The missing value is obtained using the equation:

$$y' = \frac{nB' + kT' - G'}{(n-1)(k-1)} \qquad (3.2.6.\text{-}1)$$

where B' is the sum of the responses in the block containing the missing value, T' the corresponding treatment total and G' is the sum of all responses recorded in the assay.

Latin square design

The missing value y' is obtained from:

$$y' = \frac{k(B' + C' + T') - 2G'}{(k-1)(k-2)} \qquad (3.2.6.\text{-}2)$$

where B' and C' are the sums of the responses in the row and column containing the missing value. In this case $k = n$.

Cross-over design

If an accident leading to loss of values occurs in a cross-over design, a book on statistics should be consulted (e.g. D.J. Finney, see Section 10), because the appropriate formulae depend upon the particular treatment combinations.

3.3. THE SLOPE-RATIO MODEL

3.3.1. INTRODUCTION

This model is suitable, for example, for some microbiological assays when the independent variable is the concentration of an essential growth factor below the optimal concentration of the medium. The slope-ratio model is illustrated in Figure 3.3.1.-I.

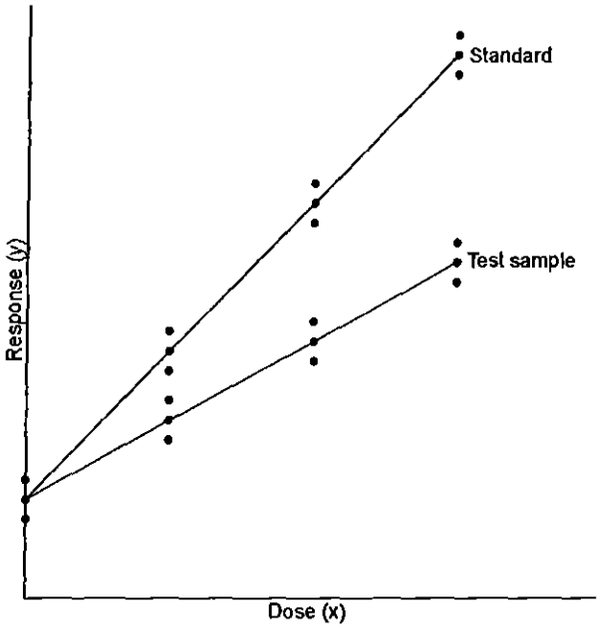


Figure 3.3.1.-I. – *The slope-ratio model for a 2 × 3 + 1 assay*

The doses are represented on the horizontal axis with zero concentration on the left and the highest concentration on the right. The responses are indicated on the vertical axis. The individual responses to each treatment are indicated with black dots. The 2 lines are the calculated dose-response relationship for the standard and the unknown under the assumption that they intersect each other at zero-dose. Unlike the parallel-line model, the doses are not transformed to logarithms.

Just as in the case of an assay based on the parallel-line model, it is important that the assumed potency is close to the true potency, and to prepare equipotent dilutions of the test preparations and the standard (if feasible). The more nearly correct the assumed potency, the closer the 2 lines will be together. The ratio of the slopes represents the "true" potency of the unknown, relative to its assumed potency. If the slope of the unknown preparation is steeper than that of the standard, the potency was underestimated and the calculations will indicate an estimated potency higher than the assumed potency. Similarly, if the slope of the unknown is less steep than that of the standard, the potency was overestimated and the calculations will result in an estimated potency lower than the assumed potency.

In setting up an experiment, all responses should be examined for the fulfilment of the conditions 1, 2 and 3 in

Section 3.1. The analysis of variance to be performed in routine is described in Section 3.3.3 so that compliance with conditions 4B and 5B of Section 3.1 may be examined.

3.3.2. ASSAY DESIGN

The use of the statistical analysis presented below imposes the following restrictions on the assay:

a) the standard and the test preparations must be tested with the same number of equally spaced dilutions,

b) an extra group of experimental units receiving no treatment may be tested (the blanks),

c) there must be an equal number of experimental units to each treatment.

As already remarked in Section 3.1.3, assay designs not meeting these restrictions may be both possible and correct, but the simple statistical analyses presented here are no longer applicable and either expert advice should be sought or suitable software should be used.

A design with 2 doses per preparation and 1 blank, the "common zero ($2h + 1$)-design", is usually preferred, since it gives the highest precision combined with the possibility to check validity within the constraints mentioned above. However, a linear relationship cannot always be assumed to be valid down to zero-dose. With a slight loss of precision a design without blanks may be adopted. In this case 3 doses per preparation, the "common zero ($3h$)-design", are preferred to 2 doses per preparation. The doses are thus given as follows:

1) the standard is given in a high dose, near to but not exceeding the highest dose giving a mean response on the straight portion of the dose-response line,

2) the other doses are uniformly spaced between the highest dose and zero dose,

3) the test preparations are given in corresponding doses based on the assumed potency of the material.

A completely randomised, a randomised block or a Latin square design may be used, such as described in Section 3.2.2. The use of any of these designs necessitates an adjustment to the error sum of squares as described for assays based on the parallel-line model. The analysis of an assay of one or more test preparations against a standard is described below.

3.3.3. ANALYSIS OF VARIANCE

3.3.3.1. The ($hd + 1$)-design

The responses are verified as described in Section 3.1 and, if necessary, transformed. The responses are then averaged over each treatment and each preparation as shown in Table 3.3.3.1.-I. Additionally, the mean response for blanks (B) is calculated.

The sums of squares in the analysis of variance are calculated as shown in Tables 3.3.3.1.-I to 3.3.3.1.-III. The sum of squares due to non-linearity can only be calculated if at least 3 doses of each preparation have been included in the assay. The residual error is obtained by subtracting the variations allowed for in the design from the total variation in response (Table 3.3.3.1.-IV).

The analysis of variance is now completed as follows. Each sum of squares is divided by the corresponding number of degrees of freedom to give mean squares. The mean square for each variable to be tested is now expressed as a ratio to the residual error (s^2) and the significance of these values (known as F-ratios) are assessed by use of Table 8.1 or a suitable sub-routine of a computer program.

3.3.3.2. The (hd)-design

The formulae are basically the same as those for the ($hd + 1$)-design, but there are some slight differences.

— B is discarded from all formulae.

— $K = \frac{n(P_S + P_T + \ldots)^2}{hd}$

— SS_{blank} is removed from the analysis of variance.

— The number of degrees of freedom for treatments becomes $hd - 1$.

— The number of degrees of freedom of the residual error and the total variance is calculated as described for the parallel-line model (see Table 3.2.3.-IV).

Validity of the assay, potency and confidence interval are found as described in Sections 3.3.4 and 3.3.5.

3.3.4. TESTS OF VALIDITY

Assay results are said to be "statistically valid" if the outcome of the analysis of variance is as follows:

1) the variation due to blanks in ($hd + 1$)-designs is not significant, i.e. the calculated probability is not smaller than 0.05. This indicates that the responses of the blanks do not significantly differ from the common intercept and the linear relationship is valid down to zero dose;

2) the variation due to intersection is not significant, i.e. the calculated probability is not less than 0.05. This indicates that condition 5B, Section 3.1 is satisfied;

3) in assays including at least 3 doses per preparation, the variation due to non-linearity is not significant, i.e. the calculated probability is not less than 0.05. This indicates that condition 4B, Section 3.1 is satisfied.

A significant variation due to blanks indicates that the hypothesis of linearity is not valid near zero dose. If this is likely to be systematic rather than incidental for the type of assay, the (hd-design) is more appropriate. Any response to blanks should then be disregarded.

When these tests indicate that the assay is valid, the potency is calculated with its confidence limits as described in Section 3.3.5.

3.3.5. ESTIMATION OF POTENCY AND CONFIDENCE LIMITS

3.3.5.1. The ($hd + 1$)-design

The common intersection a' of the preparations can be calculated from:

$$a' = \frac{(2d+1)B + (2d-3)ha}{h(2d-3) + 2d + 1} \qquad (3.3.5.1.\text{-}1)$$

The slope of the standard, and similarly for each of the other preparations, is calculated from:

$$b'_S = \frac{6L_S - 3d(d+1)a'}{2d^3 + 3d^2 + d} \qquad (3.3.5.1.\text{-}2)$$

The potency ratio of each of the test preparations can now be calculated from:

$$R'_T = \frac{b'_T}{b'_S} \qquad (3.3.5.1.\text{-}3)$$

which has to be multiplied by A_T, the assumed potency of the test preparation, in order to find the estimated potency R_T. If the step between adjacent doses was not identical for the standard and the test preparation, the potency has to be multiplied by I_S/I_T. Note that, unlike the parallel-line analysis, no antilogarithms are calculated.

Table 3.3.3.1.-I. – *Formulae for slope-ratio assays with d doses of each preparation and a blank*

	Standard (S)	1st Test sample (T)	2nd Test sample (U, etc.)
Mean response lowest dose	S_1	T_1	U_1
Mean response 2nd dose	S_2	T_2	U_2
...	...	...	...
Mean response highest dose	S_d	T_d	U_d
Total preparation	$P_S = S_1 + S_2 + ... + S_d$	$P_T = T_1 + T_2 + ... + T_d$	$P_U = ...$
Linear product	$L_S = 1S_1 + 2S_2 + ... + dS_d$	$L_T = 1T_1 + 2T_2 + ... + dT_d$	$L_U = ...$
Intercept value	$a_S = (4d+2)P_S - 6L_S$	$a_T = (4d+2)P_T - 6L_T$	$a_U = ...$
Slope value	$b_S = 2L_S - (d+1)P_S$	$b_T = 2L_T - (d+1)P_T$	$b_U = ...$
Treatment value	$G_S = S_1^2 + ... + S_d^2$	$G_T = T_1^2 + ... + T_d^2$	$G_U = ...$
Non-linearity*	$J_S = G_S - \frac{P_S^2}{d} - \frac{3b_S^2}{d^3 - d}$	$J_T = G_T - \frac{P_T^2}{d} - \frac{3b_T^2}{d^3 - d}$	$J_U = ...$
* Not calculated for 2-dose assays			

Table 3.3.3.1.-II. – *Additional formulae for the construction of the analysis of variance*

$H_B = \frac{nhd^2 - nhd}{hd^2 - hd + 4d + 2}$	$H_I = \frac{n}{4d^3 - 2d^2 - 2d}$	$a = \frac{a_S + a_T + ...}{h(d^2 - d)}$	$K = \frac{n(B + P_S + P_T + ...)^2}{hd + 1}$

Table 3.3.3.1.-III. – *Formulae to calculate the sum of squares and degrees of freedom*

Source of variation	Degrees of freedom (f)	Sum of squares
Regression	h	$SS_{reg} = SS_{treat} - SS_{blank} - SS_{int} - SS_{lin}$
Blanks	1	$SS_{blank} = H_B(B - a)^2$
Intersection	$h - 1$	$SS_{int} = H_I\left((a_S^2 + a_T^2 + ...) - h(d^2 - d)^2 a^2\right)$
Non-linearity*	$h(d - 2)$	$SS_{lin} = n(J_S + J_T + ...)$
Treatments	hd	$SS_{treat} = n(B^2 + G_S + G_T + ...) - K$
* Not calculated for 2-dose assays		

Table 3.3.3.1.-IV. – *Estimation of the residual error*

Source of variation		Degrees of freedom	Sum of squares
Blocks (rows)*		$n - 1$	$SS_{block} = hd\left(R_1^2 + ... + R_n^2\right) - K$
Columns**		$n - 1$	$SS_{col} = hd\left(C_1^2 + ... + C_n^2\right) - K$
Residual error***	Completely randomised	$(hd + 1)(n - 1)$	$SS_{res} = SS_{tot} - SS_{treat}$
	Randomised block	$hd(n - 1)$	$SS_{res} = SS_{tot} - SS_{treat} - SS_{block}$
	Latin square	$(hd - 1)(n - 1)$	$SS_{res} = SS_{tot} - SS_{treat} - SS_{block} - SS_{col}$
Total		$nhd + n - 1$	$SS_{tot} = \sum(y - \bar{y})^2$

For Latin square designs, these formulae are only applicable if $n = hd$
* Not calculated for completely randomised designs
** Only calculated for Latin square designs
*** Depends on the type of design

The confidence interval for R_T' is calculated from:

$$CR_T' - K' \pm \sqrt{(C-1)(CR_T'2+1) + K'(K' - 2CR_T')} \qquad (3.3.5.1.-4)$$

where $C = \frac{b_S'2}{b_S'2 - s^2 t^2 V_1}$ and $K' = (C-1)V_2$

V_1 and V_2 are related to the variance and covariance of the numerator and denominator of R_T. They can be obtained from:

$$V_1 = \frac{6}{n(2d+1)}\left(\frac{1}{d(d+1)} + \frac{3}{2(2d+1)+hd(d-1)}\right) \qquad (3.3.5.1.-5)$$

$$V_2 = \frac{3d(d+1)}{(3d+1)(d+2)+hd(d-1)} \qquad (3.3.5.1.-6)$$

The confidence limits are multiplied by A_T, and if necessary by I_S/I_T.

3.3.5.2. The (*hd*)-design

The formulae are the same as for the $(hd + 1)$-design, with the following modifications:

$$d' = a \qquad (3.3.5.2.-1)$$

$$V_1 = \frac{6}{nd(2d+1)}\left(\frac{1}{d+1} + \frac{3}{h(d-1)}\right) \qquad (3.3.5.2.-2)$$

$$V_2 = \frac{3(d+1)}{3(d+1)+h(d-1)} \qquad (3.3.5.2.-3)$$

3.4. EXTENDED SIGMOID DOSE-RESPONSE CURVES

This model is suitable, for example, for some immunoassays when analysis is required of extended sigmoid dose-response curves. This model is illustrated in Figure 3.4.-I.

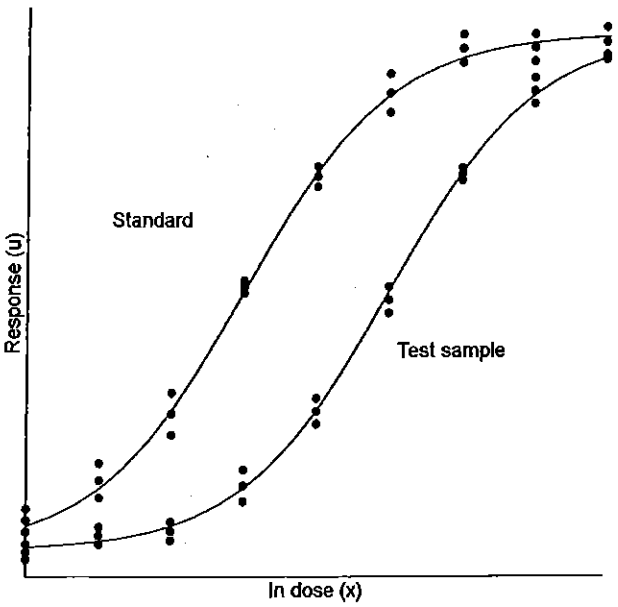


Figure 3.4.-I. – *The four-parameter logistic curve model*

The logarithms of the doses are represented on the horizontal axis with the lowest concentration on the left and the highest concentration on the right. The responses are indicated on the vertical axis. The individual responses to each treatment are indicated with black dots. The 2 curves are the calculated ln(dose)-response relationship for the standard and the test preparation.

The general shape of the curves can usually be described by a logistic function but other shapes are also possible. Each curve can be characterised by 4 parameters: The upper asymptote (α), the lower asymptote (δ), the slope-factor (β), and the horizontal location (γ). This model is therefore often referred to as a four-parameter model. A mathematical representation of the ln(dose)-response curve is:

$$u = \delta + \frac{\alpha - \delta}{1 + e^{-\beta(x-\gamma)}}$$

For a valid assay it is necessary that the curves of the standard and the test preparations have the same slope-factor, and the same maximum and minimum response level at the extreme parts. Only the horizontal location (γ) of the curves may be different. The horizontal distance between the curves is related to the "true" potency of the unknown. If the assay is used routinely, it may be sufficient to test the condition of equal upper and lower response levels when the assay is developed, and then to retest this condition directly only at suitable intervals or when there are changes in materials or assay conditions.

The maximum-likelihood estimates of the parameters and their confidence intervals can be obtained with suitable computer programs. These computer programs may include some statistical tests reflecting validity. For example, if the maximum likelihood estimation shows significant deviations from the fitted model under the assumed conditions of equal upper and lower asymptotes and slopes, then one or all of these conditions may not be satisfied.

The logistic model raises a number of statistical problems which may require different solutions for different types of assays, and no simple summary is possible. A wide variety of possible approaches is described in the relevant literature. Professional advice is therefore recommended for this type of analysis. A simple example is nevertheless included in Section 5.4 to illustrate a "possible" way to analyse the data presented. A short discussion of alternative approaches and other statistical considerations is given in Section 7.5.

If professional advice or suitable software is not available, alternative approaches are possible: 1) if "reasonable" estimates of the upper limit (α) and lower limit (δ) are available, select for all preparations the doses with mean of the responses (u) falling between approximately 20 per cent and 80 per cent of the limits, transform responses of the selected doses to:

$$y = \ln\left(\frac{u-\delta}{\alpha-u}\right)$$

and use the parallel line model (Section 3.2) for the analysis; 2) select a range of doses for which the responses (u) or suitably transformed responses, for example ln u, are approximately linear when plotted against ln(dose); the parallel line model (Section 3.2) may then be used for analysis.

4. ASSAYS DEPENDING UPON QUANTAL RESPONSES

4.1. INTRODUCTION

In certain assays it is impossible or excessively laborious to measure the effect on each experimental unit on a quantitative scale. Instead, an effect such as death or

hypoglycaemic symptoms may be observed as either occurring or not occurring in each unit, and the result depends on the number of units in which it occurs. Such assays are called quantal or all-or-none.

The situation is very similar to that described for quantitative assays in Section 3.1, but in place of n separate responses to each treatment a single value is recorded, i.e. the fraction of units in each treatment group showing a response. When these fractions are plotted against the logarithms of the doses the resulting curve will tend to be sigmoid (S-shaped) rather than linear. A mathematical function that represents this sigmoid curvature is used to estimate the dose-response curve. The most commonly used function is the cumulative normal distribution function. This function has some theoretical merit, and is perhaps the best choice if the response is a reflection of the tolerance of the units. If the response is more likely to depend upon a process of growth, the logistic distribution model is preferred, although the difference in outcome between the 2 models is usually very small.

The maximum likelihood estimators of the slope and location of the curves can be found only by applying an iterative procedure. There are many procedures which lead to the same outcome, but they differ in efficiency due to the speed of convergence. One of the most rapid methods is direct optimisation of the maximum-likelihood function (see Section 7.1), which can easily be performed with computer programs having a built-in procedure for this purpose. Unfortunately, most of these procedures do not yield an estimate of the confidence interval, and the technique to obtain it is too complicated to describe here. The technique described below is not the most rapid, but has been chosen for its simplicity compared to the alternatives. It can be used for assays in which one or more test preparations are compared to a standard. Furthermore, the following conditions must be fulfilled:

1) the relationship between the logarithm of the dose and the response can be represented by a cumulative normal distribution curve,

2) the curves for the standard and the test preparation are parallel, i.e. they are identically shaped and may only differ in their horizontal location,

3) in theory, there is no natural response to extremely low doses and no natural non-response to extremely high doses.

4.2. THE PROBIT METHOD

The sigmoid curve can be made linear by replacing each response, i.e. the fraction of positive responses per group, by the corresponding value of the cumulative standard normal distribution. This value, often referred to as "normit", ranges theoretically from $-\infty$ to $+\infty$. In the past it was proposed to add 5 to each normit to obtain "probits". This facilitated the hand-performed calculations because negative values were avoided. With the arrival of computers the need to add 5 to the normits has disappeared. The term "normit method" would therefore be better for the method described below. However, since the term "probit analysis" is so widely spread, the term will, for historical reasons, be maintained in this text.

Once the responses have been linearised, it should be possible to apply the parallel-line analysis as described in Section 3.2. Unfortunately, the validity condition of homogeneity of variance for each dose is not fulfilled. The variance is minimal at normit = 0 and increases for positive and negative values of the normit. It is therefore necessary to give more weight to responses in the middle part of the curve, and less weight to the more extreme parts of the curve. This method, the analysis of variance, and the estimation of the potency and confidence interval are described below.

4.2.1. TABULATION OF THE RESULTS

Table 4.2.1.-I is used to enter the data into the columns indicated by numbers:

(1) the dose of the standard or the test preparation,

(2) the number n of units submitted to that treatment,

(3) the number of units r giving a positive response to the treatment,

(4) the logarithm x of the dose,

(5) the fraction $p = r/n$ of positive responses per group.

The first cycle starts here.

(6) column Y is filled with zeros at the first iteration,

(7) the corresponding value $\Phi = \Phi(Y)$ of the cumulative standard normal distribution function (see also Table 8.4).

The columns (8) to (10) are calculated with the following formulae:

(8) $$Z = \frac{e^{-Y^2/2}}{\sqrt{2\pi}} \qquad (4.2.1.-1)$$

(9) $$y = Y + \frac{p - \Phi}{Z} \qquad (4.2.1.-2)$$

(10) $$w = \frac{nZ^2}{\Phi - \Phi^2} \qquad (4.2.1.-3)$$

The columns (11) to (15) can easily be calculated from columns (4), (9) and (10) as wx, wy, wx^2, wy^2 and wxy respectively, and the sum (Σ) of each of the columns (10) to (15) is calculated separately for each of the preparations.

The sums calculated in Table 4.2.1.-I are transferred to columns (1) to (6) of Table 4.2.1.-II and 6 additional columns (7) to (12) are calculated as follows:

(7) $$S_{xx} = \sum wx^2 - \frac{(\sum wx)^2}{\sum w} \qquad (4.2.1.-4)$$

(8) $$S_{xy} = \sum wxy - \frac{(\sum wx)(\sum wy)}{\sum w} \qquad (4.2.1.-5)$$

(9) $$S_{yy} = \sum wy^2 - \frac{(\sum wy)^2}{\sum w} \qquad (4.2.1.-6)$$

(10) $$\bar{x} = \frac{\sum wx}{\sum w} \qquad (4.2.1.-7)$$

(11) $$\bar{y} = \frac{\sum wy}{\sum w} \qquad (4.2.1.-8)$$

The common slope b can now be obtained as:

$$b = \frac{\sum S_{xy}}{\sum S_{xx}} \qquad (4.2.1.-9)$$

and the intercept a of the standard, and similarly for the test preparations is obtained as:

(12) $$a = \bar{y} - b\bar{x} \qquad (4.2.1.-10)$$

Column (6) of the first working table can now be replaced by $Y = a + bx$ and the cycle is repeated until the difference between 2 cycles has become small (e.g. the maximum

Table 4.2.1.-I. – *First working table*

	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)	(14)	(15)
	dose	n	r	x	p	Y	Φ	Z	y	w	wx	wy	wx^2	wy^2	wxy
S	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
										Σ =	Σ =	Σ =	Σ =	Σ =	Σ =
T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
										Σ =	Σ =	Σ =	Σ =	Σ =	Σ =
etc.															

Table 4.2.1.-II. – *Second working table*

	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)
	Σw	Σwx	Σwy	Σwx^2	Σwy^2	Σwxy	S_{xx}	S_{xy}	S_{yy}	$\bar{x}$	$\bar{y}$	a
S	.	.	.	.	.	.	.	.	.	.	.	.
T	.	.	.	.	.	.	.	.	.	.	.	.
etc.	.	.	.	.	.	.	.	.	.	.	.	.
							Σ =	Σ =				

difference of Y between 2 consecutive cycles is smaller than 10^{-8}).

4.2.2. TESTS OF VALIDITY

Before calculating the potencies and confidence intervals, validity of the assay must be assessed. If at least 3 doses for each preparation have been included, the deviations from linearity can be measured as follows: add a 13th column to Table 4.2.1.-II and fill it with:

$$S_{yy} - \frac{S_{xy}^2}{S_{xx}} \qquad (4.2.2.\text{-}1)$$

The column total is a measure of deviations from linearity and is approximately χ^2 distributed with degrees of freedom equal to $N - 2h$. Significance of this value may be assessed with the aid of Table 8.3 or a suitable sub-routine in a computer program. If the value is significant at the 0.05 probability level, the assay must probably be rejected (see Section 4.2.4).

When the above test gives no indication of significant deviations from linear regression, the deviations from parallelism are tested at the 0.05 significance level with:

$$\chi^2 = \sum \frac{S_{xy}^2}{S_{xx}} - \frac{(\sum S_{xy})^2}{\sum S_{xx}} \qquad (4.2.2.\text{-}2)$$

with $h - 1$ degrees of freedom.

4.2.3. ESTIMATION OF POTENCY AND CONFIDENCE LIMITS

When there are no indications for a significant departure from parallelism and linearity the ln(potency ratio) M'_T is calculated as:

$$M'_T = \frac{a_T - a_S}{b} \qquad (4.2.3.\text{-}1)$$

and the antilogarithm is taken. Now let $t = 1.96$ and $s = 1$. Confidence limits are calculated as the antilogarithms of:

$$CM'_T - (C-1)(\bar{x}_S - \bar{x}_T) \pm \sqrt{(C-1)\left(V \sum S_{xx} + C(M'_T - \bar{x}_S + \bar{x}_T)^2\right)} \qquad (4.2.3.\text{-}2)$$

where $C = \frac{b^2 \sum S_{xx}}{b^2 \sum S_{xx} - s^2 t^2}$ and $V = \frac{1}{\sum_S w} + \frac{1}{\sum_T w}$

4.2.4. INVALID ASSAYS

If the test for deviations from linearity described in Section 4.2.2 is significant, the assay should normally be rejected. If there are reasons to retain the assay, the formulae are slightly modified. t becomes the t-value ($p = 0.05$) with the same number of degrees of freedom as used in the check for linearity and s^2 becomes the χ^2 value divided by the same number of degrees of freedom (and thus typically is greater than 1).

The test for parallelism is also slightly modified. The χ^2 value for non-parallelism is divided by its number of degrees of freedom. The resulting value is divided by s^2 calculated above to obtain an F-ratio with h - 1 and N - $2h$ degrees of freedom, which is evaluated in the usual way at the 0.05 significance level.

4.3. THE LOGIT METHOD

As indicated in Section 4.1 the logit method may sometimes be more appropriate. The name of the method is derived from the logit function which is the inverse of the logistic distribution. The procedure is similar to that described for the probit method with the following modifications in the formulae for Φ and Z.

$$\Phi = \frac{1}{1+e^{-Y}} \qquad (4.3.-1)$$

$$Z = \frac{e^{-Y}}{(1+e^{-Y})^2} \qquad (4.3.-2)$$

4.4. OTHER SHAPES OF THE CURVE

The probit and logit method are almost always adequate for the analysis of quantal responses called for in the European Pharmacopoeia. However, if it can be made evident that the ln(dose)-response curve has another shape than the 2 curves described above, another curve Φ may be adopted. Z is then taken to be the first derivative of Φ.

For example, if it can be shown that the curve is not symmetric, the Gompertz distribution may be appropriate (the gompit method) in which case $\Phi = 1 - e^{-e^{Y}}$ and $Z = e^{Y-e^{Y}}$.

4.5. THE MEDIAN EFFECTIVE DOSE

In some types of assay it is desirable to determine a median effective dose which is the dose that produces a response in 50 per cent of the units. The probit method can be used to determine this median effective dose (ED_{50}), but since there is no need to express this dose relative to a standard, the formulae are slightly different.

Note A standard can optionally be included in order to validate the assay. Usually the assay is considered valid if the calculated ED_{50} of the standard is close enough to the assigned ED_{50}. What "close enough" in this context means depends on the requirements specified in the monograph.

The tabulation of the responses to the test samples, and optionally a standard, is as described in Section 4.2.1. The test for linearity is as described in Section 4.2.2. A test for parallelism is not necessary for this type of assay. The ED_{50} of test sample T, and similarly for the other samples, is obtained as described in Section 4.2.3, with the following modifications in formulae 4.2.3.-1 and 4.2.3.-2:

$$M'_T = \frac{-a_T}{b} \qquad (4.5.-1)$$

$$GM'_T - (C-1)(\bar{x}_T) \pm \sqrt{(C-1)\left(V\sum S_{xx} + C(M'_T - \bar{x}_T)^2\right)} \qquad (4.5.-2)$$

where $V = \frac{1}{\sum_T w}$ and C is left unchanged

5. EXAMPLES

This section consists of worked examples illustrating the application of the formulae. The examples have been selected primarily to illustrate the statistical method of calculation. They are not intended to reflect the most suitable method of assay, if alternatives are permitted in the individual monographs. To increase their value as program checks, more decimal places are given than would usually be necessary. It should also be noted that other, but equivalent methods of calculation exist. These methods should lead to exactly the same final results as those given in the examples.

5.1. PARALLEL-LINE MODEL

5.1.1. TWO-DOSE MULTIPLE ASSAY WITH COMPLETELY RANDOMISED DESIGN

An assay of corticotrophin by subcutaneous injection in rats

The standard preparation is administered at 0.25 and 1.0 units per 100 g of body mass. Two preparations to be examined are both assumed to have a potency of 1 unit per milligram and they are administered in the same quantities as the standard. The individual responses and means per treatment are given in Table 5.1.1.-I. A graphical presentation (Figure 5.1.1.-I) gives no rise to doubt the homogeneity of variance and normality of the data, but suggests problems with parallelism for preparation *U*.

Table 5.1.1.-I. *Response metameter y: mass of ascorbic acid (mg) per 100 g of adrenal gland*

	Standard S		Preparation T		Preparation U	
	S_1	S_2	T_1	T_2	U_1	U_2
	300	289	310	230	250	236
	310	221	290	210	268	213
	330	267	360	280	273	283
	290	236	341	261	240	269
	364	250	321	241	307	251
	328	231	370	290	270	294
	390	229	303	223	317	223
	360	269	334	254	312	250
	342	233	295	216	320	216
	306	259	315	235	265	265
Mean	332.0	248.4	323.9	244.0	282.2	250.0

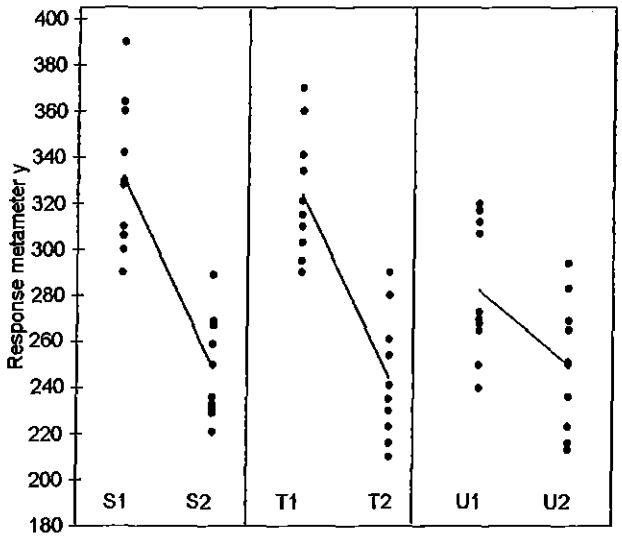


Figure 5.1.1.-I.

The formulae in Tables 3.2.3.-I and 3.2.3.-II lead to:

P_S	=	580.4	L_S	=	−41.8
P_T	=	567.9	L_T	=	−39.95
P_U	=	532.2	L_U	=	−16.1
H_P	=	$\frac{10}{2} = 5$	H_L	=	$\frac{120}{6} = 20$

The analysis of variance can now be completed with the formulae in Tables 3.2.3-III and 3.2.3.-IV. This is shown in Table 5.1.1.-II.

Table 5.1.1.-II. *Analysis of variance*

Source of variation	Degrees of freedom	Sum of squares	Mean square	*F*-ratio	Probability
Preparations	2	6256.6	3128.3		
Regression	1	63 830.8	63 830.8	83.38	0.000
Non-parallelism	2	8218.2	4109.1	5.37	0.007
Treatments	5	78 305.7			
Residual error	54	41 340.9	765.57		
Total	59	119 646.6			

The analysis confirms a highly significant linear regression. Departure from parallelism, however, is also significant ($p = 0.0075$) which was to be expected from the graphical observation that preparation *U* is not parallel to the standard. This preparation is therefore rejected and the analysis repeated using only preparation *T* and the standard (Table 5.1.1.-III).

Table 5.1.1.-III. *Analysis of variance without sample U*

Source of variation	Degrees of freedom	Sum of squares	Mean square	*F*-ratio	Probability
Preparations	1	390.6	390.6		
Regression	1	66 830.6	66 830.6	90.5	0.000
Non-parallelism	1	34.2	34.2	0.05	0.831
Treatments	3	67 255.5			
Residual error	36	26 587.3	738.54		
Total	39	93 842.8			

The analysis without preparation *U* results in compliance with the requirements with respect to both regression and parallelism and so the potency can be calculated.
The formulae in Section 3.2.5 give:
— for the common slope:

$$b = \frac{20(-41.8 - 39.95)}{\ln 4 \times 10 \times 2} = -58.970$$

— the ln(potency ratio) is:

$$M'_T = \frac{567.9 - 580.4}{2 \times (-58.970)} = 0.1060$$

$$C = \frac{66\,830.6}{66\,830.6 - 738.54 \times 2.028^2} = 1.0476$$

$$V = \frac{66\,830.6}{(-58.970)^2 \times 2 \times 10} = 0.9609$$

— and ln(confidence limits) are:

$$1.0476 \times 0.1060 \pm \sqrt{0.0476 \times (1.0476 \times 0.1060^2 + 2 \times 0.9609)} = 0.1110 \pm 0.3034$$

By taking the antilogarithms we find a potency ratio of 1.11 with 95 per cent confidence limits from 0.82-1.51.

Multiplying by the assumed potency of preparation *T* yields a potency of 1.11 units/mg with 95 per cent confidence limits from 0.82 to 1.51 units/mg.

5.1.2. THREE-DOSE LATIN SQUARE DESIGN

Antibiotic agar diffusion assay using a rectangular tray

The standard has an assigned potency of 4855 IU/mg. The test preparation has an assumed potency of 5600 IU/mg. For the stock solutions 25.2 mg of the standard is dissolved in 24.5 mL of solvent and 21.4 mg of the test preparation is dissolved in 23.95 mL of solvent. The final solutions are prepared by first diluting both stock solutions to 1/20 and further using a dilution ratio of 1.5.

A Latin square is generated with the method described in Section 8.6 (see Table 5.1.2.-I). The responses of this routine assay are shown in Table 5.1.2.-II (inhibition zones in mm × 10). The treatment mean values are shown in Table 5.1.2.-III. A graphical representation of the data (see Figure 5.1.2.-I) gives no rise to doubt the normality or homogeneity of variance of the data.

The formulae in Tables 3.2.3.-I and 3.2.3.-II lead to:

$$P_S = 529.667 \qquad L_S = 35.833$$
$$P_T = 526.333 \qquad L_T = 39.333$$
$$H_P = \frac{6}{3} = 2 \qquad H_L = \frac{72}{24} = 3$$

The analysis of variance can now be completed with the formulae in Tables 3.2.3.-III and 3.2.3.-IV. The result is shown in Table 5.1.2.-IV.

The analysis shows significant differences between the rows. This indicates the increased precision achieved by using a Latin square design rather than a completely randomised design. A highly significant regression and no significant departure of the individual regression lines from parallelism and linearity confirms that the assay is satisfactory for potency calculations.

Table 5.1.2.-I. *Distribution of treatments over the plate*

	1	2	3	4	5	6
1	S_1	T_1	T_2	S_3	S_2	T_3
2	T_1	T_3	S_1	S_2	T_2	S_3
3	T_2	S_3	S_2	S_1	T_3	T_1
4	S_3	S_2	T_3	T_1	S_1	T_2
5	S_2	T_2	S_3	T_3	T_1	S_1
6	T_3	S_1	T_1	T_2	S_3	S_2

Table 5.1.2.-II. *Measured inhibition zones in mm × 10*

	1	2	3	4	5	6	Row mean
1	161	160	178	187	171	194	175.2 = R_1
2	151	192	150	172	170	192	171.2 = R_2
3	162	195	174	161	193	151	172.7 = R_3
4	194	184	199	160	163	171	178.5 = R_4
5	176	181	201	202	154	151	177.5 = R_5
6	193	166	161	186	198	182	181.0 = R_6
Col. Mean	172.8 = C_1	179.7 = C_2	177.2 = C_3	178.0 = C_4	174.8 = C_5	173.5 = C_6	

Table 5.1.2.-III. *Means of the treatments*

	Standard *S*			Preparation *T*		
	S_1	S_2	S_3	T_1	T_2	T_3
Mean	158.67	176.50	194.50	156.17	174.67	195.50

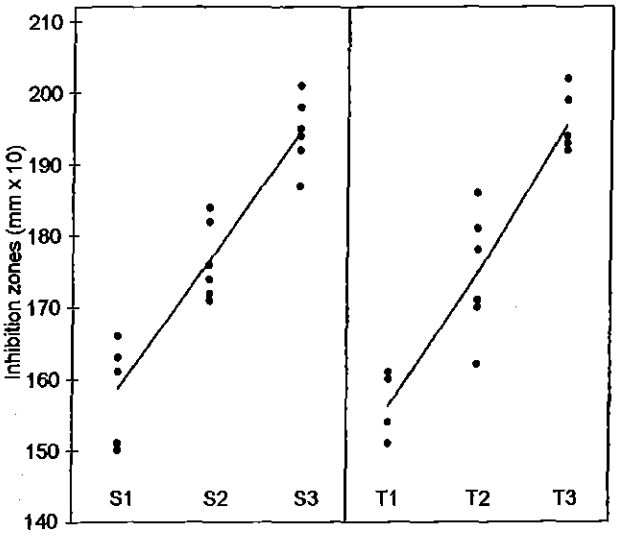


Figure 5.1.2.-I.

Table 5.1.2.-IV. *Analysis of variance*

Source of variation	Degrees of freedom	Sum of squares	Mean square	*F*-ratio	Probability
Preparations	1	11.1111	11.1111		
Regression	1	8475.0417	8475.0417	408.1	0.000
Non-parallelism	1	18.3750	18.3750	0.885	0.358
Non-linearity	2	5.4722	2.7361	0.132	0.877
Treatments	5	8510			
Rows	5	412	82.40	3.968	0.012
Columns	5	218.6667	43.73	2.106	0.107
Residual error	20	415.3333	20.7667		
Total	35	9556			

The formulae in Section 3.2.5 give:

— for the common slope:

$$b = \frac{3 \times (35.833 + 39.333)}{\ln(1.5) \times 6 \times 2} = 46.346$$

— the ln(potency ratio) is:

$$M'_T = \frac{526.333 - 529.667}{3 \times 46.346} = -0.023974$$

$$C = \frac{8475.0417}{8475.0417 - 20.7667 \times 2.086^2} = 1.0108$$

$$V = \frac{8475.0417}{46.346^2 \times 3 \times 6} = 0.2192$$

— and ln(confidence limits) are:

$$1.0108 \times (-0.0240) \pm \sqrt{0.0108 \times (1.0108 \times (-0.0240)^2 + 2 \times 0.2192)}$$

$$= -0.02423 \pm 0.06878$$

The potency ratio is found by taking the antilogarithms, resulting in 0.9763 with 95 per cent confidence limits from 0.9112-1.0456.

A correction factor of:

$$\frac{4855 \times 25.2/24.5}{5600 \times 21.4/23.95} = 0.99799$$

is necessary because the dilutions were not exactly equipotent on the basis of the assumed potency. Multiplying by this correction factor and the assumed potency of 5600 IU/mg yields a potency of 5456 IU/mg with 95 per cent confidence limits from 5092 to 5843 IU/mg.

5.1.3. FOUR-DOSE RANDOMISED BLOCK DESIGN

Antibiotic turbidimetric assay

This assay is designed to assign a potency in international units per vial. The standard has an assigned potency of 670 IU/mg. The test preparation has an assumed potency of 20 000 IU/vial. On the basis of this information the stock solutions are prepared as follows. 16.7 mg of the standard is dissolved in 25 mL solvent and the contents of one vial of the test preparation are dissolved in 40 mL solvent. The final solutions are prepared by first diluting to 1/40 and further using a dilution ratio of 1.5. The tubes are placed in a water-bath in a randomised block arrangement (see Section 8.5). The responses are listed in Table 5.1.3.-I.

Inspection of Figure 5.1.3.-I gives no rise to doubt the validity of the assumptions of normality and homogeneity of variance of the data. The standard deviation of S_3 is somewhat high but is no reason for concern.

Table 5.1.3.-I. *Absorbances of the suspensions (× 1000)*

	Standard *S*				Preparation *T*				
Block	S_1	S_2	S_3	S_4	T_1	T_2	T_3	T_4	Mean
1	252	207	168	113	242	206	146	115	181.1
2	249	201	187	107	236	197	153	102	179.0
3	247	193	162	111	246	197	148	104	176.0
4	250	207	155	108	231	191	159	106	175.9
5	235	207	140	98	232	186	146	95	167.4
Mean	246.6	203.0	162.4	107.4	237.4	195.4	150.4	104.4	

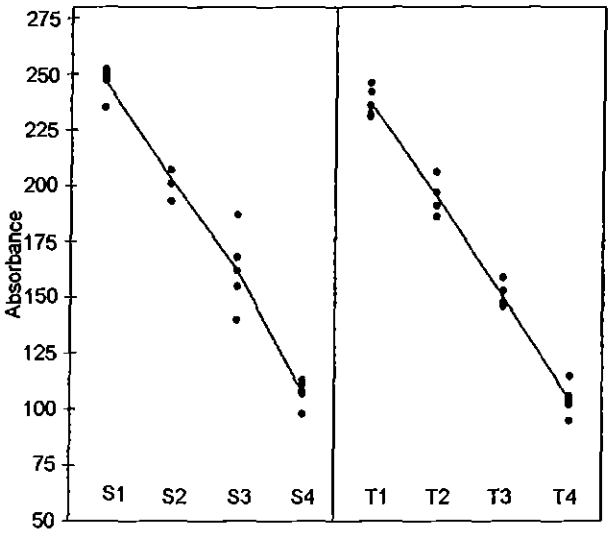


Figure 5.1.3.-I.

The formulae in Tables 3.2.3.-I and 3.2.3.-II lead to:

$P_S = 719.4$ $L_S = -229.1$
$P_T = 687.6$ $L_T = -222$
$H_P = \frac{5}{4} = 1.25$ $H_L = \frac{60}{60} = 1$

The analysis of variance is constructed with the formulae in Tables 3.2.3.-III and 3.2.3.-IV. The result is shown in Table 5.1.3.-II.

Table 5.1.3.-II. *Analysis of variance*

Source of variation	Degrees of freedom	Sum of squares	Mean square	*F*-ratio	Probability
Preparations	1	632.025	632.025		
Regression	1	101 745.6	101 745.6	1887.1	0.000
Non-parallelism	1	25.205	25.205	0.467	0.500
Non-linearity	4	259.14	64.785	1.202	0.332
Treatments	7	102 662			
Blocks	4	876.75	219.188	4.065	0.010
Residual error	28	1509.65	53.916		
Total	39	105 048.4			

A significant difference is found between the blocks. This indicates the increased precision achieved by using a randomised block design. A highly significant regression and no significant departure from parallelism and linearity confirms that the assay is satisfactory for potency calculations. The formulae in Section 3.2.5 give:
— for the common slope:

$$b = \frac{1 \times (-229.1 - 222)}{\ln(1.5) \times 5 \times 2} = 111.255$$

— the ln(potency ratio) is:

$$M'_T = \frac{687.6 - 719.4}{4 \times (-111.255)} = 0.071457$$

$$C = \frac{101\ 745.6}{101\ 745.6 - 53.916 \times 2.048^2} = 1.00223$$

$$V = \frac{101\ 745.6}{(-111.255)^2 \times 4 \times 5} = 0.4110$$

— and ln(confidence limits) are:

$$1.00223 \times 0.0715 \pm \sqrt{0.00223 \times (1.00223 \times 0.0715^2 + 2 \times 0.4110)}$$

$$= 0.07162 \pm 0.04293$$

The potency ratio is found by taking the antilogarithms, resulting in 1.0741 with 95 per cent confidence limits from 1.0291 to 1.1214. A correction factor of:

$$\frac{670 \times 16.7/25}{20\ 000 \times 1/40} = 0.89512$$

is necessary because the dilutions were not exactly equipotent on the basis of the assumed potency. Multiplying by this correction factor and the assumed potency of 20 000 IU/vial yields a potency of 19 228 IU/vial with 95 per cent confidence limits from 18 423-20 075 IU/vial.

5.1.4. FIVE-DOSE MULTIPLE ASSAY WITH COMPLETELY RANDOMISED DESIGN

An in-vitro assay of three hepatitis B vaccines against a standard

Three independent two-fold dilution series of 5 dilutions were prepared from each of the vaccines. After some additional steps in the assay procedure, absorbances were measured. They are shown in Table 5.1.4.-I.

Table 5.1.4.-I. *Optical densities*

Dilution	Standard *S*			Preparation *T*		
1:16 000	0.043	0.045	0.051	0.097	0.097	0.094
1:8000	0.093	0.099	0.082	0.167	0.157	0.178
1:4000	0.159	0.154	0.166	0.327	0.355	0.345
1:2000	0.283	0.295	0.362	0.501	0.665	0.576
1:1000	0.514	0.531	0.545	1.140	1.386	1.051

Dilution	Preparation *U*			Preparation *V*		
1:16 000	0.086	0.071	0.073	0.082	0.082	0.086
1:8000	0.127	0.146	0.133	0.145	0.144	0.173
1:4000	0.277	0.268	0.269	0.318	0.306	0.316
1:2000	0.586	0.489	0.546	0.552	0.551	0.624
1:1000	0.957	0.866	1.045	1.037	1.039	1.068

The logarithms of the optical densities are known to have a linear relationship with the logarithms of the doses.
The mean responses of the ln-transformed optical densities are listed in Table 5.1.4.-II. No unusual features are discovered in a graphical presentation of the data (Figure 5.1.4.-I).

Table 5.1.4.-II. *Means of the ln-transformed absorbances*

S_1	−3.075	T_1	−2.344	U_1	−2.572	V_1	−2.485
S_2	−2.396	T_2	−1.789	U_2	−2.002	V_2	−1.874
S_3	−1.835	T_3	−1.073	U_3	−1.305	V_3	−1.161
S_4	−1.166	T_4	−0.550	U_4	−0.618	V_4	−0.554
S_5	−0.635	T_5	0.169	U_5	−0.048	V_5	0.047

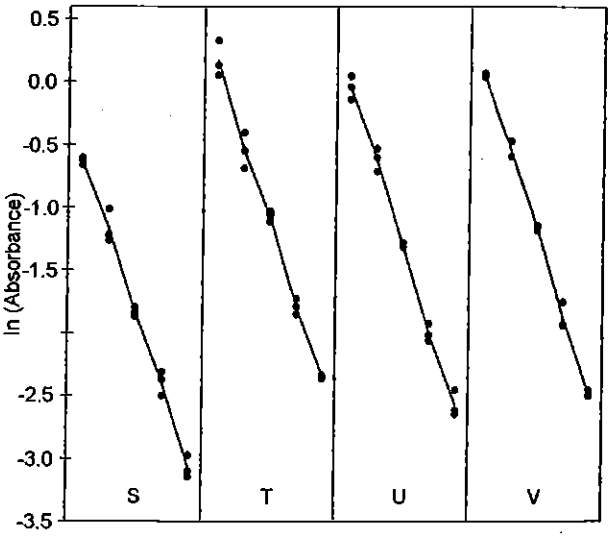


Figure 5.1.4.-I.

The formulae in Tables 3.2.3.-I and 3.2.3.-II give:

$P_S = -9.108$ $L_S = 6.109$
$P_T = -5.586$ $L_T = 6.264$
$P_U = -6.544$ $L_U = 6.431$
$P_V = -6.027$ $L_V = 6.384$
$H_P = \frac{3}{5} = 0.6$ $H_L = \frac{36}{120} = 0.3$

The analysis of variance is completed with the formulae in Tables 3.2.3.-III and 3.2.3.-IV. This is shown in Table 5.1.4.-III.

Table 5.1.4.-III. *Analysis of variance*

Source of variation	Degrees of freedom	Sum of squares	Mean square	*F*-ratio	Probability
Preparations	3	4.475	1.492		
Regression	1	47.58	47.58	7126	0.000
Non-parallelism	3	0.0187	0.006	0.933	0.434
Non-linearity	12	0.0742	0.006	0.926	0.531
Treatments	19	52.152			
Residual error	40	0.267	0.0067		
Total	59	52.42			

A highly significant regression and a non-significant departure from parallelism and linearity confirm that the potencies can be safely calculated. The formulae in Section 3.2.5 give:

— for the common slope:

$$b = \frac{0.3 \times (6.109 + 6.264 + 6.431 + 6.384)}{\ln 2 \times 3 \times 4} = 0.90848$$

— the ln(potency ratio) for preparation T is:

$$M'_T = \frac{-5.586 - (-9.108)}{5 \times 0.90848} = 0.7752$$

$$C = \frac{47.58}{47.58 - 0.0067 \times 2.021^2} = 1.00057$$

$$V = \frac{47.58}{0.9085^2 \times 5 \times 3} = 3.8436$$

— and ln(confidence limits) for preparation T are:

$$1.00057 \times 0.7752 \pm$$

$$\sqrt{0.00057 \times (1.00057 \times 0.7752^2 + 2 \times 3.8436)}$$

$$= 0.7756 \pm 0.0689$$

By taking the antilogarithms a potency ratio of 2.171 is found with 95 per cent confidence limits from 2.027 to 2.327. All samples have an assigned potency of 20 µg protein/mL and so a potency of 43.4 µg protein/mL is found for test preparation T with 95 per cent confidence limits from 40.5-46.5 µg protein/mL.

The same procedure is followed to estimate the potency and confidence interval of the other test preparations. The results are listed in Table 5.1.4.-IV.

Table 5.1.4.-IV. *Final potency estimates and 95 per cent confidence intervals of the test vaccines (in µg protein/mL)*

	Lower limit	Estimate	Upper limit
Vaccine *T*	40.5	43.4	46.5
Vaccine *U*	32.9	35.2	37.6
Vaccine *V*	36.8	39.4	42.2

5.1.5. TWIN CROSS-OVER DESIGN

Assay of insulin by subcutaneous injection in rabbits

The standard preparation was administered at 1 unit and 2 units per millilitre. Equivalent doses of the unknown preparation were used based on an assumed potency of 40 units per millilitre. The rabbits received subcutaneously 0.5 mL of the appropriate solutions according to the design in Table 5.1.5.-I and responses obtained are shown in Table 5.1.5.-II and Figure 5.1.5.-I. The large variance illustrates the variation between rabbits and the need to employ a cross-over design.

Table 5.1.5.-I. *Arrangements of treatments*

	Group of rabbits			
	1	2	3	4
Day 1	S_1	S_2	T_1	T_2
Day 2	T_2	T_1	S_2	S_1

Table 5.1.5.-II. *Response y: sum of blood glucose readings (mg/100 mL) at 1 h and 2.5 h*

	Group 1		Group 2		Group 3		Group 4	
	S_1	T_2	S_2	T_1	T_1	S_2	T_2	S_1
	112	104	65	72	105	91	118	144
	126	112	116	160	83	67	119	149
	62	58	73	72	125	67	42	51
	86	63	47	93	56	45	64	107
	52	53	88	113	92	84	93	117
	110	113	63	71	101	56	73	128
	116	91	50	65	66	55	39	87
	101	68	55	100	91	68	31	71
Mean	95.6	82.8	69.6	93.3	89.9	66.6	72.4	106.8

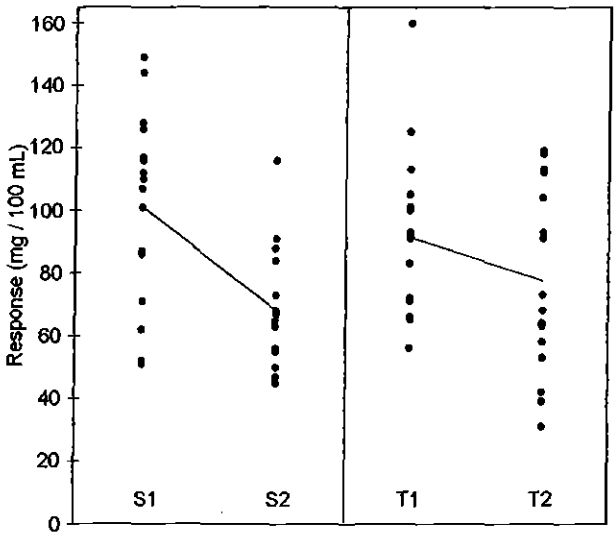


Figure 5.1.5.-I.

The analysis of variance is more complicated for this assay than for the other designs given because the component of the sum of squares due to parallelism is not independent of the component due to rabbit differences. Testing of the

parallelism of the regression lines involves a second error-mean-square term obtained by subtracting the parallelism component and 2 "interaction" components from the component due to rabbit differences.

Three "interaction" components are present in the analysis of variance due to replication within each group:

days × preparation; days × regression; days × parallelism.

These terms indicate the tendency for the components (preparations, regression and parallelism) to vary from day to day. The corresponding F-ratios thus provide checks on these aspects of assay validity. If the values of F obtained are significantly high, care should be exercised in interpreting the results of the assay and, if possible, the assay should be repeated.

The analysis of variance is constructed by applying the formulae given in Tables 3.2.3.-I to 3.2.3.-III separately for both days and for the pooled set of data. The formulae in Tables 3.2.3.-I and 3.2.3.-II give:

Day 1:	P_S	=	165.25	L_S	=	−13
	P_T	=	162.25	L_T	=	−8.75
	H_P	=	$\frac{8}{2}=4$	H_L	=	$\frac{96}{6}=16$
Day 2:	P_S	=	173.38	L_S	=	−20.06
	P_T	=	176.00	L_T	=	−5.25
	H_P	=	$\frac{8}{2}=4$	H_L	=	$\frac{96}{6}=16$
Pooled:	P_S	=	169.31	L_S	=	−16.53
	P_T	=	169.13	L_T	=	−7.00
	H_P	=	$\frac{16}{2}=8$	H_L	=	$\frac{192}{6}=32$

and with the formulae in Table 3.2.3.-III this leads to:

Day 1			Day 2			Pooled		
SS_{prep}	=	18.000	SS_{prep}	=	13.781	SS_{prep}	=	0.141
SS_{reg}	=	3784.5	SS_{reg}	=	5125.8	SS_{reg}	=	8859.5
SS_{par}	=	144.5	SS_{par}	=	1755.3	SS_{par}	=	1453.5

The interaction terms are found as Day 1 + Day 2 - Pooled.

$$SS_{\text{days}\times\text{prep}} = 31.64$$

$$SS_{\text{days}\times\text{reg}} = 50.77$$

$$SS_{\text{days}\times\text{par}} = 446.27$$

In addition the sum of squares due to day-to-day variation is calculated as:

$$SS_{\text{days}} = \frac{1}{2}N(D_1^2+D_2^2) - K = 478.52$$

and the sum of squares due to blocks (the variation between rabbits) as:

$$SS_{\text{block}} = 2\sum B_i^2 - K = 39\,794.7$$

where B_i is the mean response per rabbit.

The analysis of variance can now be completed as shown in Table 5.1.5.-III.

Table 5.1.5.-III. *Analysis of variance*

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-ratio	Probability
Non-parallelism	1	1453.5	1453.5	1.064	0.311
Days × Prep.	1	31.6	31.6	0.023	0.880
Days × Regr.	1	50.8	50.8	0.037	0.849
Residual error between rabbits	28	38 258.8	1366.4		
Rabbits	31	39 794.7	1283.7		
Preparations	1	0.14	0.14	0.001	0.975
Regression	1	8859.5	8859.5	64.532	0.000
Days	1	478.5	478.5	3.485	0.072
Days × non-par.	1	446.3	446.3	3.251	0.082
Residual error within rabbits	28	3844.1	137.3		
Total	63	53 423.2			

The analysis of variance confirms that the data fulfil the necessary conditions for a satisfactory assay: a highly significant regression, no significant departures from parallelism, and none of the three interaction components is significant.

The formulae in Section 3.2.5 give:

— for the common slope:

$$b = \frac{32\times(-16.53-7)}{\ln 2\times 16\times 2} = -33.95$$

— the ln(potency ratio) is:

$$M'_T = \frac{169.13-169.31}{2\times(-33.95)} = 0.00276$$

$$C = \frac{8859.5}{8859.5 - 137.3\times 2.048^2} = 1.0695$$

$$V = \frac{8859.5}{(-33.95)^2\times 2\times 16} = 0.2402$$

— and ln(confidence limits) are:

$$1.0695\times 0.00276 \pm \sqrt{0.0695\times(1.0695\times 0.00276^2 + 2\times 0.2402)} = 0.00295 \pm 0.18279$$

By taking the antilogarithms a potency ratio of 1.003 with 95 per cent confidence limits from 0.835 to 1.204 is found. Multiplying by A_T = 40 yields a potency of 40.1 units per millilitre with 95 per cent confidence limits from 33.4-48.2 units per millilitre.

5.2. SLOPE-RATIO MODEL

5.2.1. A COMPLETELY RANDOMISED (0,3,3)-DESIGN

An assay of factor VIII

A laboratory carries out a chromogenic assay of factor VIII activity in concentrates. The laboratory has no experience with the type of assay but is trying to make it operational. Three equivalent dilutions are prepared of both the standard and the test preparation. In addition a blank is prepared, although a linear dose-response relationship is not expected

for low doses. 8 replicates of each dilution are prepared, which is more than would be done in a routine assay.

A graphical presentation of the data shows clearly that the dose-response relationship is indeed not linear at low doses. The responses to blanks will therefore not be used in the calculations (further assays are of course needed to justify this decision). The formulae in Tables 3.3.3.1.-I and 3.3.3.1.-II yield:

$P_S = 0.6524$ $P_T = 0.5651$
$L_S = 1.4693$ $L_T = 1.2656$
$a_S = 0.318$ $a_T = 0.318$
$b_S = 0.329$ $b_T = 0.271$
$G_S = 0.1554$ $G_T = 0.1156$
$J_S = 4.17 \cdot 10^{-8}$ $J_T = 2.84 \cdot 10^{-6}$

And

$H_I = 0.09524$ $a' = 0.05298$ $K = 1.9764$

and the analysis of variance is completed with the formulae in Tables 3.3.3.1.-III and 3.3.3.1.-IV.

A highly significant regression and no significant deviations from linearity and intersection indicate that the potency can be calculated.

Slope of standard:

$$b'_S = \frac{6 \times 1.469 - 36 \times 0.0530}{84} = 0.0822$$

Slope of test sample:

$$b'_T = \frac{6 \times 1.266 - 36 \times 0.0530}{84} = 0.0677$$

Formula 3.3.5.1.-3 gives:

$$R = \frac{0.0677}{0.0822} = 0.823$$

$$C = \frac{0.0822^2}{0.0822^2 - 3.86 \cdot 10^{-6} \times 2.018^2 \times 0.0357} = 1.000083$$

$$K' = 0.000083 \times 0.75 = 0.000062$$

and the 95 per cent confidence limits are:

$$0.823 \pm \sqrt{0.000083 \times 1.678 + 0.000062 \times (-1.646)} = 0.823 \pm 0.006$$

The potency ratio is thus estimated as 0.823 with 95 per cent confidence limits from 0.817 to 0.829.

Table 5.2.1.-I. *Absorbances*

	Blank	Standard S (in IU/mL)			Preparation T (in IU/mL)		
Conc.	B	S_1 0.01	S_2 0.02	S_3 0.03	T_1 0.01	T_2 0.02	T_3 0.03
	0.022	0.133	0.215	0.299	0.120	0.188	0.254
	0.024	0.133	0.215	0.299	0.119	0.188	0.253
	0.024	0.131	0.216	0.299	0.118	0.190	0.255
	0.026	0.136	0.218	0.297	0.120	0.190	0.258
	0.023	0.137	0.220	0.297	0.120	0.190	0.257
	0.022	0.136	0.220	0.305	0.121	0.191	0.257
	0.022	0.138	0.219	0.299	0.121	0.191	0.255
	0.023	0.137	0.218	0.302	0.121	0.190	0.254
Mean	0.0235	0.1351	0.2176	0.2996	0.1200	0.1898	0.2554

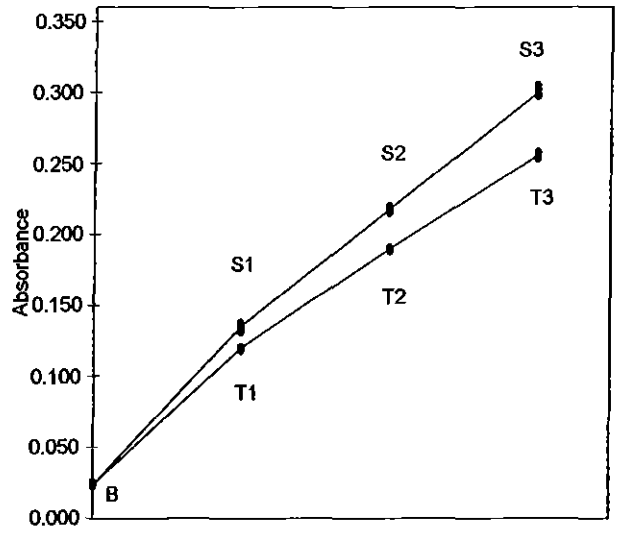


Figure 5.2.1.-I.

Table 5.2.1.-II. *Analysis of variance*

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-ratio	Probability
Regression	2	0.1917	0.0958	24 850	0.000
Intersection	1	$3 \cdot 10^{-9}$	$3 \cdot 10^{-9}$	$7 \cdot 10^{-4}$	0.978
Non-linearity	2	$2 \cdot 10^{-5}$	$1 \cdot 10^{-5}$	2.984	0.061
Treatments	5	0.1917			
Residual error	42	$1.62 \cdot 10^{-4}$	$3.86 \cdot 10^{-6}$		
Total	47	0.1919			

5.2.2. A COMPLETELY RANDOMISED (0,4,4,4)-DESIGN

An in-vitro assay of influenza vaccines

The haemagglutinin antigen (HA) content of 2 influenza vaccines is determined by single radial immunodiffusion. Both have a labelled potency of 15 µg HA per dose, which is

equivalent with a content of 30 µg HA/mL. The standard has an assigned content of 39 µg HA/mL.

Standard and test vaccines are applied in 4 duplicate concentrations which are prepared on the basis of the assigned and the labelled contents. When the equilibrium between the external and the internal reactant is established, the zone of the annulus precipitation area is measured. The results are shown in Table 5.2.2.-I.

Table 5.2.2.-I. *Zone of precipitation area (mm^2)*

Conc.	Standard *S*		Preparation *T*		Preparation *U*	
(µg/mL)	I	II	I	II	I	II
7.5	18.0	18.0	15.1	16.8	15.4	15.7
15.0	22.8	24.5	23.1	24.2	20.2	18.6
22.5	30.4	30.4	28.9	27.4	24.2	23.1
30.0	35.7	36.6	34.4	37.8	27.4	27.0

A graphical presentation of the data shows no unusual features (see Figure 5.2.2.-I). The formulae in Tables 3.3.3.1.-I and 3.3.3.1.-II yield:

P_S	=	108.2	P_T	=	103.85	P_U	=	85.8
L_S	=	301.1	L_T	=	292.1	L_U	=	234.1
a_S	=	141.0	a_T	=	116.7	a_U	=	139.8
b_S	=	61.2	b_T	=	64.95	b_U	=	39.2
G_S	=	3114.3	G_T	=	2909.4	G_U	=	1917.3
J_S	=	0.223	J_T	=	2.227	J_U	=	0.083

and

H_I	=	0.0093	a'	=	11.04	K	=	14 785.8

and the analysis of variance is completed with the formulae in Tables 3.3.3.1.-III and 3.3.3.1.-IV. This is shown in Table 5.2.2.-II.

A highly significant regression and no significant deviations from linearity and intersection indicate that the potency can be calculated.

Slope of standard:

$$b'_S = \frac{6 \times 301.1 - 60 \times 11.04}{180} = 6.356$$

Slope of *T* is:

$$b'_T = \frac{6 \times 292.1 - 60 \times 11.04}{180} = 6.056$$

Slope of *U* is:

$$b'_U = \frac{6 \times 234.1 - 60 \times 11.04}{180} = 4.123$$

This leads to a potency ratio of 6.056/6.356 = 0.953 for vaccine *T* and 4.123/6.356 = 0.649 for vaccine *U*.

$$C = \frac{6.356^2}{6.356^2 - 1.068 \times 2.179^2 \times 0.0444} = 1.0056$$

$$K' = 0.0056 \times 0.625 = 0.0035$$

And the confidence limits are found with formula 3.3.5.1.-4.

For vaccine *T*:

$$0.955 \pm \sqrt{0.0056 \times 1.913 + 0.0035 \times (-1.913)} = 0.955 \pm 0.063$$

For vaccine *U*:

$$0.649 \pm \sqrt{0.0056 \times 1.423 + 0.0035 \times (-1.301)} = 0.649 \pm 0.058$$

The HA content in µg/dose can be found by multiplying the potency ratios and confidence limits by the assumed content of 15 µg/dose. The results are given in Table 5.2.2.-III.

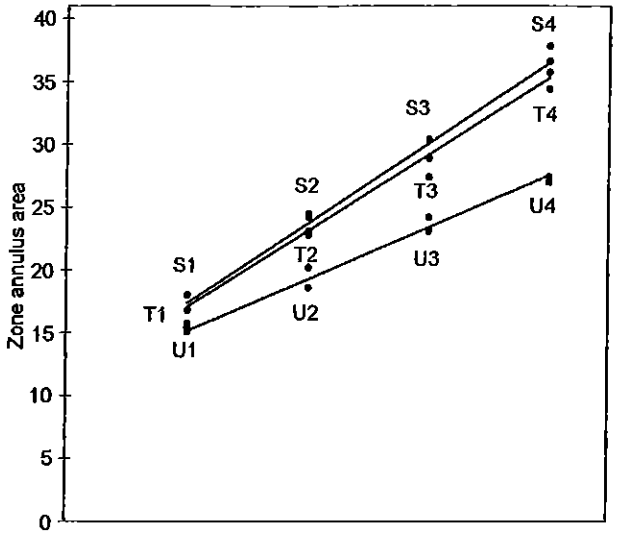


Figure 5.2.2.-I.

Table 5.2.2.-II. *Analysis of variance*

Source of variation	Degrees of freedom	Sum of squares	Mean square	*F*-ratio	Probability
Regression	3	1087.7	362.6	339.5	0.000
Intersection	2	3.474	1.737	1.626	0.237
Non-linearity	6	5.066	0.844	0.791	0.594
Treatments	11	1096.2			
Residual error	12	12.815	1.068		
Total	23	1109.0			

Table 5.2.2.-III. *Estimates of HA content* (µg/dose)

	Lower limit	Estimate	Upper limit
Vaccine *T*	13.4	14.3	15.3
Vaccine *U*	8.9	9.7	10.6

5.3. QUANTAL RESPONSES

5.3.1. PROBIT ANALYSIS OF A TEST PREPARATION AGAINST A REFERENCE

An in-vivo assay of a diphtheria vaccine

A diphtheria vaccine (assumed potency 140 IU/vial) is assayed against a standard (assigned potency 132 IU/vial). On the basis of this information, equivalent doses are prepared and randomly administered to groups of guinea-pigs. After a given period, the animals are challenged with

diphtheria toxin and the number of surviving animals recorded as shown in Table 5.3.1.-I.

Table 5.3.1.-I. *Raw data from a diphtheria assay in guinea-pigs*

Standard (*S*) Assigned potency 132 IU/vial			Test preparation (*T*) Assumed potency 140 IU/vial		
dose (IU/mL)	challenged	protected	dose (I.U./mL)	challenged	protected
1.0	12	0	1.0	11	0
1.6	12	3	1.6	12	4
2.5	12	6	2.5	11	8
4.0	11	10	4.0	11	10

The observations are transferred to the first working table and the subsequent columns are computed as described in Section 4.2.1. Table 5.3.1.-II shows the first cycle of this procedure.

The sums of the last 6 columns are then calculated per preparation and transferred to the second working table (see Table 5.3.1.-III). The results in the other columns are found with formulae 4.2.1.-4 to 4.2.1.-10. This yields a common slope b of 1.655.

The values for Y in the first working table are now replaced by $a + bx$ and a second cycle is carried out (see Table 5.3.1.-IV).

The cycle is repeated until the difference between 2 consecutive cycles has become small. The second working table should then appear as shown in Table 5.3.1.-V.

Linearity is tested as described in Section 4.2.2. The χ^2-value with 4 degrees of freedom is 0.851 + 1.070 = 1.921 representing a p-value of 0.750 which is not significant.

Since there are no significant deviations from linearity, the test for parallelism can be carried out as described in the same section. The χ^2-value with 1 degree of freedom is:

$$(16.71 + 17.27) - \frac{14.15^2}{5.89} = 0.001$$

representing a p-value of 0.974 which is not significant.

The ln(potency ratio) can now be estimated as described in Section 4.2.3.

$$M'_T = \frac{-1.721 - (-2.050)}{2.401} = 0.137$$

Further:

$$C = \frac{2.401^2 \times 5.893}{2.401^2 \times 5.893 - 1^2 \times 1.960^2} = 1.127$$

$$V = \frac{1}{18.37} + \frac{1}{17.96} = 0.110$$

So ln confidence limits are:

$$0.155 - 0.013 \pm \sqrt{0.127(0.649 + 1.127 \times 0.036^2)} = 0.142 \pm 0.288$$

The potency and confidence limits can now be found by taking the antilogarithms and multiplying these by the assumed potency of 140 IU/vial. This yields an estimate of 160.6 IU/vial with 95 per cent confidence limits from 121.0-215.2 IU/vial.

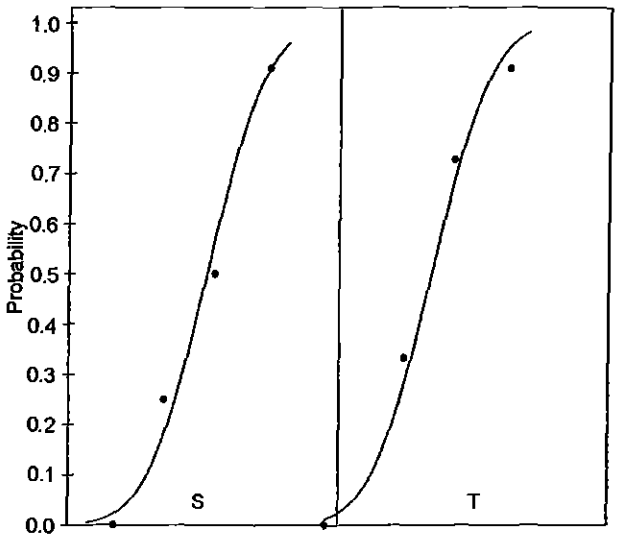


Figure 5.3.1.-I.

5.3.2. LOGIT ANALYSIS AND OTHER TYPES OF ANALYSES OF A TEST PREPARATION AGAINST A REFERENCE

Results will be given for the situation where the logit method and other "classical" methods of this family are applied to the data in Section 5.3.1. This should be regarded as an exercise rather than an alternative to the probit method in this specific case. Another shape of the curve may be adopted only if this is supported by experimental or theoretical evidence. See Table 5.3.2.-I.

Table 5.3.2.-I. *Results by using alternative curves*

	Logit	Gompit	Angle*
Φ	$\frac{1}{1+e^{-Y}}$	$1-e^{-e^{Y}}$	$\frac{1}{2}\sin Y + \frac{1}{2}$
Z	$\frac{e^{-Y}}{(1+e^{-Y})^2}$	$e^{Y-e^{Y}}$	$\frac{1}{2}\cos Y$
slope b	4.101	2.590	1.717
χ^2 lin	2.15	3.56	1.50
χ^2 par	0.0066	0.168	0.0010
Potency	162.9	158.3	155.8
Lower limit	121.1	118.7	122.6
Upper limit	221.1	213.3	200.7

* If $Y - \frac{1}{2}\pi$ then $\Phi = 0$ and $Z = 0$
If $Y \frac{1}{2}\pi$ then $\Phi = 1$ and $Z = 0$

5.3.3. THE ED_{50} DETERMINATION OF A SUBSTANCE USING THE PROBIT METHOD

An in-vitro assay of oral poliomyelitis vaccine

In an ED_{50} assay of oral poliomyelitis vaccine with 10 different dilutions in 8 replicates of 50 µL on an ELISA-plate, results were obtained as shown in Table 5.3.3.-I.

The observations are transferred to the first working table and the subsequent columns are computed as described in Section 4.2.1. Table 5.3.3.-II shows the first cycle of this procedure.

Table 5.3.1.-II. *First working table in the first cycle*

Vaccine	Dose	n	r	x	p	Y	Φ	Z	y	w	wx	wy	wx^2	wy^2	wxy
S	1.0	12	0	0.000	0.000	0	0.5	0.399	−1.253	7.64	0.00	−9.57	0.00	12.00	0.00
	1.6	12	3	0.470	0.250	0	0.5	0.399	−0.627	7.64	3.59	−4.79	1.69	3.00	−2.25
	2.5	12	6	0.916	0.500	0	0.5	0.399	0.000	7.64	7.00	0.00	6.41	0.00	0.00
	4.0	11	10	1.386	0.909	0	0.5	0.399	1.025	7.00	9.71	7.18	13.46	7.36	9.95
T	1.0	11	0	0.000	0.000	0	0.5	0.399	−1.253	7.00	0.00	−8.78	0.00	11.00	0.00
	1.6	12	4	0.470	0.333	0	0.5	0.399	−0.418	7.64	3.59	−3.19	1.69	1.33	−1.50
	2.5	11	8	0.916	0.727	0	0.5	0.399	0.570	7.00	6.42	3.99	5.88	2.27	3.66
	4.0	11	10	1.386	0.909	0	0.5	0.399	1.025	7.00	9.71	7.18	13.46	7.36	9.95

Table 5.3.1.-III. *Second working table in the first cycle*

Vaccine	Σw	Σwx	Σwy	Σwx^2	Σwy^2	Σwxy	S_{xx}	S_{xy}	S_{yy}	$\bar{x}$	$\bar{y}$	a
S	29.92	20.30	−7.18	21.56	22.36	7.70	7.79	12.58	20.64	0.68	−0.24	−1.36
T	28.65	19.72	−0.80	21.03	21.97	12.11	7.46	12.66	21.95	0.69	−0.03	−1.17

Table 5.3.1.-IV. *First working table in the second cycle*

Vaccine	Dose	n	r	x	p	Y	Φ	Z	y	w	wx	wy	wx^2	wy^2	wxy
S	1.0	12	0	0.000	0.000	−1.36	0.086	0.158	−1.911	3.77	0.00	−7.21	0.00	13.79	0.00
	1.6	12	3	0.470	0.250	−0.58	0.279	0.336	−0.672	6.74	3.17	−4.53	1.49	3.04	−2.13
	2.5	12	6	0.916	0.500	0.15	0.561	0.394	−0.001	7.57	6.94	−0.01	6.36	0.00	−0.01
	4.0	11	10	1.386	0.909	0.93	0.824	0.258	1.260	5.07	7.03	6.39	9.75	8.05	8.86
T	1.0	11	0	0.000	0.000	−1.17	0.122	0.202	−1.769	4.20	0.00	−7.43	0.00	13.14	0.00
	1.6	12	4	0.470	0.333	−0.39	0.349	0.370	−0.430	7.23	3.40	−3.11	1.60	1.34	−1.46
	2.5	11	8	0.916	0.727	0.35	0.637	0.375	0.591	6.70	6.14	3.96	5.62	2.34	3.63
	4.0	11	10	1.386	0.909	1.13	0.870	0.211	1.311	4.35	6.03	5.70	8.36	7.48	7.90

Table 5.3.1.-V. *Second working table after sufficient cycles*

Vaccine	Σw	Σwx	Σwy	Σwx^2	Σwy^2	Σwxy	S_{xx}	S_{xy}	S_{yy}	$\bar{x}$	$\bar{y}$	a
S	18.37	14.80	−2.14	14.85	17.81	5.28	2.93	7.00	17.56	0.81	−0.12	−2.05
T	17.96	12.64	−0.55	11.86	18.35	6.76	2.96	7.15	18.34	0.70	−0.03	−1.72

Table 5.3.3.-I. *Dilutions (10^x µL of the undiluted vaccine)*

−3.5	−4.0	−4.5	−5.0	−5.5	−6.0	−6.5	−7.0	−7.5	−8.0
+	+	+	+	−	−	−	−	−	−
+	+	+	+	−	−	−	−	−	−
+	+	−	−	−	−	−	−	−	−
+	+	+	+	−	−	−	−	−	−
+	+	+	−	−	−	−	−	−	−
+	+	+	+	+	−	−	−	−	−
+	+	+	+	+	−	+	−	−	−
+	+	+	+	−	+	−	−	−	−

The sums of the last 6 columns are calculated and transferred to the second working table (see Table 5.3.3.-III). The results in the other columns are found with formulae 4.2.1.-4 to 4.2.1.-10. This yields a common slope b of −0.295.

The values for Y in the first working table are now replaced by $a + bx$ and a second cycle is carried out. The cycle is repeated until the difference between 2 consecutive cycles has become small. The second working table should then appear as shown in Table 5.3.3.-IV.

Linearity is tested as described in Section 4.2.2. The χ^2-value with 8 degrees of freedom is 2.711 representing a p-value of 0.951 which is not significant.

The potency ratio can now be estimated as described in Section 4.5.

$$M_T' = \frac{-(-7.931)}{-0.646} = -12.273$$

Further:

Table 5.3.3.-II. *First working table in the first cycle*

Vaccine	Dose	n	r	x	p	Y	Φ	Z	y	w	wx	wy	wx^2	wy^2	wxy
T	$10^{-3.5}$	8	0	−8.06	0.000	0.00	0.5	0.399	−1.253	5.09	−41.04	−6.38	330.8	8.00	51.4
	$10^{-4.0}$	8	0	−9.21	0.000	0.00	0.5	0.399	−1.253	5.09	−46.91	−6.38	432.0	8.00	58.8
	$10^{-4.5}$	8	1	−10.36	0.125	0.00	0.5	0.399	−0.940	5.09	−52.77	−4.79	546.8	4.50	49.6
	$10^{-5.0}$	8	2	−11.51	0.250	0.00	0.5	0.399	−0.627	5.09	−58.63	−3.19	675.1	2.00	36.7
	$10^{-5.5}$	8	6	−12.66	0.750	0.00	0.5	0.399	0.627	5.09	−64.50	3.19	816.8	2.00	−40.4
	$10^{-6.0}$	8	7	−13.82	0.875	0.00	0.5	0.399	0.940	5.09	−70.36	4.79	972.1	4.50	−66.1
	$10^{-6.5}$	8	7	−14.97	0.875	0.00	0.5	0.399	0.940	5.09	−76.23	4.79	1140.8	4.50	−71.7
	$10^{-7.0}$	8	8	−16.12	1.000	0.00	0.5	0.399	1.253	5.09	−82.09	6.38	1323.1	8.00	−102.9
	$10^{-7.5}$	8	8	−17.27	1.000	0.00	0.5	0.399	1.253	5.09	−87.95	6.38	1518.9	8.00	−110.2
	$10^{-8.0}$	8	8	−18.42	1.000	0.00	0.5	0.399	1.253	5.09	−93.82	6.38	1728.2	8.00	−117.6

Table 5.3.3.-III. *Second working table in the first cycle*

Vaccine	Σw	Σwx	Σwy	Σwx^2	Σwy^2	Σwxy	S_{xx}	S_{xy}	S_{yy}	$\bar{x}$	$\bar{y}$	a
T	50.93	−674.3	11.17	9484.6	57.50	−312.32	556.92	−164.43	55.05	−13.24	0.219	−3.690

Table 5.3.3.-IV. *Second working table after sufficient cycles*

Vaccine	Σw	Σwx	Σwy	Σwx^2	Σwy^2	Σwxy	S_{xx}	S_{xy}	S_{yy}	$\bar{x}$	$\bar{y}$	a
T	19.39	−238.2	0.11	2981.1	26.05	−37.45	55.88	−36.11	26.05	−12.28	0.006	−7.931

$$C = \frac{(-0.646)^2 \times 55.883}{(-0.646)^2 \times 55.883 - 1^2 \times 1.960^2} = 1.197$$

$$V = \frac{1}{19.39} = 0.052$$

So ln confidence limits are:

$$-14.692 - (-2.420) \pm \sqrt{0.197 \times (2.882 + 1.197 \times 0.009^2)}$$

$$= -12.272 \pm 0.754$$

This estimate is still expressed in terms of the ln(dilutions). In order to obtain estimates expressed in ln(ED_{50})/mL the values are transformed to:

$$-M'_T + \ln\left(\frac{1000}{50}\right)$$

Since it has become common use to express the potency of this type of vaccine in terms of $\log_{10}(ED_{50})$/mL, the results have to be divided by ln(10). The potency is thus estimated as 6.63 $\log_{10}(ED_{50})$/mL with 95 per cent confidence limits from 6.30 to 6.96 $\log_{10}(ED_{50})$/mL.

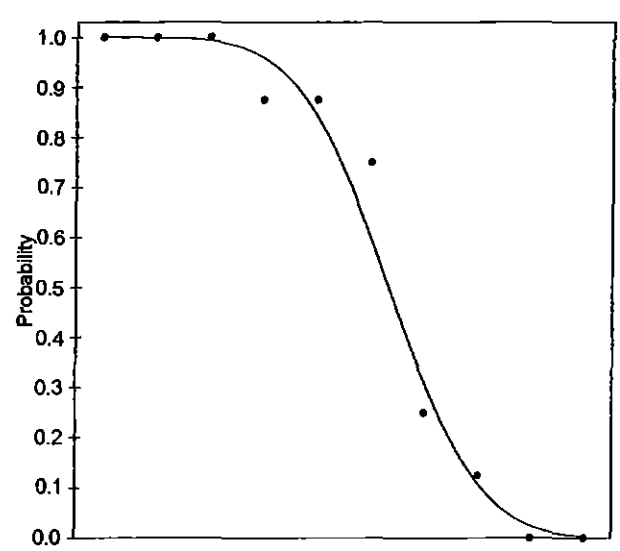


Figure 5.3.3.-I.

5.4. EXTENDED SIGMOID DOSE-RESPONSE CURVES

5.4.1. FOUR-PARAMETER LOGISTIC CURVE ANALYSIS

A serological assay of tetanus sera

As already stated in Section 3.4, this example is intended to illustrate a "possible" way to analyse the data presented, but not necessarily to reflect the "only" or the "most appropriate" way. Many other approaches can be found in the literature, but in most cases they should not yield dramatically different outcomes. A short discussion of

alternative approaches and other statistical considerations is given in Section 7.5.

A guinea-pig antiserum is assayed against a standard serum (0.4 IU/mL) using an enzyme-linked immunosorbent assay technique (ELISA). 10 two-fold dilutions of each serum were applied on a 96-well ELISA plate. Each dilution was applied twice. The observed responses are listed in Table 5.4.1.-I.

Table 5.4.1.-I. *Observed responses*

Standard S			Preparation to be examined T		
Dil.	Obs. 1	Obs. 2	Dil.	Obs. 1	Obs. 2
1/10	2.912	2.917	1/10	3.017	2.987
1/20	2.579	2.654	1/20	2.801	2.808
1/40	2.130	2.212	1/40	2.401	2.450
1/80	1.651	1.638	1/80	1.918	1.963
1/160	1.073	0.973	1/160	1.364	1.299
1/320	0.585	0.666	1/320	0.861	0.854
1/640	0.463	0.356	1/640	0.497	0.496
1/1280	0.266	0.234	1/1280	0.340	0.344
1/2560	0.228	0.197	1/2560	0.242	0.217
1/5120	0.176	0.215	1/5120	0.178	0.125

For this example, it will be assumed that the laboratory has validated conditions 1 to 3 in Section 3.1.1 when the assay was being developed for routine use. In addition, the laboratory has validated that the upper limit and lower limit of the samples can be assumed to be equal.

No unusual features are discovered in a graphical representation. A least squares method of a suitable computer program is used to fit the parameters of the logistic function, assuming that the residual error terms are independent and identically distributed normal random variables. In this case, 3 parameters (α, β and δ) are needed to describe the common slope-factor and the common lower and upper asymptotes. 2 additional parameters (γ_S and γ_T) are needed to describe the horizontal location of the 2 curves.

The following estimates of the parameters are returned by the program:

$\alpha = 3.196$ $\quad \gamma_S = -4.307$

$\beta = 1.125$ $\quad \gamma_T = -4.684$

$\delta = 0.145$

In addition, the estimated residual variance (s^2) is returned as 0.001429 with 20 degrees of freedom (within-treatments variation).

In order to obtain confidence limits, and also to check for parallelism and linearity, the observed responses (u) are linearised and submitted to a weighted parallel-line analysis by the program. This procedure is very similar to that described in Section 4.2 for probit analysis with the following modifications:

$$Y = \beta(x_I - \gamma)$$

$$\Phi = \frac{1}{1 + e^{-Y}}$$

$$Z = \frac{e^{-Y}}{(1 + e^{-Y})^2}$$

$$y = Y + \frac{\left(\frac{u-\delta}{\alpha-\delta}\right) - \Phi}{Z}$$

$$w = \frac{Z^2(\alpha-\delta)^2}{s^2}$$

The resulting weighted analysis of variance of the transformed responses (y) using weights (w) is shown in Table 5.4.1.-II.

Table 5.4.1.-II *Weighted analysis of variance*

Source of variation	Degrees of freedom	χ^2	Probability
Preparations	1	0.529653	0.467
Regression	1	6599.51	0.000
Non-parallelism	1	0.0458738	0.830
Non-linearity	16	8.89337	0.918
Treatments	19	6608.98	0.000
Residual error	20	20.0000	
Total	39	6628.98	

There are no significant deviations from parallelism and linearity and thus the assay is satisfactory for potency calculations. If the condition of equal upper and lower asymptotes is not fulfilled, significant deviations from linearity and/or parallelism are likely to occur because the tests for linearity and parallelism reflect the goodness of fit of the complete four-parameter model. The residual error in the analysis of variance is always equal to 1 as a result of the transformation. However, a heterogeneity factor (analogous to that for the probit model) can be computed.

The relative potency of the test preparation can be obtained as the antilogarithm of $\gamma_S - \gamma_T$. Multiplying by the assigned potency of the standard yields an estimate of 1.459 × 0.4 = 0.584 IU/mL. Formula 4.2.3.-2 gives 95 per cent confidence limits from 0.557-0.612 IU/mL.

6. COMBINATION OF ASSAY RESULTS

6.1. INTRODUCTION

Replication of independent assays and combination of their results is often needed to fulfil the requirements of the European Pharmacopoeia. The question then arises as to whether it is appropriate to combine the results of such assays and if so in what way.

Two assays may be regarded as mutually independent when the execution of either does not affect the probabilities of the possible outcomes of the other. This implies that the random errors in all essential factors influencing the result (for example, dilutions of the standard and of the preparation to be examined, the sensitivity of the biological indicator) in 1 assay must be independent of the corresponding random errors in the other one. Assays on successive days using the original and retained dilutions of the standard therefore are not independent assays.

There are several methods for combining the results of independent assays, the most theoretically acceptable being the most difficult to apply. Three simple, approximate methods are described below; others may be used provided the necessary conditions are fulfilled.

Before potencies from assays based on the parallel-line or probit model are combined they must be expressed in logarithms; potencies derived from assays based on the slope-ratio model are used as such. As the former models are more common than those based on the slope-ratio model, the symbol M denoting ln potency is used in the formulae in this section; by reading R (slope-ratio) for M, the analyst may use the same formulae for potencies derived from assays based on the slope-ratio model. All estimates of potency must be corrected for the potency assigned to the reference preparation before they are combined.

6.2. COMBINATION OF INDEPENDENT ASSAY RESULTS

This method can be used provided the following conditions are fulfilled:

1) the potency estimates are derived from independent assays;

2) for each assay C is close to 1 (say less than 1.1);

3) the number of degrees of freedom of the individual residual errors is not smaller than 6, but preferably larger than 15.

When these conditions are not fulfilled, an independent combination of the results cannot be applied. The method described in Section 6.3 may then be used to obtain the best estimate of the mean potency to be adopted in further assays as an assumed potency.

6.2.1. HOMOGENEITY OF POTENCY ESTIMATES

It is assumed that the results of each of the n' assays have been analysed to give n' values of M with associated confidence limits. For each assay the logarithmic confidence interval L is obtained by subtracting the lower limit from the upper. A weight W for each value of M is calculated from equation 6.2.1.-1, where t has the same value as that used in the calculation of confidence limits.

$$W = \frac{4t^2}{L^2} \qquad (6.2.1.\text{-}1)$$

By squaring the deviation of each value of M from the weighted mean, multiplying by the appropriate weight and summing over all assays, a statistic is obtained which is approximately distributed as χ^2 (see Table 8.3) and which may be used to test the homogeneity of a set of ln potency estimates:

$$\chi^2 \approx \sum_{n'} W(M - \overline{M})^2 \text{ where } \overline{M} = \frac{\sum WM}{\sum W} \qquad (6.2.1.\text{-}2)$$

If the calculated χ^2 is smaller than the tabulated value corresponding to $(n' - 1)$ degrees of freedom the potencies are homogeneous and the mean potency and limits obtained in Section 6.2.2 will be meaningful.

If the calculated value of this statistic is greater than the tabulated value, the potencies are heterogeneous. This means that the variation between individual estimates of M is greater than would have been predicted from the estimates of the confidence limits, i.e. that there is a significant variability between the assays. Under these circumstances the equations in Section 6.2.2 are no longer applicable. Instead, the formulae in Section 6.2.3 may be used.

6.2.2. WEIGHTED COMBINATION (BASED ON THE INTRA-ASSAY VARIATION)

The products WM are formed for each assay and their sum divided by the total weight for all assays to give the logarithm of the weighted mean potency.

$$\overline{M} = \frac{\sum WM}{\sum W} \qquad (6.2.2.\text{-}1)$$

The standard error of the ln (mean potency) is taken to be the square root of the reciprocal of the total weight:

$$s_{\overline{M}} = \sqrt{\frac{1}{\sum W}} \qquad (6.2.2.\text{-}2)$$

and approximate confidence limits are obtained from the antilogarithms of the value given by:

$$\overline{M} \pm t \times s_{\overline{M}} \qquad (6.2.2.\text{-}3)$$

where the number of degrees of freedom of t equals the sum of the number of degrees of freedom for the error mean squares in the individual assays.

6.2.3. SEMI-WEIGHTED COMBINATION (BASED ON THE INTRA- AND INTER-ASSAY VARIATION)

When results of several repeated assays are combined, the χ^2-value may be significant. The observed variation is then considered to have two components:

— the intra-assay variation:

$$s_M^2 = 1/W$$

— the inter-assay variation:

$$s_m^2 = \frac{\sum(M - \overline{M})^2}{(n' - 1)} - \frac{\sum s_M^2}{n'}$$

where M is the unweighted mean. The intra-assay variation varies from assay to assay whereas the inter-assay variation is common to all M.

For each M a weighting coefficient is then calculated as:

$$W' = \frac{1}{s_M^2 + s_m^2}$$

which replaces W in Section 6.2.2. where t is taken to be approximately 2.

Note: if s_m^2 is negative, then it is replaced by 0.

6.3. UNWEIGHTED COMBINATION OF ASSAY RESULTS

The method described below may be used when the conditions described in Section 6.2 are not fulfilled.

To combine the n' estimates of M from n' assays, the mean is calculated and an estimate of its standard error is obtained by calculating:

$$s_m^2 = \frac{\sum(M - \overline{M})^2}{(n' - 1)} - \frac{\sum s_M^2}{n'} \qquad (6.3.\text{-}1)$$

and the limits are:

$$\overline{M} \pm ts_{\overline{M}} \qquad (6.3.\text{-}2)$$

where t has $(n' - 1)$ degrees of freedom. The number n' of estimates of M is usually small, and hence the value of t is quite large.

6.4. EXAMPLE OF A WEIGHTED MEAN POTENCY WITH CONFIDENCE LIMITS

Table 6.4.-I lists 6 independent potency estimates of the same preparation together with their 95 per cent confidence limits and the number of degrees of freedom of their error variances. Conditions 1, 2 and 3 in Section 6.2. are met. The ln potencies and the weights are calculated as described in Section 6.2.

Table 6.4.-I. – *Potency estimates and confidence intervals of 6 independent assays*

Potency estimate (IU/vial)	Lower limit (IU/vial)	Upper limit (IU/vial)	Degrees of freedom	ln potency M	Weight W
18 367	17 755	19 002	20	9.8183	3777.7
18 003	17 415	18 610	20	9.7983	3951.5
18 064	17 319	18 838	20	9.8017	2462.5
17 832	17 253	18 429	20	9.7887	4003.0
18 635	17 959	19 339	20	9.8328	3175.6
18 269	17 722	18 834	20	9.8130	4699.5

Homogeneity of potency estimates is assessed with formula 6.2.1.-2 which gives a χ^2 of 4.42 with 5 degrees of freedom. This is not significant ($p = 0.49$) and thus, a weighted mean potency is calculated with formula 6.2.2.-1. which yields 9.8085.

Formula 6.2.2.-2 gives a standard error of 0.00673 and approximate 95 per cent confidence limits of 9.7951 and 9.8218 are calculated with formula 6.2.2.-3 where t has 120 degrees of freedom.

By taking the antilogarithms a potency of 18 187 IU/vial is found with 95 per cent confidence limits from 17 946-18 431 IU/vial.

7. BEYOND THIS ANNEX

It is impossible to give a comprehensive treatise of statistical methods in a pharmacopoeial text. However, the methods described in this annex should suffice for most pharmacopoeial purposes. This section tries to give a more abstract survey of alternative or more general methods that have been developed. The interested reader is encouraged to further explore the existing literature in this area. The use of more specialised statistical methods should, in any case, be left to qualified personnel.

7.1. GENERAL LINEAR MODELS

The methods given in this annex can be described in terms of general linear models (or generalised linear models to include the probit and logit methods). The principle is to define a linear structure matrix X (or design matrix) in which each row represents an observation and each column a linear effect (preparation, block, column, dose). For example: the Latin square design in example 5.1.2 would involve a matrix with 36 rows and 13 columns. 1 column for each of the preparations, 1 column for the doses, 5 columns for each block except the first, and 5 columns for each row except the first. All columns, except the one for doses, are filled with 0 or 1 depending on whether or not the observation relates to the effect. A vector Y is filled with the (transformed) observations. The effects are estimated with the formula $(X^TX)^{-1}X^TY$ from which the potency estimate m can easily be derived as a ratio of relevant effects. Confidence intervals are calculated from Fieller's theorem:

$$m_L,\ m_U = \frac{\left[m - \frac{gv_{12}}{v_{22}} \pm \frac{t}{b}\sqrt{v_{11} - 2mv_{12} + m^2v_{22} - g\left(v_{11} - \frac{v_{12}^2}{v_{22}}\right)}\right]}{(1-g)}$$

where $g = \frac{t^2s^2v_{22}}{b^2}$

and v_{11}, v_{22}, v_{12} represent the variance multipliers for the numerator, the denominator and their covariance multiplier respectively. These are taken directly from $(X^TX)^{-1}$ or indirectly by noting that:

$$Var(a_1 - a_2) = Var(a_1) + Var(a_2) - 2Cov(a_1, a_2)$$

and

$$Cov(a_1 - a_2, b) = Cov(a_1, b) - Cov(a_2, b)$$

A full analysis of variance in which all components are partitioned is slightly more complicated as it involves a renewed definition of X with more columns to relax the assumptions of parallelism and linearity, after which the linear hypotheses can be tested. For assays depending upon quantal responses the linear effects (intercepts a_S, a_T etc. and the common slope b are found by maximising the sum over treatments of $n\ln\Phi(a_i + bx) + (n - r)\ln(1 - \Phi(a_i + bx))$ where x is the ln(dose), Φ denotes the shape of the distribution and $i \in \{S, T, \ldots\}$.

7.2. HETEROGENEITY OF VARIANCE

Heterogeneity of variance cannot always be solved by simply transforming the responses. A possible way to cope with this problem is to perform a weighted linear regression. In order to obtain an unbiased estimate, the weight of the observations is taken to be proportional to the reciprocal of the error variances. Since the true error variance is not always known, an iterative reweighted linear procedure may be followed. However, the calculation of the confidence interval involves new problems.

7.3. OUTLIERS AND ROBUST METHODS

The method of least squares described in this annex has the disadvantage of being very sensitive to outliers. A clear outlier may completely corrupt the calculations. This problem is often remedied by discarding the outlying result from the dataset. This policy can lead to arbitrary rejection of data and is not always without danger. It is not easy to give a general guideline on how to decide whether or not a specific observation is an outlier and it is for this reason that many robust methods have been developed. These methods are less sensitive to outliers because they give less weight to observations that are far away from the predicted value. New problems usually arise in computing confidence intervals or defining a satisfactory function to be minimised.

7.4. CORRELATED ERRORS

Absolute randomisation is not always feasible or very undesirable from a practical point of view. Thus, subsequent doses within a dilution series often exhibit correlated errors leading to confidence limits that are far too narrow. Some methods have been developed that take account of this autocorrelation effect.

7.5. EXTENDED NON-LINEAR DOSE-RESPONSE CURVES

Analysis of extended non-linear dose-response curves raises a number of statistical questions which require consideration, and for which professional advice is recommended. Some of these are indicated below.

1) An example using the four-parameter logistic function has been shown. However, models based on functions giving other sigmoid curves may also be used. Models incorporating additional asymmetry parameters have been suggested.

2) Heterogeneity of variance is common when responses cover a wide range. If the analysis ignores the heterogeneity, interpretation of results may not be correct and estimates may be biased. Use of the reciprocal of the error variances as weights is unlikely to be reliable with limited numbers of replicates. It may be appropriate to estimate a function which relates variance to mean response.

3) The statistical curve-fitting procedures may give different estimates depending on assumptions made about the homogeneity of the variance and on the range of responses used.

4) In principle, equality of upper and lower response limits for the different preparations included in an assay can be directly tested in each assay. However, interpretation of the results of these tests may not be straightforward. The tests for linearity and parallelism given by the simplified method of analysis (Example 5.4.1) indirectly incorporate tests for equality and accuracy of upper and lower limits.

5) Many assays include "controls" which are intended to identify the upper and/or lower response limits. However, these values may not be consistent with the statistically fitted upper and lower response limits based on the extended dose-response curve.

6) The simplified method of analysis given in Example 5.4.1 provides approximate confidence intervals. Other methods may also be used, for example intervals based on lack-of-fit of the completely specified model. For typical assay data, with responses covering the complete range for each preparation tested, all methods give similar results.

7.6. NON-PARALLELISM OF DOSE-RESPONSE CURVES

Similarity of dose-response relationships is a fundamental criterion for assessing whether an assay may be regarded as a dilution assay and hence whether the estimation of relative potency is valid (see Section 3.1.1).

This criterion is frequently met by showing that dose-response curves for standard and test samples do not deviate significantly from parallelism. Underestimation of the residual error can lead to excess rejection of assays due to significant deviations from parallelism and/or linearity. This is often an artefact of inappropriate assay design or analysis. Minor modifications to assay designs might in many cases substantially improve the estimation of the residual error. Analysis allowing for the actual level of replication may also improve the situation, e.g. by accounting for multiple applications of the same dilution or correlation of errors between serial dilutions. If estimation of the relevant residual error is not feasible for individual assays, for example because it is impractical to create independent doses and/or replicates, it might be possible to obtain a more correct estimate of the residual error during the assay validation process. In such a case it may be justified to replace the residual error, estimated from the individual assay, by an average (pooled) residual error, determined during the validation process and agreed by the competent authorities.

Where not specified by relevant procedures, curve similarity may be assessed by another approach described as "equivalence testing", whereby, during the validation process, a range of the allowable difference between slopes or other measures of curve similarity, an equivalence acceptance criterion, is determined. If supported by appropriate documentation and agreed by the competent authorities, meeting the equivalence acceptance criteria may be used to satisfy the curve similarity requirement.

There may be cases where the assay system is sufficiently precise to detect genuine non-parallelism. If there is true non-parallelism this needs to be recognised and a suitable solution adopted. A solution might, for example, require a suitable standard that is similar in composition to, and therefore parallel to, the test samples. If the assay system is responding in a non-specific manner to extraneous components of the standard or test samples, then a more specific assay system that does not respond to the irrelevant components may be the solution. No simple, generally applicable statistical solution exists to overcome these fundamental problems. The appropriate action has to be decided on a case-by-case basis with the help of statistical expertise.

8. TABLES AND GENERATING PROCEDURES

The tables in this section list the critical values for the most frequently occurring numbers of degrees of freedom. If a critical value is not listed, reference should be made to more extensive tables. Many computer programs include statistical functions and their use is recommended instead of the tables in this section. Alternatively, the generating procedures given below each table can be used to compute the probability corresponding to a given statistic and number of degrees of freedom.

8.1. THE *F*-DISTRIBUTION

If an observed value is higher than the value in Table 8.1.-I, it is considered to be significant (upper lines, $p = 0.05$) or highly significant (lower lines, $p = 0.01$). *df*1 is the number of degrees of freedom of the numerator and *df*2 is the number of degrees of freedom of the denominator.

Generating procedure Let F be the *F*-ratio and df1 and df2 as described above. Let pi = π = 3.14159265358979... The procedure in Table 8.1.-II will then generate the *p*-value.

8.2. THE *t*-DISTRIBUTION

If an observed value is higher than the value in Table 8.2.-I, it is considered to be significant ($p = 0.05$) or highly significant ($p = 0.01$).

Table 8.2.-I *Critical values of the t-distribution*

df	$p = 0.05$	$p = 0.01$	df	$p = 0.05$	$p = 0.01$
1	12.706	63.656	22	2.074	2.819
2	4.303	9.925	24	2.064	2.797
3	3.182	5.841	26	2.056	2.779
4	2.776	4.604	28	2.048	2.763
5	2.571	4.032	30	2.042	2.750
6	2.447	3.707	35	2.030	2.724
7	2.365	3.499	40	2.021	2.704
8	2.306	3.355	45	2.014	2.690
9	2.262	3.250	50	2.009	2.678
10	2.228	3.169	60	2.000	2.660
12	2.179	3.055	70	1.994	2.648
14	2.145	2.977	80	1.990	2.639
16	2.120	2.921	90	1.987	2.632
18	2.101	2.878	100	1.984	2.626
20	2.086	2.845	∞	1.960	2.576

Generating procedures The *p*-value for a given *t* with df degrees of freedom can be found with the procedures in Section 8.1 where $F = t^2$, df1 = 1 and df2 = df.

Table 8.1.-I *Critical values of the F-distribution*

df1 → df2 ↓	1	2	3	4	5	6	8	10	12	15	20	∞
10	4.965	4.103	3.708	3.478	3.326	3.217	3.072	2.978	2.913	2.845	2.774	2.538
	10.044	7.559	6.552	5.994	5.636	5.386	5.057	4.849	4.706	4.558	4.405	3.909
12	4.747	3.885	3.490	3.259	3.106	2.996	2.849	2.753	2.687	2.617	2.544	2.296
	9.330	6.927	5.953	5.412	5.064	4.821	4.499	4.296	4.155	4.010	3.858	3.361
15	4.543	3.682	3.287	3.056	2.901	2.790	2.641	2.544	2.475	2.403	2.328	2.066
	8.683	6.359	5.417	4.893	4.556	4.318	4.004	3.805	3.666	3.522	3.372	2.868
20	4.351	3.493	3.098	2.866	2.711	2.599	2.447	2.348	2.278	2.203	2.124	1.843
	8.096	5.849	4.938	4.431	4.103	3.871	3.564	3.368	3.231	3.088	2.938	2.421
25	4.242	3.385	2.991	2.759	2.603	2.490	2.337	2.236	2.165	2.089	2.007	1.711
	7.770	5.568	4.675	4.177	3.855	3.627	3.324	3.129	2.993	2.850	2.699	2.169
30	4.171	3.316	2.922	2.690	2.534	2.421	2.266	2.165	2.092	2.015	1.932	1.622
	7.562	5.390	4.510	4.018	3.699	3.473	3.173	2.979	2.843	2.700	2.549	2.006
50	4.034	3.183	2.790	2.557	2.400	2.286	2.130	2.026	1.952	1.871	1.784	1.438
	7.171	5.057	4.199	3.720	3.408	3.186	2.890	2.698	2.563	2.419	2.265	1.683
∞	3.841	2.996	2.605	2.372	2.214	2.099	1.938	1.831	1.752	1.666	1.571	1.000
	6.635	4.605	3.782	3.319	3.017	2.802	2.511	2.321	2.185	2.039	1.878	1.000

Table 8.1.-II *Generating procedure for the F-distribution*

If df1 is even	If df1 is odd and df2 is even	If df1 and df2 are odd
x=df1/(df1+df2/F)	x=df2/(df2+df1*F)	x=atn(sqr(df1*F/df2))
s=1	s=1	ca=cos(x)
t=1	t=1	sn=sin(x)
for i=2 to(df1-2) step 2	for i=2 to(df2-2) step 2	x=x/2
t=t*x*(df2+i-2)/i	t=t*x*(df1+i-2)/i	s=0
s=s+t	s=s+t	t=sn*cs/2
next i	next i	v=0
p=s*(1-x)^(df2/2)	p=1-s*(1-x)^(df1/2)	w=1
		for i=2 to(df2-1) step 2
		s=s+t
		t=t*i/(i+1)*cs*cs
		next i
		for i=1 to(df1-2) step 2
		v=v+w
		w=w*(df2+i)/(i+2)*sn*sn
		next i
		p=1+(t*df2*v-x-s)/pi*4

The t-value ($p = 0.05$) for a given number of degrees of freedom df can be found with the procedure in Table 8.2.-II, which should be accurate up to 6 decimal places.

Table 8.2.-II *Generating procedure for the t-distribution*

```
t = 1.959964+
    2.37228/df+
    2.82202/df^2+
    2.56449/df^3+
    1.51956/df^4+
    1.02579/df^6+
    0.44210/df^7
```

8.3. THE χ^2-DISTRIBUTION

Table 8.3.-I *Critical values of the χ^2-distribution*

df	$p = 0.05$	$p = 0.01$	df	$p = 0.05$	$p = 0.01$
1	3.841	6.635	11	19.675	24.725
2	5.991	9.210	12	21.026	26.217
3	7.815	11.345	13	22.362	27.688
4	9.488	13.277	14	23.685	29.141
5	11.070	15.086	15	24.996	30.578
6	12.592	16.812	16	26.296	32.000
7	14.067	18.475	20	31.410	37.566
8	15.507	20.090	25	37.652	44.314
9	16.919	21.666	30	43.773	50.892
10	18.307	23.209	40	55.758	63.691

If an observed value is higher than the value in Table 8.3.-I, it is considered to be significant ($p = 0.05$) or highly significant ($p = 0.01$).

Generating procedure Let X2 be the χ^2-value and df as described above. The procedure in Table 8.3.-II will then generate the p-value.

Table 8.3.-II *Generating procedure for the χ^2-distribution*

If df is even	If df is odd
s=0	x=sqr(x2)
t=exp(-x2/2)	s=0
for i=2 to df step 2	t=x*exp(-x2/2)/sqr(pi/2)
s=s+t	for i=3 to df step 2
t=t*x2/i	s=s+t
next i	t=t*x2/i
p=1-s	next i
	p=1-s-2*phi(x)

In this procedure phi is the cumulative standard normal distribution function Φ (see Section 8.4).

8.4. THE Φ-DISTRIBUTION (THE CUMULATIVE STANDARD NORMAL DISTRIBUTION)

The Φ-value for negative x is found from Table 8.4.-I as $1 - \Phi(-x)$.

Table 8.4.-I *Values of the Φ-distribution*

x	Φ	x	Φ	x	Φ
0.00	0.500	1.00	0.841	2.00	0.977
0.05	0.520	1.05	0.853	2.05	0.980
0.10	0.540	1.10	0.864	2.10	0.982
0.15	0.560	1.15	0.875	2.15	0.984
0.20	0.579	1.20	0.885	2.20	0.986
0.25	0.599	1.25	0.894	2.25	0.988
0.30	0.618	1.30	0.903	2.30	0.989
0.35	0.637	1.35	0.911	2.35	0.991
0.40	0.655	1.40	0.919	2.40	0.992
0.45	0.674	1.45	0.926	2.45	0.993
0.50	0.691	1.50	0.933	2.50	0.994
0.55	0.709	1.55	0.939	2.55	0.995
0.60	0.726	1.60	0.945	2.60	0.995
0.65	0.742	1.65	0.951	2.65	0.996
0.70	0.758	1.70	0.955	2.70	0.997
0.75	0.773	1.75	0.960	2.75	0.997
0.80	0.788	1.80	0.964	2.80	0.997
0.85	0.802	1.85	0.968	2.85	0.998
0.90	0.816	1.90	0.971	2.90	0.998
0.95	0.829	1.95	0.974	2.95	0.998

Generating procedure Let x be the x-value.
The procedure in Table 8.4.-II will generate the corresponding Φ-value if $0 \leq x \leq 8.15$. If x is greater than 8.15 the Φ-value can be set to 1. If x is negative, the formula given above can be used. This procedure assumes that the computer can represent about 15 decimal places. If less digits or more digits can be represented, the procedure needs some trivial modifications.

Table 8.4.-II *Generating procedure for the Φ-distribution*

```
s=0
t=x
i=1
repeat
s=s+t
i=i+2
t=t*x*x/i
until t<1E-16
phi=0.5+s*exp(-x*x/2)/sqr(2*pi)
```

8.5. RANDOM PERMUTATIONS

Random permutations are needed in randomised block designs. The following algorithm shows how the built-in random generator of a computer can be used to create random permutations of N treatments.

Step 1. Write the N possible treatments down in a row.

Step 2. Obtain a random integer r such that $1 \leq r \leq N$.

Step 3. Exchange the r-th treatment with the N-th treatment in the row.

Step 4. Let $N = N - 1$ and repeat steps 2 to 4 until $N = 1$.

An example with 6 treatments will illustrate this algorithm.

1.	$N = 6$	S_1	S_2	S_3	T_1	T_2	T_3
2.	$r = 2$		→				←
3.		S_1	T_3	S_3	T_1	T_2	S_2
4.	$N = 5$						
2.	$r = 4$				→	←	
3.		S_1	T_3	S_3	T_2	T_1	S_2
4.	$N = 4$						
2.	$r = 4$				↓		
3.		S_1	T_3	S_3	T_2	T_1	S_2
4.	$N = 3$						
2.	$r = 1$	→		←			
3.		S_3	T_3	S_1	T_2	T_1	S_2
4.	$N = 2$						
2.	$r = 1$	→	←				
3.		T_3	S_3	S_1	T_2	T_1	S_2
4.	$N = 1$						

8.6. LATIN SQUARES

The following example shows how 3 independent permutations can be used to obtain a Latin square.

1) Generate a random permutation of the N possible treatments (see Section 8.5):

T_3	S_3	S_1	T_2	T_1	S_2

2) A simple Latin square can now be constructed by "rotating" this permutation to the right. This can be done as follows. Write the permutation found in step 1 down on the first row. The second row consists of the same permutation, but with all treatments shifted to the right. The rightmost treatment is put on the empty place at the left. This is repeated for all the rows until all the treatments appear once in each column:

T_3	S_3	S_1	T_2	T_1	S_2
S_2	T_3	S_3	S_1	T_2	T_1
T_1	S_2	T_3	S_3	S_1	T_2
T_2	T_1	S_2	T_3	S_3	S_1
S_1	T_2	T_1	S_2	T_3	S_3
S_3	S_1	T_2	T_1	S_2	T_3

3) Generate 2 independent random permutations of the figures 1 to N:
— one for the rows:

2	3	6	1	4	5

— and one for the columns:

3	4	6	2	5	1

4) The Latin square can now be found by sorting the rows and columns of the simple Latin square according to the 2 permutations for the rows and columns:

	3	4	6	2	5	1
2	T_3	S_3	S_1	T_2	T_1	S_2
3	S_2	T_3	S_3	S_1	T_2	T_1
6	T_1	S_2	T_3	S_3	S_1	T_2
1	T_2	T_1	S_2	T_3	S_3	S_1
4	S_1	T_2	T_1	S_2	T_3	S_3
5	S_3	S_1	T_2	T_1	S_2	T_3

	1	2	3	4	5	6
1	S_1	T_3	T_2	T_1	S_3	S_2
2	S_2	T_2	T_3	S_3	T_1	S_1
3	T_1	S_1	S_2	T_3	T_2	S_3
4	S_3	S_2	S_1	T_2	T_3	T_1
5	T_3	T_1	S_3	S_1	S_2	T_2
6	T_2	S_3	T_1	S_2	S_1	T_3

9. GLOSSARY OF SYMBOLS

Symbol	Definition
a	Intersection of linear regression of responses on dose or ln (dose)
b	Slope of linear regression of responses on dose or on ln (dose)
d	Number of dose levels for each preparation (excluding the blank in slope-ratio assays)
e	Base of natural logarithms (= 2.71828182845905...)
g	Statistic used in Fieller's theorem: $g = \frac{C-1}{C}$
h	Number of preparations in an assay, including the standard preparation
m	Potency estimate obtained as a ratio of effects in general linear models
n	Number of replicates for each treatment
p	Probability of a given statistic being larger than the observed value. Also used as the ratio r/n in probit analysis
r	The number of responding units per treatment group in assays depending upon quantal responses
s	Estimate of standard deviation (= $\sqrt{s^2}$)
s^2	Estimate of residual variance given by error mean square in analysis of variance
t	Student's statistic (Table 8.2.)
u	Observed response in four-parameter analysis
v_{11}, v_{12}, v_{22}	(Co)variance multipliers for numerator and denominator of ratio m in Fieller's theorem
w	Weighting coefficient
x	The ln(dose)
y	Individual response or transformed response
A	Assumed potencies of test preparations when making up doses
B	Mean response to blanks in slope-ratio assays
C	Statistic used in the calculation of confidence intervals: $C = \frac{1}{1-g}$
$C_1, \ldots, C_n$	Mean response to each column of a Latin square design
D_1, D_2	Mean response on time 1 or time 2 in the twin cross-over design
F	Ratio of 2 independent estimates of variance following an F-distribution (Table 8.1.)
$G_S, G_T, \ldots$	Treatment values used in the analysis of variance for slope-ratio assays
H_P, H_L	Multipliers used in the analysis of variance for parallel-line assays
H_B, H_I	Multipliers used in the analysis of variance for slope-ratio assays
I	In parallel-line assays, the ln of the ratio between adjacent doses. In slope-ratio assays, the interval between adjacent doses
$J_S, J_T, \ldots$	Linearity values used in the analysis of variance for slope-ratio assays
K	Correction term used in the calculation of sums of squares in the analysis of variance
L	Width of confidence interval in logarithms
$L_S, L_T, \ldots$	Linear contrasts of standard and test preparations
M	ln potency
M'	ln potency ratio of a given test preparation
N	Total number of treatments in the assay (= dh)
$P_S, P_T, \ldots$	Sum of standard and test preparations
R	Estimated potency of a given test preparation
R'	Potency ratio of a given test preparation
$R_1, \ldots, R_n$	Mean response in each of rows 1 to n of a Latin square design, or in each block of a randomised block design
S	Standard preparation
$S_1, \ldots, S_d$	Mean response to the lowest dose 1 up to the highest dose d of the standard preparation S
SS	Sum of squares due to a given source of variation
$T, U, V, \ldots$	Test preparations
$T_1, \ldots, T_d$	Mean response to the lowest dose 1 up to the highest dose d of test preparation T
V	Variance coefficient in the calculation of confidence limits
W	Weighting factor in combination of assay results
X	Linear structure or design matrix used in general linear models
Y	Vector representing the (transformed) responses in general linear models
Z	The first derivative of Φ
α	Upper asymptote of the ln(dose)-response curve in four-parameter analysis
β	Slope-factor of the ln(dose)-response curve in four-parameter analysis
γ	The ln(dose) giving 50 per cent response in the four-parameter analysis
δ	Lower asymptote of the ln(dose)-response curve in four-parameter analysis
π	3.14159265358979323 8...
Φ	Cumulative standard normal distribution function (Table 8.4.)
χ^2	Chi-square statistic (Table 8.3.)

10. LITERATURE

This section lists some recommended literature for further study.

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H. Materials Used in Chromatographic Tests

Introduction

In pharmacopoeial monographs elaborated by the British Pharmacopoeia Commission, the brand name of a TLC plate, a chromatographic column or column packing material that has been found to be suitable for the purpose is usually included in the text. The inclusion of such information does not imply that a different but equivalent commercial brand may not be used.

In monographs from the European Pharmacopoeia, references to commercial sources of, *inter alia*, TLC plates and chromatographic columns are not given. However, records of the materials used during development of the monograph are included when the draft monograph is published for comment in *Pharmeuropa*.

Liquid chromatographic tests

Defining an appropriate chromatographic system for liquid chromatographic tests is complicated by the existence of numerous variants of stationary phases, particularly amongst the chemically-bonded reverse phase materials for which not only brand to brand, but also occasionally batch to batch variations occur that can influence a separation. To assist analysts in the choice of column, the European Pharmacopoeia Commission has prepared a table of the characteristics of different types of octadecylsilyl stationary phases. The Table was published in the 3rd edition of the Technical Guide to the Elaboration of Monographs [special issue of *Pharmeuropa*; December 1999] and is reproduced below. The Table lists columns that have been widely used in the development of pharmacopoeial tests, but does not imply that a different but equivalent column cannot be used.

J. Control of Impurities in Substances for Pharmaceutical Use

(Ph. Eur. general texts 5.10)

Preamble

The monographs of the European Pharmacopoeia on substances for pharmaceutical use are designed to ensure acceptable quality for users. The role of the Pharmacopoeia in public health protection requires that adequate control of impurities be provided by monographs. The quality required is based on scientific, technical and regulatory considerations.

Requirements concerning impurities are given in specific monographs and in the general monograph *Substances for pharmaceutical use (2034)*. Specific monographs and the general monograph are complementary: specific monographs prescribe acceptance criteria for impurities whereas the general monograph deals with the need for qualification, identification and reporting of any organic impurities that occur in *active substances*.

The thresholds for reporting, identification and qualification contained in the general monograph *Substances for pharmaceutical use (2034)* apply to all related substances. However, if a monograph does not contain a related substances test based on a quantitative method, any new impurities occurring above a threshold may be overlooked since the test is not capable to detect those impurities.

The provisions of the Related substances section of the general monograph *Substances for pharmaceutical use (2034)*, notably those concerning thresholds, do not apply to excipients; also excluded from the provisions of this section are: biological and biotechnological products; oligonucleotides; radiopharmaceuticals; fermentation products and semi-synthetic products derived therefrom; herbal products and crude products of animal and plant origin. Although the thresholds stated in the general monograph do not apply, the general concepts of reporting, identification (wherever possible) and qualification of impurities are equally valid for these classes.

Basis for the elaboration of monographs of the European Pharmacopoeia

European Pharmacopoeia monographs are elaborated on substances that are present in medicinal products that have been authorised by the competent authorities of Parties to the *European Pharmacopoeia Convention*. Consequently, these monographs do not necessarily cover all sources of substances for pharmaceutical use on the world market.

Organic and inorganic impurities present in those substances that have been evaluated by the competent authorities are qualified with respect to safety at the maximum authorised content (at the maximum daily dose) unless new safety data that become available following evaluation justify lower limits.

European Pharmacopoeia monographs on substances for pharmaceutical use are elaborated by groups of experts and working parties collaborating with national pharmacopoeia authorities, the competent authorities for marketing authorisation, national control laboratories and the European Pharmacopoeia laboratory; they are also assisted by the producers of the substances and/or the pharmaceutical manufacturers that use these substances.

Control of impurities in substances for pharmaceutical use

The quality with respect to impurities is controlled by a set of tests within a monograph. These tests are intended to cover organic and inorganic impurities that are relevant in view of the sources of active substances in authorised medicinal products.

Control of residual solvents is provided by the general monograph *Substances for pharmaceutical use (2034)* and general chapter *5.4. Residual solvents*. The certificate of suitability of a monograph of the European Pharmacopoeia for a given source of a substance indicates the residual solvents that are controlled together with the specified acceptance criteria and the validated control method where this differs from those described in general chapter *2.4.24. Identification and control of residual solvents*.

Monographs on organic chemicals usually have a test entitled "Related substances" that covers relevant organic impurities. This test may be supplemented by specific tests where the general test does not control a given impurity or where there

Characteristics of Different Reverse-phase (C-18) Silica Columns for Liquid Chromatography

Name	Particle type	Particle size (µm)	Specific surface area (m^2/g)	Pore size (nm)	% Carbon	Endcapped	Base deactivated
Bondapak	Irregular	15 – 20	330	12.5	10	+	–
Bondapak	Irregular	15 – 20	100	30	3.5	+	–
Bondapak (µ)	Irregular	10	330	12.5	9.8	+	–
Delta-Pak 100	Spherical	5 and 15	300	10	17	+	–
Delta-Pak 300	Spherical	5 and 15	125	30	6.8	+	–
Genesis		4 and 3	300	12	18	+	+
Hypersil	Spherical	5	300	10	15.9	+	–
Hypersil BDS	Spherical	3 and 5	170	13	11	+	+
Hypersil ODS-1	Spherical	3, 5 and 10	170	12	8.1	–	–
Hypersil ODS-2	Spherical	3, 5 and 10	170	12	9.1	+	–
Inertsil ODS1	Spherical	5	350	10	14	–	–
Inertsil ODS2	Spherical	5	320	15	18.5	–	–
Inertsphere	Spherical		500	8	18 – 22	–	–
Intersil	Irregular	5	540	6	18.5	+	–
Kromasil	Spherical	5 and 10	340	10	19	+	–
Lichrosorb	Irregular	5 and 10	300	10	16.2	–	–
Lichrospher 100	Spherical	5 and 10	350	10	21	–	–
Nova-Pak	Spherical	4 and 6	120	6	7.3	+	–
Nucleosil	Spherical	3, 5 and 10	350	10	14	+	–
Nucleosil	Spherical	5	100	10	6.0	+	–
Partisil ODS	Irregular	10	350	8.5	5.0	–	–
Partisil ODS-2	Irregular	10	350	8.5	15	+	–
Partisil ODS-3	Irregular	5 and 10	350	8.5	10.5	+	–
Resolve	Spherical	5 and 10	200	9	10.2	–	–
Vydac HS	Spherical	5 and 10	500	8	13.5	+	–
Vydax TP	Spherical	5 and 10	80	30	8	+	–
Waters Spherisorb ODS	Spherical	3, 5 and 10	220	8	6.2	–	–
Waters Spherisorb ODS2	Spherical	3, 5 and 10	220	8	11.5	+	–
Waters Spherisorb ODSB	Spherical	5	220	8	11.5	+	+
Zorbax	Spherical	5	330	7.5	16	+	–

are particular reasons (for example, safety reasons) for requiring special control.

Where a monograph has no Related substances (or equivalent) test but only specific tests, the user of a substance must nevertheless ensure that there is suitable control of organic impurities; those occurring above the identification threshold are to be identified (wherever possible) and, unless justified, those occurring above the qualification threshold are to be qualified (see also under Recommendations to users of monographs of active substances).

Where the monograph covers substances with different impurity profiles, it may have a single related substances test to cover all impurities mentioned in the Impurities section or several tests may be necessary to give control of all known profiles. Compliance may be established by carrying out only the tests relevant to the known impurity profile for the source of the substance.

Instructions for control of impurities may be included in the Production section of a monograph, for example where the only analytical method appropriate for the control of a given impurity is to be performed by the manufacturer since the method is too technically complex for general use or cannot be applied to the final drug substance and/or where validation of the production process (including the purification step) will give sufficient control.

Impurities section in monographs on active substances

The Impurities section in a monograph includes impurities (chemical structure and name wherever possible), which are usually organic, that are known to be detected by the tests prescribed in the monograph. It is based on information available at the time of elaboration or revision of the monograph and is not necessarily exhaustive. The section includes specified impurities and, where so indicated, other detectable impurities.

Specified impurities Have an acceptance criterion not greater than that authorised by the competent authorities.

Other detectable impurities Are potential impurities with a defined structure but not known to be normally present above the identification threshold in substances used in medicinal products that have been authorised by the competent authorities of Parties to the Convention. They are given in the Impurities section for information.

Where an impurity other than a specified impurity is found in an active substance it is the responsibility of the user of the substance to check whether it has to be identified/qualified, depending on its content, nature, maximum daily dose and relevant identification/qualification threshold, in accordance with the general monograph on *Substances for pharmaceutical use (2034)*, Related substances section.

It should be noted that specific thresholds are applied to substances exclusively for veterinary use.

Interpretation of the test for related substances in the monographs on active substances

A specific monograph on a substance for pharmaceutical use is to be read and interpreted in conjunction with the general monograph on *Substances for pharmaceutical use (2034)*.

Where a general acceptance criterion for impurities ("any other impurity", "other impurities", "any impurity") equivalent to a nominal content greater than the applicable identification threshold (see the general monograph on *Substances for pharmaceutical use (2034)*) is prescribed, this is valid only for specified impurities mentioned in the Impurities section. The need for identification (wherever possible), reporting, specification and qualification of other impurities that occur must be considered according to the requirements of the general monograph. It is the responsibility of the user of the substance to determine the validity of the acceptance criteria for impurities not mentioned in the Impurities section and for those indicated as other detectable impurities.

Acceptance criteria for the related substances test are presented in different ways in existing monographs; the decision tree (Figure 5.10.-1) may be used as an aid in the interpretation of general acceptance criteria and their relation with the Impurities section of the monograph.

General acceptance criteria for "other" impurities are expressed in various ways in the monographs: "any other impurity", "other impurities", "any impurity", "any spot", "any band", etc. The general acceptance criteria may apply to certain specified impurities only or to unspecified impurities and certain specified impurities, depending on the nature of the active substance and the applicable identification threshold. Pending editorial adaptation of already published monographs using unequivocal terminology, the decision tree (Figure 5.10.-1) may be used to determine the acceptance criterion to be applied.

Recommendations to users of monographs of active substances

Monographs give a specification for suitable quality of substances with impurity profiles corresponding to those taken into account during elaboration and/or revision of the monograph. It is the responsibility of the user of the substance to check that the monograph provides adequate control of impurities for a substance for pharmaceutical use from a given source, notably by using the procedure for certification of suitability of the monographs of the European Pharmacopoeia.

A monograph with a related substances test based on a quantitative method (such as liquid chromatography, gas chromatography and capillary electrophoresis) provides adequate control of impurities for a substance from a given source if impurities present in amounts above the applicable identification threshold are specified impurities mentioned in the Impurities section.

If the substance contains impurities other than those mentioned in the Impurities section, it has to be verified that these impurities are detectable by the method described in the monograph, otherwise a new method must be developed and revision of the monograph must be requested. Depending on the contents found and the limits proposed, the identification and/or the qualification of these impurities must be considered.

Where a single related substances test covers different impurity profiles, only impurities for the known profile from a single source need to be reported in the certificate of analysis unless the marketing authorisation holder uses active substances with different impurity profiles.

Identification of impurities (peak assignment)

Where a monograph has an individual limit for an impurity, it is often necessary to define means of identification, for example using a reference substance, a representative chromatogram or relative retention. The user of the substance may find it necessary to identify impurities other than those for which the monograph provides a means of identification, for example to check the suitability of the specification for a given impurity profile by comparison with the Impurities section. The European Pharmacopoeia does not provide reference substances, representative chromatograms or information on relative retentions for this purpose, unless prescribed in the monograph. Users will therefore have to apply the available scientific techniques for identification.

New impurities/Specified impurities above the specified limit

Where a new manufacturing process or change in an established process leads to the occurrence of a new impurity, it is necessary to apply the provisions of the general monograph on *Substances for pharmaceutical use (2034)* regarding identification and qualification and to verify the suitability of the monograph for control of the impurity. A certificate of suitability is a means for confirming for a substance from a given source that the new impurity is adequately controlled or the certificate contains a method for control with a defined acceptance criterion. In the latter case revision of the monograph will be initiated.

Where a new manufacturing process or change in an established process leads to the occurrence of a specified impurity above the specified limit, it is necessary to apply the provisions of the general monograph on *Substances for pharmaceutical use (2034)* regarding qualification.

Expression of acceptance criteria

The acceptance criteria for related substances are expressed in monographs either in terms of comparison of peak areas (comparative tests) or as numerical values.

Chromatographic methods

General chapter *2.2.46. Chromatographic separation techniques* deals with various aspects of impurities control.

Information is available via the EDQM website on commercial names for columns and other reagents and equipment found suitable during monograph development, where this is considered useful.

GLOSSARY

Disregard limit

In chromatographic tests, the nominal content at or below which peaks/signals are not taken into account for calculating a sum of impurities. The numerical values for the disregard limit and the reporting threshold are usually the same.

Identification threshold

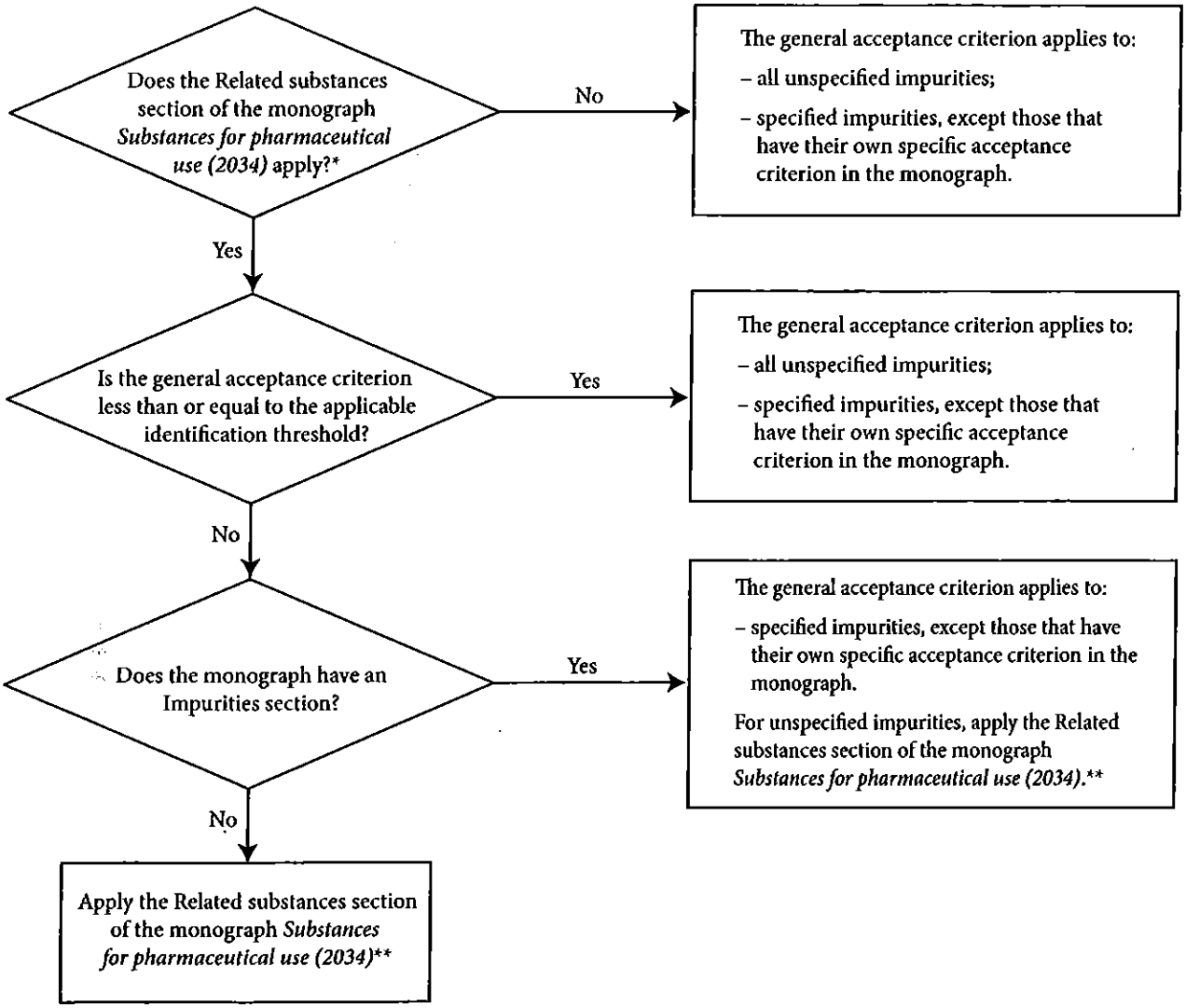
A limit above which an impurity is to be identified.

Identified impurity

An impurity for which structural characterisation has been achieved.

Impurity

Any component of a substance for pharmaceutical use that is not the chemical entity defined as the substance.



* The requirements of this section apply to active substances with the exception of: biological and biotechnological products; oligonucleotides; radiopharmaceuticals; products of fermentation and semi-synthetic products derived therefrom; crude products of animal or plant origin; herbal products.

** To apply the Related substances section of the monograph *Substances for pharmaceutical use (2034)*:
- an individual acceptance criterion must be defined for any impurity that may be present above the identification threshold;
- any impurity with an acceptance criterion above the identification threshold must wherever possible be identified;
- any impurity with an acceptance criterion above the qualification threshold must be qualified.

Figure 5.10.-1. – *Decision tree for interpretation of general acceptance criteria for 'other' impurities in monographs*

Nominal concentration
Concentration calculated on the basis of the concentration of the prescribed reference and taking account of the prescribed correction factor.

Other detectable impurities
Potential impurities with a defined structure that are known to be detected by the tests in a monograph but not known to be normally present above the identification threshold in substances used in medicinal products that have been authorised by the competent authorities of Parties to the Convention. They are unspecified impurities and are thus limited by a general acceptance criterion.

Potential impurity
An impurity that theoretically can arise during manufacture or storage. It may or may not actually appear in the substance. Where a potential impurity is known to be detected by the tests in a monograph but not known to be normally present in substances used in medicinal products that have been authorised by the competent authorities of Parties to the Convention, it will be included in the Impurities section under *Other detectable impurities* for information.

Qualification
The process of acquiring and evaluating data that establishes the biological safety of an individual impurity or a given impurity profile at the level(s) specified.

Qualification threshold
A limit above which an impurity is to be qualified.

Related substances
Title used in monographs for general tests for organic impurities.

Reporting threshold
A limit above which an impurity is to be reported. Synonym: reporting level.

Specified impurity
An impurity that is individually listed and limited with a specific acceptance criterion in a monograph. A specified impurity can be either identified or unidentified.

Unidentified impurity
An impurity for which a structural characterisation has not been achieved and that is defined solely by qualitative analytical properties (for example, relative retention).

Unspecified impurity
An impurity that is limited by a general acceptance criterion and not individually listed with its own specific acceptance criterion.

K. Characters Section in Monographs

(Ph. Eur. general texts 5.11)

The General Notices indicate that the statements included in the Characters section are not to be interpreted in a strict sense and are not requirements. For information of users, the methods recommended to authors of monographs as the basis for statements concerning hygroscopicity, crystallinity and solubility are given below.

HYGROSCOPICITY

This method is to be carried out on a substance that complies with the test for loss on drying or water content of the monograph. The method gives an indication of the degree of hygroscopicity rather than a true determination.

Use a glass weighing vessel 50 mm in external diameter and 15 mm high. Weigh the vessel and stopper (m_1). Place the amount of substance prescribed for the test for loss on drying or water in the vessel and weigh (m_2). Place the unstoppered vessel in a desiccator at 25 °C containing a saturated solution of ammonium chloride or ammonium sulfate or place it in a climatic cabinet set at 25 ± 1 °C and 80 ± 2 per cent relative humidity. Allow to stand for 24 h. Stopper the weighing vessel and weigh (m_3).

Calculate the percentage increase in mass using the expression:

$$\frac{m_3 - m_2}{m_2 - m_1} \times 100$$

The result is interpreted as follows:
- *deliquescent*: sufficient water is absorbed to form a liquid,
- *very hygroscopic*: increase in mass is equal to or greater than 15 per cent,
- *hygroscopic*: increase in mass is less than 15 per cent and equal to or greater than 2 per cent,
- *slightly hygroscopic*: increase in mass is less than 2 per cent and equal to or greater than 0.2 per cent.

CRYSTALLINITY

This method is employed to establish the crystalline or amorphous nature of a substance.

Mount a few particles of the substance to be examined in mineral oil on a clean glass slide. Examine under a polarising microscope. Crystalline particles exhibit birefringence and extinction positions when the microscope stage is revolved.

SOLUBILITY

For this test a maximum of 111 mg of substance (for each solvent) and a maximum of 30 mL of each solvent are necessary.

Dissolving procedure
Shake vigorously for 1 min and place in a constant temperature device, maintained at a temperature of 25.0 ± 0.5 °C for 15 min. If the substance is not completely dissolved, repeat the shaking for 1 min and place the tube in the constant temperature device for 15 min.

Method
Weigh 100 mg of finely powdered substance (90) (*2.9.12*) in a stoppered tube (16 mm in internal diameter and 160 mm long), add 0.1 mL of the solvent and proceed as described under Dissolving Procedure. If the substance is completely dissolved, it is *very soluble*.

If the substance is not completely dissolved, add 0.9 mL of the solvent and proceed as described under Dissolving Procedure. If the substance is completely dissolved, it is *freely soluble*.

If the substance is not completely dissolved, add 2.0 mL of the solvent and proceed as described under Dissolving Procedure. If the substance is completely dissolved, it is *soluble*.

If the substance is not completely dissolved, add 7.0 mL of the solvent and proceed as described under Dissolving Procedure. If the substance is completely dissolved, it is *sparingly soluble*.

If the substance is not completely dissolved, weigh 10 mg of finely powdered substance (90) (*2.9.12*) in a stoppered tube, add 10.0 mL of the solvent and proceed as described under Dissolving Procedure. If the substance is completely dissolved, it is *slightly soluble*.

If the substance is not completely dissolved, weigh 1 mg of finely powdered substance (90) (*2.9.12*) in a stoppered tube, add 10.0 mL of the solvent and proceed as described under Dissolving Procedure. If the substance is completely dissolved, it is *very slightly soluble*.

L. Alternative Methods for Control of Microbiological Quality

(Ph. Eur. general texts 5.1.6)

The following chapter is published for information.

1 GENERAL INTRODUCTION

The objective of this chapter is to facilitate the implementation and use of alternative microbiological methods where this can lead to efficient microbiological control and improved assurance for the quality of pharmaceutical products.

The microbiological methods described in the European Pharmacopoeia have been used for over a century and these methods for detecting, enumerating and identifying micro-organisms still serve microbiologists well. Over the years, these methods have been invaluable for the production of microbiologically safe pharmaceutical products. However, these microbiological methods are slow, and in the case of sterility tests, results are not available before an incubation period of 14 days. Consequently, the results from these methods seldom enable proactive corrective action to be taken.

Alternative methods for the control of microbiological quality have shown potential for real-time or near real-time results with the possibility of earlier corrective action. These new methods, if validated and adapted for routine use, can also offer significant improvements in the quality of testing.

Alternative methods may be used for in-process samples of pharmaceutical products, particularly for the application of Process Analytical Technology (PAT), for environmental monitoring and for industrial utilities (e.g. production and distribution of water, steam etc.), thereby contributing to the quality control of these products.

In this chapter, alternative microbiological methods for pharmaceutical application are described. For each method, the basic principle is stated and the advantages and disadvantages of the method are discussed along with any critical aspects to be considered. Potential uses that may be envisaged based on the principles of the method concerned are given, but it is not intended to suggest that such applications have been realised or that the list provided is exhaustive.

It is not the intention of this chapter to recommend one method over another, nor is it the intention to provide an exclusive or exhaustive list of alternative methods that can be used for pharmaceutical microbiological control.
The information herein may be used, however, in the process of choosing an alternative microbiological method as a supplement or as an alternative to pharmacopoeial microbiological methods and to give guidance on validation of the chosen method. If a suitable method is described in the Pharmacopoeia, this method is the reference method.
In this rapidly developing field, other methods are likely to appear and the guidance offered herein may be equally applicable in these cases.

There are 3 major types of determination specific to microbiological tests:
— qualitative tests for the presence or absence of micro-organisms;
— quantitative tests for enumeration of micro-organisms;
— identification tests.

1-1 QUALITATIVE TESTS FOR THE PRESENCE OR ABSENCE OF MICRO-ORGANISMS

In conventional microbiological analysis, this type of test is characterised by the use of turbidity or other growth-related changes in a culture medium as evidence of the presence of viable micro-organisms in the test sample. The most common example of this test is the test for sterility (*2.6.1*). Other examples include those tests designed to evaluate the presence or absence of a particular type of viable micro-organism in a sample. The conventional sterility test may be replaced by, for example, tests based on bioluminescence or solid phase cytometry, gas detection or autofluorescence. Nucleic acid amplification techniques (NAT) (*2.6.21*) may also be used for the detection of mycoplasmas (*2.6.7*).

1-2 QUANTITATIVE TESTS FOR ENUMERATION OF MICRO-ORGANISMS

Membrane filtration and plate count methods are conventional methods used to estimate the number of viable micro-organisms present in a sample. The Most Probable Number (MPN) method is another example of such methods and was developed as a means of estimating the number of viable micro-organisms present in a sample not amenable to direct plating. Examples of alternative methods for enumeration include autofluorescence, flow cytometry, direct epifluorescent filter technique (DEFT) and solid phase cytometry.

1-3 IDENTIFICATION TESTS

Biochemical and morphological characterisation of an unknown micro-organism is the classical approach to identification. Recently developed methods have streamlined and automated aspects of this identification, especially in the areas of data handling, analysis and storage. Several alternative approaches that have been integrated into these methods include biochemical reactions, carbon substrate utilisation, characterisation of fatty acid composition, mass spectroscopy and Raman spectroscopy, restriction endonuclease banding patterns and the use of genome sequencing methods such as 16S rRNA gene sequence analysis for prokaryotes.

Traditional biochemical and phenotypic techniques have been shown to be less accurate and precise than genotypic methods.

Pure cultures are required for a precise identification and such cultures must be fresh and cultivated in appropriate media.

Databases are part of the systems and are included in the primary validation. As identification methods depend on the use of databases, the extent of coverage of the database with respect to the range of micro-organisms of interest must be taken into account during validation. Appropriate software allows customisation of the database, thereby allowing the user to add micro-organisms not previously included. This possibility must be considered during the validation.

2 GENERAL PRINCIPLES OF ALTERNATIVE METHODS

Alternative microbiological methods employ direct and indirect methods of detection; in some instances amplification of the signal is achieved by enrichment methods. In recognition of these differences, and for convenience within this chapter, alternative methods for the control of microbiological quality are divided into 3 categories:
— growth-based methods, where a detectable signal is usually achieved by a period of culture;
— direct measurement, where individual cells are differentiated and/or imaged;
— cell component analysis, where the expression of specific cell components offers an indirect measure of microbial presence and identification of micro-organisms.

In some instances, these distinctions are artificial, but enable a working classification to be created.

2-1 GROWTH-BASED METHODS

2-1-1 General critical aspects of methods based on early detection of growth

Such methods are critically dependent on microbial growth in order to provide an indication of the presence and/or number of micro-organisms. For the typically low levels of microbial contamination seen in pharmaceutical products, detection may take 24 h or longer. Increased sensitivity can be achieved with filtered products. In this case, after filtration, the membrane filter is incubated in or on the medium and the result is expressed as presence or absence in the quantity corresponding to the filtered volume. These systems, if they use an incubation step in liquid media, do not offer quantitative information, but a presence/absence determination in the quantity analysed. Analysis of more than one sample quantity may offer a semi-quantitative estimation (limit test). The major benefit of early detection methods compared to classical methods is often the capacity to simultaneously process a large number of samples and the potential to obtain a result in a shorter time.

The methods described below can be used for quantitative, semi-quantitative or qualitative analyses. They are also non-destructive, therefore subsequent identification of the micro-organism is possible.

2-1-2 Electrochemical methods

Principles of measurement Micro-organisms multiplying and metabolising in appropriate growth media produce highly charged ionic metabolites from weakly charged organic nutrients leading to the modification of electrical properties in such media. These changes in impedance (measured by conductance or capacitance) are monitored with electrodes included in the culture vessels and in contact with the culture medium. The measurable end-point is the time taken to detect a predetermined impedance change; for particular types of micro-organisms, the detection time is inversely proportional to the initial inoculum size. For yeasts and moulds, which only produce small changes in electrical impedance, an indirect measurement of conductance can be used. Direct measurement of capacitance can also be carried out.

Critical aspects There is no direct relationship between the original microbial level and the detectable end-point.

Potential uses Microbiological assay of antibiotics, efficacy of antimicrobial preservation and presence/absence testing.

2-1-3 Measurement of consumption or production of gas

Principles of measurement Appropriate growth media is utilised by actively multiplying and metabolising micro-organisms, leading to the production of metabolites or the elimination of specific nutrients. These methods detect microbial growth either by changes in the electrical properties of a sensor in response to a change in gas composition or by colorimetric changes of a sensor in response to physico-chemical changes in the growth medium in contact with that sensor. The systems are based on non-destructive techniques which enable subsequent identification or strain typing of the micro-organisms. Bacteria and/or fungi may be grown in closed containers and continuous monitoring can be performed using automated instruments that measure gas evolution (e.g. CO_2) or consumption (e.g. O_2) as surrogate markers of microbial growth. Furthermore, the production of metabolites or elimination of nutrients can lead to changes in pH or redox potential. All of these changes can be measured either directly or indirectly as changes in colorimetric markers in the growth medium.

Critical aspects There is no direct relationship between the original microbial level and the detectable end-point. The incubation temperature, the physiological state and type of micro-organism, the initial load and the algorithm for data processing can significantly affect the results or the time to detection.

Potential uses Presence/absence testing of filterable or non-filterable samples (e.g. final drug products, in-process control samples, media fill or container closure integrity testing).

2-1-4 Bioluminescence

Principles of measurement Adenosine triphosphate (ATP) is a well-documented marker of cell viability. In this method, ATP first needs to be released from the micro-organisms using an appropriate extractant, followed by an assay using the luciferin/luciferase enzyme system, which emits light in proportion to the ATP present. The signal-to-noise ratio can be increased by addition of ADP and converting this ADP into released ATP.

Qualitative method: micro-organisms are cultivated in liquid medium. The emitted light is measured with a bioluminometer and is expressed in relative light units (RLU) (e.g. bioluminescence in a tube or a well of a microtitre plate). The RLU obtained from the sample is compared with a pre-determined threshold value. The result is positive if the RLU obtained with the analysed sample exceeds the threshold value.

Quantitative method: micro-organisms are captured on a membrane and cultivated by incubation on agar medium. Using a charge coupled device (CCD) camera, the ATP released from microcolonies can be detected by light emission and a quantitative determination is possible.

Critical aspects If the sample has a high level of bacterial contamination, the detection is rapid. For low levels of contamination, it is necessary to increase the number of micro-organisms using an incubation step in culture media (liquid or solid). The yield of ATP varies from one micro-organism to another and can depend on several factors including the species, the growth phase of the cell, the nutritional status, the cellular stress or the cellular age. Additional factors such as turbidity, sample colour or product matrix effects can also influence bioluminescence measurements. Extraction of ATP is generally a destructive process which should be considered with respect to any subsequent need for identification of detected micro-organisms.

Potential uses Presence/absence testing of filterable or non-filterable samples (e.g. final drug products, in-process control samples, media fill), total aerobic microbial count (TAMC), environmental and water monitoring, testing for efficacy of antimicrobial preservation.

2-1-5 Turbidimetry

Principles of measurement Microbial growth leads to detectable changes in medium opacity, which can be accurately quantified by optical density measurements at a specified wavelength. In its simplest form, such measurements are performed using a standard spectrophotometer, generally over a wavelength range of 420-615 nm. Alternative automated systems employ microtitre plate readers offering a continuous readout with early detection of optical density change.

Critical aspects Attempts have been made to extrapolate the initial microbial contamination from the time to detection, but this is limited to healthy micro-organisms with reproducible growth characteristics.

Potential uses By means of calibration graphs, determination of the inoculum size of microbial suspensions for use in pharmacopoeial tests. In automated mode, microbiological assay of antibiotics and testing for efficacy of antimicrobial preservation.

2-1-6 Growth detection using selective and/or indicative media

Principles of measurement The ability to detect the presence of specific enzymes using suitable chromogenic substrates has led to the development of a large number of methods for the identification of micro-organisms employing either manual or automated techniques. The incorporation of such substrates into a selective or non-selective primary isolation medium can eliminate the need for further subculture and biochemical testing for the identification of certain micro-organisms. Consequently, chromogenic liquid or solid culture media are designed to reveal specific enzymatic activities for detection and differentiation of micro-organisms. In these particular media, defined substrates are

introduced into the formulation and are metabolised by the specific cell enzyme of a given bacterium or fungus during growth. These substrates, which are linked to coloured indicators, are chosen according to the diagnostic enzymatic activity sought. Furthermore, chromogenic broth can be used for early or improved detection of contamination (e.g. in media fill or broth-based detection methods).

The use of innovative media presents several advantages, namely improved discrimination of colonies in a mixed culture, ease of use and ease of interpretation. In addition, response times are shorter as the growth and identification of the micro-organism are simultaneous.

Critical aspects Validation of the media must be undertaken carefully to ensure a combination of specificity, selectivity and robustness. The quality of the signal is based not only on the careful choice of the enzymes or indicators used as the basis of detection (as these enzymes may be present in different micro-organism genera), but also on the physico-chemical characteristics of the medium, e.g. pH.

Potential uses Detection of specified micro-organisms and qualitative testing (e.g. media fill and container closure integrity testing) and quantitative testing (e.g. water testing).

2-2 DIRECT MEASUREMENT

2-2-1 Solid phase cytometry

Principles of measurement Micro-organisms are stained for viability by exposure to a conjugated, initially non-fluorogenic, fluorophore. An intact cellular membrane is required to retain and accumulate the fluorophore within the cytoplasm. Inside metabolically-active microbial cells, the conjugate is enzymatically cleaved and the fluorescent derivative is released intracellularly. Micro-organisms are collected on a membrane filter either before or after viability staining.

Membrane surfaces retaining vital-stained cells are then scanned by a laser beam and epifluorescent excitation allows the detection of single, viable fluorescent micro-organisms. Appropriate software allows differentiation of viable micro-organisms from autofluorescent particles. The high sensitivity and rapidity of the method permit detection of microbial contaminants within a few hours. Total cell counts (viable and non-viable) can be obtained using fluorescent staining.

Critical aspects Metabolically active, fastidious and viable non-culturable micro-organisms can all be detected. This may result in reappraisal of the microbial limits established for the samples under evaluation. Spores require initiation of germination to enable detection. Single cell detection may be achievable, but identification of isolates might not be possible. False positives may occur due to autofluorescent particles that can be difficult to differentiate from micro-organisms. Signal discrimination and enhancement can be aided by microcolony growth.

Potential uses Rapid and sensitive method for the non-specific evaluation of microbial contamination.

2-2-2 Flow cytometry

Principles of measurement Fluorophore-labelled micro-organisms can be detected in suspension as they pass through a flow cytometer. Viable micro-organisms can be differentiated from non-viable particles by use of a viability-indicating fluorophore (see 2-2-1). The cell suspension stream is dispersed into a narrow channel and exposed to a laser which excites the fluorophore. Micro-organisms and particles are then counted in different channels depending on whether or not they contain a fluorescent cell.

Critical aspects Direct flow cytometry may be applied to the microbiological analysis of both filterable and non-filterable materials, and after possible enrichment in the case of the low contamination levels. It gives near real-time detection, but is not as sensitive as solid phase cytometry. To increase sensitivity for use in the pharmaceutical field, it is often necessary to add an incubation step in culture media, in which case the method becomes a combination of a growth-based method and a direct detection method. Particle size and number may have a significant effect on performance, and samples may require serial dilution. With the exception of filterability, similar considerations to those in solid phase cytometry apply. Clumping of bacteria can be a problem (e.g. *Staphylococcus aureus*).

Potential uses In contrast to solid phase cytometry, this method offers the potential to detect and enumerate microbial contamination in materials containing particulate matter and if the material cannot be filtered. If a pre-incubation step is needed, the method becomes a qualitative determination.

2-2-3 Direct epifluorescent filtration technique (DEFT)

Principles of measurement This technique may be considered a forerunner of solid phase cytometry. Micro-organisms, concentrated by filtration of the sample, are stained with a fluorescent dye (formerly acridine orange and now more commonly 4′,6-diamidino-2-phenylindole (DAPI)), that can be detected by epifluorescent illumination. Fluorescent vital staining techniques, as employed in solid phase cytometry (see 2-2-1), are amenable to DEFT, and fluorescent redox dyes such as 5-cyano-2,3-ditolyltetrazolium chloride (CTC) can be used to highlight respiring cells. Coupled with microscopy, the method allows rapid detection of micro-organisms with an absolute sensitivity that is dependent on the volume of product filtered and the number of fields of view examined. Semi-automated auto-focusing systems coupled to image analysis have served to improve the utility of this method. A modification of the principle involves sampling using an adhesive sheet (which permits collection of cells from surfaces), subsequent staining on the sheet itself, followed by direct observation using an epifluorescence microscope.

Critical aspects The distribution of micro-organisms on the membrane affects method robustness. The intensity of fluorescence can be influenced by the staining process and the metabolic status of the micro-organisms. Fluorescence is not necessarily an indicator of viability. A brief period of culture on the filter surface prior to staining allows microcolony formation; these microcolonies stain readily, can be easily enumerated and are demonstrable evidence of viability.

Potential uses DEFT is generally limited to low viscosity fluids, although pre-dilution or pre-filtration has occasionally been applied to viscous or particulate products. Monitoring of microbial contamination has been successfully applied to aqueous pharmaceuticals.

2-2-4 Autofluorescence

Principles of measurement The presence of endogenous autofluorescent molecules and metabolites (e.g. NADPH, flavoproteins) within micro-organisms allows the early detection and quantitative enumeration of microcolonies or single cells. For direct measurements, the laser-induced autofluorescence of a single micro-organism is captured by a detector, while for growth-based systems, automated sequential imaging of the membrane surface on agar medium over the incubation period is employed and image overlay

allows differentiation of growing microcolonies from fluorescent particulates. The emitted light is detected by a CCD camera. Non-destructive detection allows identification of contaminants at the end of the incubation period.

Critical aspects For a non-growth based measurement, viable, but non-culturable, micro-organisms might be detected. It may be difficult to distinguish between culturable micro-organisms, viable but non-culturable micro-organisms and/or other particles.

Potential uses Environmental monitoring, filterable in-process samples, water testing and product release for both sterile and non-sterile applications.

2-3 CELL COMPONENT ANALYSIS

2-3-1 Phenotypic techniques

2-3-1-1 Immunological methods

Principles of measurement Antibody-antigen reactions can be employed to detect unique cellular determinants of specific micro-organisms. These reactions can be linked to agglutination phenomena and colorimetric or fluorimetric end-points, which offer both quantitative and qualitative detection. Enzyme-linked immunosorbent assays (ELISA) offer simple solid-phase methodologies.

Critical aspects Immunological detection methods depend on the unique expression of specific identifiers, but do not necessarily demonstrate the presence of viable micro-organisms.

Potential uses Detection and identification of specified micro-organisms.

2-3-1-2 Fatty acid profiles

Principles of measurement The fatty acid composition of micro-organisms is stable, well conserved and shows a high degree of homogeneity within different taxonomic groups. The isolate is grown on a standard medium and harvested. The fatty acids are saponified, methylated and extracted, and the occurrence and amount of the resulting fatty acid methyl esters are measured using high-resolution gas chromatography. The fatty acid composition of an unknown isolate is compared with a database of known isolates for a possible match and identification.

Critical aspects The use of fatty acid profiles for microbial identification requires a high degree of standardisation. It is critical for the fatty acid composition of microbial cells that isolates are grown using standardised media and standard incubation conditions. Standard conditions for operation of the gas chromatograph must also be employed, with frequent runs of calibration standards and known isolates being very important.

Potential uses Identification or characterisation of environmental and product microbial contamination (for contaminant tracing and detection of specified micro-organisms).

2-3-1-3 Fourier transform infrared (FTIR) spectroscopy

Principles of measurement A Fourier transformation of the infrared spectrum of whole micro-organisms gives a stable, recognisable pattern typical of the taxonomic groups of micro-organisms. The analysis of the FTIR pattern can be performed with commercially available instruments.
The isolate is grown on a standard medium and harvested. Cell mass is transferred to a carrier, and the infrared spectrum is recorded. The Fourier transformation is calculated and the pattern is compared with a database of known isolates for a possible match and identification.

Critical aspects The use of FTIR patterns for microbial identification requires a high degree of standardisation. It is critical for the FTIR pattern of microbial cells that isolates are grown using standardised media and standard incubation conditions. The cells must be in the same state of the growth cycle when analysed, and particular attention must be paid to this in the validation process.

Potential uses Identification or characterisation of environmental and product microbial contamination (for contaminant tracing and detection of specified micro-organisms).

2-3-1-4 Mass spectrometry

Principles of measurement Ionised particles released by exposing microbial isolates to a laser in a vacuum can be analysed by mass spectrometry, providing characteristic spectra. Similarly, intact microbial cells, when subject to intense ionisation under matrix-assisted laser desorption ionisation-time of flight (MALDI-TOF) mass spectrometry, release a distinctive pattern of charged species. Such spectra can be compared with known profiles.

Critical aspects The isolates must be cultured under standardised conditions prior to analysis.

Potential uses Identification or characterisation of environmental and product microbial contaminants (for contaminant tracing and detection of specified micro-organisms).

2-3-1-5 Biochemical assays based on physiological reactions

Principles of measurement Systems capable of performing biochemical assays based on physiological reactions are used for the identification of micro-organisms. In the presence of a pure colony, the five basic steps for these assays are preparation, inoculation, incubation, readings and interpretation. These steps are usually preceded by a description of the colony morphology, a differentiation test (e.g. Gram stain), a description of the cellular morphology and/or other early biochemical differentiation tests (e.g. oxidase, catalase, coagulase) in order to determine the appropriate testing protocol.

The Gram stain is often a key characteristic upon which further testing is based. Alternatives to the traditional staining method include the potassium hydroxide (KOH) string test, the aminopeptidase test, a fluorescent staining method and a limulus amoebocyte lysate (LAL) based assay. Test kits are available for the latter 3 methods. The fluorescent staining method requires a fluorescence microscope or a flow cytometer.

Microbial cell suspensions are tested using biochemical (assimilation or susceptibility) test kits (plates or strips). Anaerobic and aerobic micro-organisms develop characteristic reactions to selected biochemical substances. They are also known to utilise specific carbon, nitrogen, phosphorus and sulfur sources or to be inhibited by a specific concentration of an antimicrobial agent. The results are based on measurable changes (e.g. turbidity, chromogenic or fluorogenic reaction) due to the growth or inhibition of the micro-organism under investigation. Comparison of the metabolic and/or antimicrobial resistance profile with a database allows for identification of the culture. These methods can be performed manually or by semi- or fully automated instruments. Complementary tests can be performed in cases of poor discrimination. Subcultures can help in cases of indeterminate results.

Critical aspects A fresh physiological culture is required. The performance of the system is also dependent on the selected phenotypic parameters, which must be stable, significant and in sufficient number.

Potential uses Identification or characterisation of environmental and product microbial contamination (for contaminant tracing and detection of specified micro-organisms).

2-3-2 Genotypic techniques

Identification and detection of micro-organisms as well as characterisation of strains belonging to the same species may be achieved by direct detection of nucleotide target sequences that are unique for a particular microbial species or microbial group, and are targets of the genotypic (DNA or RNA-based) detection techniques. These detection techniques may be separated into 3 broad categories: direct hybridisation, nucleic acid amplification and genetic fingerprinting.

2-3-2-1 Direct hybridisation

General principles of measurement DNA probes are short, labelled, single-strand segments of DNA that hybridise with a complementary region of microbial DNA or RNA. The probe or target DNA is usually labelled with either radioactive, fluorescent or chromogenic molecules in order to provide a hybridisation signal. Hybridisation assays include fluorescence *in situ* hybridisation (FISH) and microarray-based techniques.

General critical aspects Hybridisation generally requires a large amount of the target DNA for analysis, which may result in lower detection sensitivity. The availability of suitable probes may be limited.

Potential uses Due to the high specificity of the sequence-based hybridisation reaction, this method may be used for both detection and identification of micro-organisms.

2-3-2-2 Nucleic acid amplification techniques (NAT)

General principles of measurement NAT relies on the reiteration of the DNA polymerisation process, leading to an exponential increase of a specific nucleic acid fragment. The polymerase chain reaction (PCR) is the most widely used method for target DNA amplification. In this cyclic process, a specific DNA fragment is copied by a thermostable DNA polymerase enzyme in the presence of nucleotides and oligonucleotide primers, previously designed to flank the target sequence and to hybridise with it (see also general chapter *2.6.21*). After PCR, the amplified nucleic acid targets can be analysed using several methods of post-amplification analysis: fragment size analysis in gel electrophoresis, DNA sequencing or specific detection by hybridisation with a fluorescent-labelled probe. Real-time PCR eliminates the need for further post-amplification processing and offers the additional advantage that the likelihood of cross-contamination is minimised. An important advantage of real-time PCR is the ability to quantify the starting amount of the DNA target sequence in the original sample, in contrast to conventional PCR techniques, which are based on end-point detection. Since the amount of PCR product detected at the beginning of the exponential phase of the amplification reaction correlates with the initial starting amount of the DNA target, modern real-time PCR techniques have been developed to measure this exponential phase of the reaction. Automated real-time PCR systems are commercially available. For identification of species, either species-specific probes or primers can be used.

RNA can also be amplified by both conventional and real-time PCR after transcription into cDNA using a reverse transcriptase enzyme. This technique is known as reverse transcriptase PCR (RT-PCR) and it enables detection and identification of RNA viruses or viable organisms. Alternatively, specific RNA-based amplification techniques, for example nucleic acid sequence-based amplification or transcription-mediated amplification, are available. Both techniques produce RNA amplicons, in contrast to PCR which only produces DNA amplicons, even when starting from an RNA target.

Types of target to be amplified Regardless of the type of NAT used, the specificity of the test is determined by the target DNA sequence under evaluation.

For identification/characterisation purposes, the 16S or 23S ribosomal RNA genes may be used as targets. The 16S rRNA gene is an evolutionary-conserved gene present in all bacterial species, and is a broad range target as it is a universal marker for bacterial detection. The 23S rRNA gene is not widely used as a single target, but the 16S-23S rRNA transcribed intergenic spacer regions can be employed to distinguish between certain closely related species and/or to identify subtypes. Alternative broad-range targets include the *groEL* and *tuf* genes. Apart from broad-range targets, species-specific sequences can be used as targets for micro-organism identification. Depending on the species, either specific surface antigens, virulence factors or genes which code toxins may be amplified to detect and identify micro-organisms.

General critical aspects:

- the target and the primers chosen must be specific for a particular micro-organism or group of micro-organisms;
- the sensitivity of the methods is highly dependent on the efficiency of the lysis protocol and how successfully the DNA targets can be purified and concentrated in the sample;
- the presence of inhibitors of the enzymatic process results in false negative reactions;
- the procedures are prone to cross-contamination from background DNA resulting in false positive results.

Depending on the aim, a choice must be made between amplification of either a DNA or an RNA target, as this target choice affects the correlation with viability.

DNA targets are generally more widely used for identification purposes, but the use of DNA as a marker has the disadvantage that dead micro-organisms can also be detected. As mRNA is rapidly degraded in dead cells, it is considered a marker for viability. Furthermore, mRNA is the obligatory target for the identification of RNA viruses.

Critical aspects of (semi-) quantitative detection by real-time PCR Quantification of the target requires generation of appropriate standards and the use of standardised procedures.

Critical aspects of RT-PCR RNA is less stable compared to DNA, so it requires more attention during processing. Depending on the quality of the RNA isolation, the efficiency of the cDNA synthesis can vary. RT-PCR can be used to specifically detect RNA if DNA contamination of the RNA sample is low.

Critical aspects of using the 16S or 23S rRNA gene as a target for species identification 16S rRNA gene sequencing is a valuable method for identification of bacteria provided that appropriate universal primers from databases are selected. Its discriminatory power depends on the variability and the length of the 16S rRNA gene within a certain species. Regarding the use of assays targeting the 16S-23S rRNA intergenic spacer regions, the choice of appropriate species-specific primers/probes is of critical

importance due to the potential polymorphism of such regions.

Potential uses of NAT Due to the high sensitivity and specificity of amplification techniques, they are suitable for both detection and identification of micro-organisms. Real-time PCR is needed for quantitative or semi-quantitative analysis of the target. Besides quantitative determinations, the real-time PCR technique allows simultaneous detection of multiple targets in a single sample, as long as appropriate primers and probes that allow for multiplexing are employed. The sequencing of different genes (e.g.16S rDNA, 23S rDNA, rpoB, Gyr) is best applied to the identification of micro-organisms.

2-3-2-3 Genetic fingerprinting

Principles of measurement Genetic fingerprinting is the identification of a strain on the basis of its DNA profile (or RNA for RNA viruses). Individual DNA profiles may be different due to genetic diversity between strains of the same species, and the aim of the fingerprinting methods is to discriminate between these strains. The classical genetic fingerprinting technique characterises micro-organisms using restriction fragments of chromosomal DNA from bacterial and fungal genomes.

Different strains from the same species may exhibit different patterns and these differences are referred to as restriction fragment length polymorphisms (RFLPs). As cutting the chromosomal DNA with restriction enzymes generates too many fragment bands to be efficiently and accurately compared, several modifications of the conventional RFLP-based method have been developed. Examples of the kind of technologies used are ribotyping, pulsed-field gel electrophoresis (PFGE) and amplified fragment length polymorphism (AFLP). Several other fingerprinting methods use PCR to selectively amplify defined subsets of DNA restriction fragments from the entire genome, for example random amplified polymorphic DNA (RAPD) and variable number tandem repeats (VNTR).

Critical aspects All fingerprinting techniques require that the micro-organism is present as a pure culture. Depending on the method, a preliminary enrichment cultivation step may be necessary if a defined quantity or a specific DNA preparation is required for the test, e.g. AFLP and PFGE. The discriminatory power, the reproducibility, the expertise needed and the labour-burden vary among techniques. The major criticism of conventional RFLP analysis is the complexity of the banding patterns. The discriminatory power of ribotyping (based on patterns of rRNA genes) is less than that of PFGE (based on patterns of the whole genomic DNA) or some PCR-based methods, but it has the advantage that it can be a highly automated system. Although PFGE is one of the most highly discriminatory fingerprinting methods, it is time-consuming and technically demanding in the laboratory as it is not automated. It also requires the use of standardised protocols. AFLP has high reproducibility, but requires technical expertise and the interpretation of results may need automated computer analysis. The reproducibility of RAPD may be poor, so it must be performed in a standardised way.

Potential uses Genetic fingerprinting methods are mainly used for strain discrimination (characterisation below species level). They are a powerful tool for investigating and tracing the source and the spread of microbial contamination.

3 VALIDATION OF ALTERNATIVE MICROBIOLOGICAL METHODS

3-1 INTRODUCTION

Validation, whilst subject to a variety of context-specific definitions, can be generally defined as a method to establish documented evidence that a process will consistently achieve its intended goal. Therefore, to validate an alternative microbiological method, it is essential to understand and define what the procedure is intended to achieve.

Typically, pharmaceutical microbiological methods use specific characteristics of micro-organisms as indicators or detection principles in order to determine microbiological quality. The information generally sought is presence/absence, number, viability and/or identity of micro-organisms in a given product or environment. Any given method will usually provide an indirect and conditional measure of microbiological quality. For example, the total number and viability of micro-organisms can be indicated by the number of colonies appearing under a certain set of conditions of sample preparation, cultivation and incubation; reproduction in classical microbiology is hence taken as the general indicator for viability. There are other parameters, however, that can be used as a viability measure, such as the level of ATP or the accumulation or metabolism of substrates in living cells. The results from different viability-indicating methods may not always be identical; micro-organisms may not be able to reproduce on a given medium, but may still accumulate and metabolise a substrate. Conversely, micro-organisms may be unable, at a given state of damage, to accumulate a substrate, but may still be able to recover and reproduce.

Similar considerations arise with the multiplicity of methods used for identification of micro-organisms. Therefore, while characterisation of the pattern of metabolic activity is frequently used for species identification, alternative methods also exist. Again, the outcomes obtained may not be fully consistent for the different identification methods, as one answer may be appropriate for the construction of a correct phylogenetic correlation tree, while another may be more useful in the context of pathogenicity or other property of the differentiated micro-organisms.

3-2 VALIDATION PROCESS

Two levels of validation must be envisaged for the application of alternative microbiological methods, namely primary validation and validation for the intended use. The supplier of the alternative technology typically performs primary validation of a method, whereas validation for the intended use, which is a verification of the suitability or applicability of the method in a given situation, must be seen as the responsibility of the user.

Where specific equipment is critical for the application of a method, the equipment, including computer hardware and software, must be fully qualified.

3-2-1 Description of the technique

In order to characterise a specific microbiological method, the principle of detection must be clearly described by the supplier. Through primary validation, the method must be fully detailed with respect to the conditions required for application, the materials and equipment needed and the expected signal. The user shall critically review the available information.

3-2-2 Risk-benefit analysis

For validation of specific alternative microbiological methods, it is critical that the purpose of the quality assurance procedure is precisely outlined, as this defines the type and

depth of information needed. The information obtained by, and the limitations of, the pharmacopoeial method and the alternative method must be considered and compared in a risk-benefit analysis.

The risk level in adopting an alternative method varies depending on the technology considered, the methodology it replaces, the nature of the measurements taken (qualitative, quantitative or identification), the particular product or process attribute being evaluated, the location of the measurement in the manufacturing process chain and various other factors.

Risk analysis tools may be utilised in order to determine which alternative method is to be implemented, to assist in the justification of its implementation or to better understand the impact of implementation on production and/or product quality. An alternative method can be justified for use if the information obtained gives a scientifically sound measure of microbiological quality, and if the limitations of the method are not more severe than those of the pharmacopoeial method.

3-2-3 Primary validation

The supplier, using a panel of test micro-organisms appropriate for the intended use, must characterise the principle of detection. Depending on the type of alternative method, relevant validation criteria shall be selected from those listed below:

— prerequisite treatment of sample or micro-organisms;
— type of response;
— specificity;
— detection limit;
— quantitation limit;
— range;
— linearity;
— accuracy and precision;
— robustness of the method in a model system.

3-2-4 Validation for the intended use

Validation for the intended use should encompass the entire process, from the decision to change any aspects of a microbiological testing programme to on-going routine use. It should consist of the following phases:

— user requirement specification (URS);
— design qualification (DQ);
— installation qualification (IQ);
— operational qualification (OQ);
— performance qualification (PQ).

The supplier and user have different tasks to perform with regard to the validation and implementation of an alternative method. These tasks are summarised in Table 5.1.6.-1.

Table 5.1.6.-1 – *Tasks to be undertaken during the validation process*

Activity	Normally carried out by	
	Supplier	User
Primary validation	+	-(1)
URS (instrument, application)	-	+
Description of the technique	+	-(2)
Risk benefit analysis	-(3)	+
Design qualification (DQ)	-	+
Installation qualification (IQ)	-(4)	+
Operational qualification (OQ)	-(4)	+
Performance qualification (PQ):		
- verification of primary validation data given by the supplier;	-	+
- verification for the intended use (e.g. sterility testing, TAMC/TYMC, ...);	-	+
- method suitability test	-	+

(1) The user performs primary validation if they employ the alternative method for a use other than that defined by the supplier.
(2) The user shall critically review information provided by the supplier.
(3) As part of commercialisation, the supplier may list advantages of the alternative method over pharmacopoeial techniques.
(4) IQ/OQ for complex equipment, IQ/OQ is often outsourced to supplier.

3-2-4-1 User requirement specification (URS)

The URS describes the functions that the method must be capable of performing and will form the basis of the method selection process. It is an essential document, as acceptance testing will be based on the requirements detailed therein. It is important to consider data management capabilities at this stage, particularly within a regulatory context. The URS shall at least address the following items:

— application of the instrument:
  — the type of analysis to be performed (e.g. quantitative, semi-quantitative, qualitative or identification).
— detection limit or quantitation limit (sensitivity):
  — the detection limit may be linked to time to detection (TTD);
  — the required level of sensitivity, which will depend on the current specification, the dilution regime and the test sample size for the existing test method under replacement.
— specificity:
  — the ability of the alternative test method to selectively detect the micro-organisms or classes of micro-organisms; this should be based on historical data generated from the pharmacopoeial test method and complemented by information from the supplier of the alternative method;
  — the ability to detect only the required viable micro-organisms;
  — for identification methods, the extent of coverage of the database with respect to the range of micro-organisms of interest.
— number and type of samples:
  — the nature of samples to be tested and the manufacturing output per batch or work-shift.
— time to detection (TTD) or time to result (TTR):

— the TTD or TTR is an important attribute for alternative microbiological methods; for monitoring purposes, a relatively short TTD (e.g. a few hours) allows corrective actions to be taken at an early stage; for quality control purposes, a short TTD may be less critical.

— data management capabilities:
  — the new instrumentation may need to have laboratory information management system (LIMS) interface capability and external server compatibility, and the data management tools should be defined; evidence of software validation and functional testing reports will be required to support each part of the software and firmware functions.

3-2-4-2 Design qualification (DQ)

The DQ provides documented evidence that the design of any associated equipment is suitable for correct performance of the method. Most alternative method systems are based on commercial off-the-shelf equipment. The DQ is most suitably performed, therefore, by the instrument developer/manufacturer. Nevertheless, the user shall verify that the equipment meets the specifications laid down in the URS for the intended application.

3-2-4-3 Installation qualification (IQ)

The IQ provides documented evidence that the equipment has been provided and installed in accordance with its specifications.

3-2-4-4 Operational qualification (OQ)

The OQ provides documented evidence that the installed equipment operates within predetermined limits when used in accordance with its operational procedures.

3-2-4-5 Performance qualification (PQ)

The PQ provides documented evidence that the method, with the equipment installed and operated according to operational procedures, consistently performs in accordance with predetermined criteria and thereby yields correct results for the method. This is typically done with a panel of micro-organisms (e.g. pharmacopoeial test strains, in-house isolates or stressed/slow-growing micro-organisms). This assures that the conditions employed by the user laboratory make it possible to satisfy the criteria described by the supplier of the method in the model system used for the primary validation.

Verification of primary validation data given by the supplier (see 3-2-3). The method is verified using the panel of test micro-organisms given by the corresponding pharmacopoeial chapter. The alternative method must be applied according to the specified procedure of the supplier, without the samples to be analysed under the responsibility of the user, and must be shown to give comparable results as characterised in the model system used by the supplier.

Verification for the intended use (e.g. sterility testing, total aerobic microbial count (TAMC)/total combined yeasts/moulds count (TYMC), etc) The following points, where applicable, should be addressed:

— compatibility of the response with the sample preparation that the user normally performs for product testing (method suitability testing);
— limit and range of detection of the method with regard to sample size and sample availability;
— specificity of the response with regard to the influence of the product ingredients;
— linearity of the response with regard to the types of samples to be analysed;
— accuracy and precision of the response with regard to the types of samples to be analysed.

Acceptance criteria for the method will need to be defined as a function of the application and the validation data.

3-3 TYPES OF MICROBIOLOGICAL TESTS

Validation of a microbiological method is the process whereby it is experimentally established by the user that the performance characteristics of the method meet the requirements of the intended application. As microbiological tests have 3 basic applications (qualitative, quantitative and identification), 3 separate sets of validation criteria are required. These criteria are described below and summarised in Table 5.1.6.-2.

Table 5.1.6.-2 – *Validation criteria for qualitative, quantitative and identification tests*

Criterion	Qualitative test	Quantitative test	Identification test
Accuracy	+(1)	+	+
Precision	-	+	-
Specificity	+	+	+
Detection limit	+	-(2)	-
Quantitation limit	-	+	-
Linearity	-	+	-
Range	-	+	-
Robustness	+	+	+
Suitability testing	+	+	-
Equivalence testing	+	+	-

(1) Performing an accuracy test of the alternate method with respect to the pharmacopoeial method can be used instead of the validation of the limit of detection test.
(2) May be needed in some cases.

3-3-1 Validation of alternative qualitative tests for the presence or absence of micro-organisms

3-3-1-1 Specificity

The specificity of an alternative qualitative method is its ability to detect only the required micro-organisms, i.e. does not generate false positive results. This can be demonstrated using a panel of appropriate micro-organisms. Where relevant for the purpose of the test, mixtures of micro-organisms are used during validation. For qualitative methods that rely on growth to demonstrate presence or absence of micro-organisms, specificity is adequately addressed by demonstrating the growth promotion properties of the media. For those methods that do not require growth as an indicator of microbial presence, the specificity assures that extraneous matter in the test system does not interfere with the test.

3-3-1-2 Detection Limit

The detection limit of an alternative qualitative method is the lowest number of micro-organisms in a sample that can be detected under the stated analytical conditions.
A microbiological limit test determines the presence or absence of micro-organisms in a defined quantity of the sample under test. Due to the nature of microbiological tests, the detection limit reflects the number of micro-organisms present in the original sample before any dilution or incubation steps. The detection limit of the alternative method must not be a number greater than that of the pharmacopoeial method.

It is essential that the detection limit is determined using a sufficient number of replicates and a number of independent determinations.

3-3-1-3 Robustness

The robustness of an alternative qualitative method is a measure of its capacity to remain unaffected by small but deliberate variations in method parameters (e.g. incubation period or incubation temperature range). Robustness is a validation parameter best suited to determination by the supplier of the method. Nevertheless, if the user modifies critical parameters, any effect on robustness must be evaluated. Robustness of a qualitative method is judged by its ability to detect the test micro-organisms after deliberate variations to the method parameters.

3-3-1-4 Suitability testing

The alternative method must be applied according to the specified procedure and with the samples to be analysed under the responsibility of the user. It must be shown that the test sample does not interfere with the system's detection capacity or microbial recovery. Specific points to be addressed are:

— the ability of the test to detect micro-organisms in the presence of the sample matrix;
— verifying if the sample matrix interferes with the alternative system (e.g. background signal or inhibiting chemical reactions).

Acceptance criteria for the method in routine use will need to be defined as a function of the application and the validation data.

3-3-1-5 Equivalence testing

Equivalence testing of 2 qualitative methods can be conducted directly on the validation parameters. This approach requires an adequate comparison experiment at low levels of inoculation (e.g. less than 5 CFU) with sufficient numbers of replicates for relevant strains of test micro-organisms. Alternatively, and in some cases additionally, equivalence testing can be carried out by the parallel testing of a predefined number of samples or for a predefined period of time. This parallel testing can be justified based on a risk assessment. The alternative method must enable an unequivocal decision as to whether compliance with the standards of the monographs would be achieved if the official method was used.

3-3-2 Validation of alternative quantitative tests for enumeration of micro-organisms

3-3-2-1 Accuracy

The accuracy of an alternative quantitative method is the closeness of the test results obtained by the alternative method to those obtained by the pharmacopoeial method. Accuracy must be demonstrated across the practical range of the test. It is usually expressed as the percentage recovery of micro-organisms by the alternative method compared to the percentage recovery using the pharmacopoeial method, taking into account statistical analysis.

Accuracy may be shown by preparing and testing a suspension of micro-organisms at the upper end of the test range and serially diluting to the lower end of the test range. For example, if the alternative method is meant to replace the pharmacopoeial plate count method for viable counts, then a reasonable range might be 10^0-10^6 CFU/mL.
If instead, it is a replacement for the MPN method, a much narrower range may be used. At least 1 suspension for each test micro-organism dilution must be analysed.

The alternative method should be shown to recover at least as many micro-organisms as the pharmacopoeial method using appropriate statistical analysis.

The protocol used to check the linearity of the method (see 3-3-2-5) may also be used to check the accuracy.
The suspensions of micro-organisms prepared for the alternative method are counted at the same time using the pharmacopoeial method.

3-3-2-2 Precision

The precision of an alternative quantitative method is the degree of agreement between individual test results when the procedure is applied repeatedly to multiple samplings of homogeneous suspensions of micro-organisms under the prescribed conditions. Precision should be split into repeatability and intermediate precision under normal or routine operating conditions. Repeatability (also referred to as within-run variability) refers to the use of the microbiological method with the same sample (replicate) in the same laboratory over a short period of time with the same analyst and the same equipment. It gives the minimum variability of the method. Intermediate precision (includes run-to-run variability and within-run variability) refers to the use of the microbiological method applied to different sample preparations of the product under test in the same laboratory with different analysts, equipment and/or on different days. It gives the maximum variability of the method.
The precision of a microbiological method is usually expressed as the standard deviation or relative standard deviation (coefficient of variation). At least 1 suspension in the middle of the test range is analysed. The number of replicates is chosen so that the entire test can be carried out during the same working session, i.e. under the same operating conditions and without any change in the suspension of micro-organisms. For intermediate precision, other working sessions are then carried out under conditions of maximum variability (different reagents, operators and/or days, etc.). The variance in the results observed in each of the working sessions is calculated. If the variances are homogeneous, the variance of the repeatability can be calculated. The inter-group variance of the results is also calculated and the resultant variance of the intermediate precision is given as the sum of the variance of the repeatability and the inter-group variance. The coefficient of variation is then calculated. Alternative methods must demonstrate precision comparable to that of the pharmacopoeial methods.

3-3-2-3 Specificity

The specificity of an alternative quantitative method is its ability to quantify only the required micro-organisms, i.e. does not generate false positive results. This may be demonstrated using a panel of appropriate micro-organisms. Where relevant for the purpose of the test, mixtures of micro-organisms are used during validation. For those methods that do not require growth as an indicator of microbial presence, the specificity assures that extraneous matter in the test system does not interfere with the test.

3-3-2-4 Quantitation limit

The quantitation limit of an alternative quantitative method is the lowest number of CFUs in a sample which can be quantitatively determined with suitable precision and accuracy. It is essential that the quantitation limit is determined from a number of replicates. The results of the linearity and accuracy studies can also be used. In this case, the lowest concentration in the linear range is considered to be the quantitation limit of the method. The quantitation

limit of the alternative method must not be greater than that of the pharmacopoeial method.

3-3-2-5 Linearity

The linearity of an alternative quantitative method is its ability (within a given range) to produce results that are proportional to the concentration of micro-organisms present in the sample. The linearity must be determined over a reasonable range (e.g. 10^0-10^6 CFU/mL) so as to correspond to the purpose of the alternative method. One approach would be to select different concentrations of each test micro-organism and test several replicates. For each concentration, an appropriate number of replicates is chosen to confirm linearity. The number of replicates is chosen so that the entire test can be carried out during the same working session. After checking the homogeneity of the variances of the results obtained for each concentration, the regression line is calculated. Linearity is demonstrated if the estimated slope is significant and if the test for deviation from linearity is non-significant (see general chapter *5.3*).

3-3-2-6 Range

The range of an alternative quantitative method is the interval between the upper and lower levels of micro-organisms as determined from the related studies of precision, accuracy and linearity using the specified method; it is dependent on the intended application.

3-3-2-7 Robustness

The robustness of an alternative quantitative method is a measure of its capacity to remain unaffected by small but deliberate variations in method parameters (e.g. incubation period or incubation temperature range). Robustness is a validation parameter best suited to determination by the supplier of the method. Nevertheless, if the user modifies critical parameters, the effects on robustness must be evaluated. Robustness of an alternative quantitative method is judged by its ability to accurately enumerate the test micro-organisms after deliberate variations to the method parameters.

3-3-2-8 Suitability testing

The alternative method must be applied according to the specified procedure and with the samples to be analysed under the responsibility of the user. It must be shown that the test sample does not interfere with the system's enumeration capacity or microbial recovery. Specific points to be addressed are:

— the ability of the test to detect micro-organisms in the presence of the sample matrix;
— verifying if the sample matrix interferes with the alternative system (e.g. background signal or inhibiting chemical reactions).

Acceptance criteria for the method are defined as a function of the application and of the validation data.

3-3-2-9 Equivalence testing

Equivalence testing of 2 quantitative methods can be conducted directly on the validation parameters. This approach requires an adequate comparison experiment at low levels of inoculation (e.g. less than 5 CFU) with sufficient numbers of replicates for relevant strains of test micro-organisms. Alternatively, and in some cases additionally, equivalence testing can be carried out by the parallel testing of a predefined number of samples or for a predefined period of time. This parallel testing can be justified based on a risk assessment.

If the result of the alternative method can be expressed as the number of CFUs per weight or per volume, statistical analysis of the results shall demonstrate that the results of the alternative method enable an unequivocal decision as to whether compliance with the standards of the monographs would be achieved if the official method was used.

If the result of the alternative method cannot be expressed as the number of CFUs, equivalence testing is performed using suitable parameters, followed by statistical analysis to demonstrate that the results of the alternative method enable an unequivocal decision as to whether compliance with the standards of the monographs would be achieved if the official method was used.

3-3-3 Validation of alternative identification tests

There is a large body of evidence that different methods vary considerably in their ability to identify micro-organisms. It must be accepted that a method of identification needs to be internally consistent, but may differ from others in its identification of micro-organisms.

3-3-3-1 Accuracy

The accuracy of an alternative identification method is its ability to identify the desired micro-organism to the required taxonomic level. It must be demonstrated using well-characterised reference micro-organisms, e.g. type strains. Accuracy of the identification method is usually expressed as the number of correct identifications divided by the total number of identifications.

3-3-3-2 Specificity

The specificity of an alternative identification method is its ability to discriminate micro-organisms actually present from interfering factors that cause false identification results. Such factors include chemical substances and mixtures of micro-organisms, which cause the test to identify micro-organisms not actually present in the sample material (e.g. the presence of mixtures of DNA material from 2 micro-organisms in a sequencing test leading to the false identification of a third micro-organism).

3-3-3-3 Robustness

The robustness of an alternative identification method is a measure of its capacity to remain unaffected by small but deliberate variations in method parameters (e.g. incubation period or incubation temperature range). Robustness is a validation parameter best suited to determination by the supplier of the method. Nevertheless, if the user modifies critical parameters, the effects on robustness have to be evaluated. Robustness of an identification method is judged by its ability to correctly identify the test micro-organisms after deliberate variations to the method parameters.

M. Reference Standards

(Ph. Eur. general texts 5.12)

This chapter is published for information.

1 INTRODUCTION

'Reference standard' is used in this chapter as a general term covering reference substances, reference preparations and reference spectra.

Reference standards are frequently necessary to achieve adequate quality control of medicinal products and their components.

Reference standards are established using suitable procedures and their continued suitability for use is monitored according to a predefined programme. Where a reference standard is needed, it is an integral part of the pharmacopoeial

monograph or the manufacturer's specification. Where a European Pharmacopoeia reference standard is referred to in a monograph or general chapter, it represents the official standard that is alone authoritative in case of doubt or dispute.

2 TERMINOLOGY

Primary standard A standard designated or widely acknowledged as having the highest metrological qualities and whose property value is accepted without reference to other standards of the same property or quantity, within a specified context. This definition does not cover international standards.

International standard A primary standard provided to enable the results of biological or immunological assay procedures to be expressed in the same way throughout the world. The value assignment is in International Units (IU) or another suitable unit. The unitage is normally assigned to a first international standard by the World Health Organization (WHO) in an arbitrary manner based on an international interlaboratory study. Activities in International Units are assigned to replacement international standards, where appropriate, by comparing them with a previous standard.

Secondary standard A standard whose property value is assigned by comparison with a primary standard of the same property or quantity.

European Pharmacopoeia reference standard A reference standard established under the aegis of and adopted by the European Pharmacopoeia Commission.

European Pharmacopoeia chemical reference substance (CRS) A substance or mixture of substances intended for use as stated in a monograph or general chapter of the European Pharmacopoeia. CRSs are in general primary standards, except for those (notably antibiotics) that are calibrated in International Units. The latter are secondary standards traceable to the international standard.

European Pharmacopoeia herbal reference standard (HRS) A herbal drug preparation (usually an extract) or a herbal drug intended for use as stated in a monograph or general chapter of the European Pharmacopoeia. Unless otherwise specified, HRSs are designated as primary reference standards for their intended use.

European Pharmacopoeia biological reference preparation (BRP) A substance or mixture of substances intended for use as stated in a monograph or general chapter of the European Pharmacopoeia. BRPs are either secondary standards calibrated in International Units or primary standards, which may be used to define a European Pharmacopoeia Unit (Ph. Eur. U.). Other assigned contents may also be used, for example, virus titre or number of bacteria.

Reference material (RM) A material sufficiently homogeneous and stable with respect to one or more specified properties, which has been established to be fit for its intended use in the measurement process.

Certified reference material (CRM) A reference material characterised by a metrologically valid procedure for one or more specified properties, accompanied by a certificate that states the value of the specified property, its associated uncertainty, and a statement of metrological traceability.

3 USE OF EUROPEAN PHARMACOPOEIA REFERENCE STANDARDS

European Pharmacopoeia reference standards are employed in the identification, purity testing and assay of articles subject to a European Pharmacopoeia monograph or general chapter. European Pharmacopoeia reference standards are shown to be suitable for their intended purpose; they are not necessarily suitable for other purposes. If a European Pharmacopoeia reference standard is to be used for any purpose other than that for which it has been established, its suitability for the new use has to be fully demonstrated and when applicable, to be described in the marketing authorisation application. Any value assigned to a reference standard is valid for the intended use and not necessarily for other uses.

A European Pharmacopoeia reference standard with an assigned content/potency for use in the assay of a substance for pharmaceutical use (see general monograph *Substances for pharmaceutical use (2034)*) may be suitable to determine the content/potency of that substance in a pharmaceutical preparation provided all of the following conditions are fulfilled:

- the chromatographic assay method described in the active substance monograph is employed;
- the applicability of the method to the particular pharmaceutical preparation (absence of interference) is verified by the user;
- any pre-treatment of the sample (e.g. extraction, filtration) is validated for the particular pharmaceutical preparation.

It is the policy of the European Pharmacopoeia to supply reference standards in adequate quantities for immediate use (i.e. the quantity needed to perform the test(s) described in the monograph or general chapter) after opening of the container. Use in other conditions is the responsibility of the analyst. If an unopened container is stored in the recommended conditions, it remains suitable for use as long as it is of the current batch. Information on current batch numbers is provided in the European Pharmacopoeia reference standards database (http://go.edqm.eu/RS). Storage of reconstituted or diluted solutions of reference standards is not recommended unless suitability has been demonstrated by the user.

Secondary standards A secondary standard is usually established to reduce the use of the primary standard and may be used for routine quality control purposes.
A secondary standard shall exhibit the same property or properties as the primary standard to which it is traceable. It shall therefore be used for the same purpose as the primary standard.

International standards are usually available in relatively limited quantities and are intended to be used for the characterisation and calibration of secondary standards; these secondary standards may then be used as working standards.

4 ESTABLISHMENT OF REFERENCE STANDARDS

4-1 PRIMARY STANDARDS

A substance or preparation to be established as a primary standard is characterised by a variety of analytical techniques chosen to demonstrate its suitability for use.

For reference standards used for the control of medicinal products and their components, relevant parts of the following test programme are usually applied.

Test programme:

- Characterisation of the substance (structural elucidation) by appropriate chemical attributes such as structural formula, empirical formula, molecular mass or composition. A number of techniques may be used including:
  - nuclear magnetic resonance spectrometry;
  - mass spectrometry;

  - infrared spectrophotometry;
  - elemental analysis.
- Determination of purity:
  - determination of the content of related substances by an appropriate separation technique and/or spectrometric method, where applicable;
  - quantitative determination of water;
  - determination of the content of residual solvents;
  - determination of loss on drying, which may in certain circumstances replace the determinations of water and residual solvents;
  - determination of inorganic impurities (e.g. sulfated ash, atomic absorption spectrometry, inductively coupled plasma spectrometry, X-ray fluorescence spectrometry); the results are usually not used to determine an assigned content, except where they would have an appreciable impact upon it;
  - determination of purity by an independent method (e.g. quantitative nuclear magnetic resonance spectrometry, differential scanning calorimetry or titration where appropriate; the results of these determinations are usually used to support and confirm the results obtained from separation techniques; they are not used in the calculation of the assigned content).

For biologicals, guidance is given in the WHO recommendations for the preparation, characterisation and establishment of international and other biological reference standards (WHO Technical Report Series).

4-2 EUROPEAN PHARMACOPOEIA CHEMICAL REFERENCE SUBSTANCES

The extent of testing and the number of laboratories involved in the establishment of a CRS depend on the use of the CRS and are tailored to ensure fitness for purpose.

Where an interlaboratory study is carried out during establishment, a protocol is provided for each participant and only valid results derived according to the protocol are used to determine an assigned content or otherwise confirm suitability.

Relevant parts of the following programme are typically applied.

4-2-1 Identification

In general, the candidate batch is shown to comply with the relevant requirements of the monograph; full structural elucidation is carried out for the first batch.

4-2-2 Related substances test

A CRS corresponding to an impurity is characterised for identity and purity. Where a CRS is used to determine the content of a given impurity, the preferred minimum content is 95.0 per cent; where this is achieved the assigned content of the CRS is not given and it is considered to be 100.0 per cent; this approximation is acceptable since there will be no appreciable effect on the determination of impurities. When this minimum content cannot be obtained, an assigned content is given to the CRS.

CRSs used to determine the content of a given impurity are normally in the same acid, base or salt form as the substance that is the subject of the corresponding monograph. Where this is not the case, unless otherwise justified, a corresponding stoichiometric conversion factor is applied.

If an impurity is not available in a sufficient quantity to establish a CRS, a number of other options exist:

- preparation of a CRS that contains a mixture of the compound(s) and the impurity or impurities;
- preparation of a CRS containing a mixture of specified impurities.

Where such a mixture is also used to determine the content of a given impurity, the content of the impurity in the CRS is determined by appropriate separation methods and a content is assigned to the reference standard.

4-2-3 Assay

4-2-3-1 Chemical assay. When a CRS is to be used for quantitative determination of a substance for pharmaceutical use (assay standard), the extent of testing is greater than when a CRS is used for other purposes. Unless the substance is of high purity, several collaborating laboratories are usually involved in testing. The results obtained are used to assign a content. It is particularly important to quantify the impurities if a selective assay is employed. In such a case, it is best to characterise the candidate substance by additional analytical procedures that are scientifically justified, including, where possible, independent methods and methods based on different principles.

For a European Pharmacopoeia chemical reference substance established for assay purposes, the assigned content is usually calculated from the values obtained from the analyses performed for the determination of impurities (organic, inorganic, water and solvents) by applying the principle of mass balance; other suitable methods may also be used. When possible, the assigned content is confirmed by comparing with the result obtained by an independent method.

If a CRS is required for a non-chromatographic assay method (e.g. colorimetry or ultraviolet spectrophotometry), the relative reactivity or relative absorbance of the impurities present in a substance must be checked to ensure that they are not markedly different from those of the substance.

Unless otherwise stated, an assigned content is given for the substance or preparation as presented in the container ('as is'), and the contents are not to be dried before use.
For assay standards prepared by lyophilisation, the content of the pure substance is indicated in milligrams or International Units per vial.

4-2-3-2 Microbiological assay. The potency is expressed in International Units or in European Pharmacopoeia Units if no international standard exists. The assigned potency together with the confidence limits are calculated from statistically valid results of an interlaboratory study, according to the usual statistical procedures (*5.3*).

4-2-3-3 Assay of components of herbal drugs and herbal drug preparations. Reference standards used in monographs on herbal drugs vary in the extent of testing depending on the type of reference standard.

An active component or marker constituent used as a CRS is usually characterised and evaluated for identity and purity; a value for content is assigned irrespective of the purity.

4-2-4 Establishment report

A report containing the results of the establishment study as well as information concerning the use of the CRS is prepared by EDQM, approved by the relevant group of experts and adopted by the European Pharmacopoeia Commission. The report for an assay standard indicates the content assigned to the substance with the rationale for this assignment. The estimated uncertainty of the assigned content is calculated, and where it is less than a predefined value, which is considered to be negligible in relation to the acceptance criteria for the assay, then the study is accepted. Otherwise, the study may be repeated, in whole or in part, or

the limits defined for the pharmaceutical substance may be widened. The uncertainty of the assigned content is usually not given as part of the information provided with the CRS, since the method performance and the uncertainty of the content assigned to the CRS are taken into account when setting the limit(s) in a monograph.

4-3 EUROPEAN PHARMACOPOEIA HERBAL REFERENCE STANDARDS

A HRS is used when the available quantity of pure constituent to be assayed is considered either insufficient or non-sustainable. HRSs may also be established for purposes other than assays, notably for use in tests for adulteration or for system suitability. A HRS is characterised by a variety of analytical techniques chosen to demonstrate its suitability for the intended use; relevant parts of the following test programme may be applied.

Test programme:

- — macroscopy;
- — microscopy;
- — thin-layer chromatography;
- — gas chromatography;
- — liquid chromatography;
- — quantitative determination of water;
- — content of residual solvents;
- — loss on drying;
- — foreign matter;
- — assay of constituents (e.g. constituents with known therapeutic activity, active markers, analytical markers) relevant to the intended use of the reference standard.

The extent of testing and the number of laboratories involved in the establishment of a HRS depend on its intended use.

For a European Pharmacopoeia herbal reference standard used for assay purposes, the assigned content is usually established by an interlaboratory study, using the assay method specified in the individual monograph in which the reference standard is intended to be used, comparing against a suitable pure sample of the constituent or constituents for which the content is to be assigned.

Establishment report

The establishment report for the HRS is prepared in the same manner as CRSs (see section 4-2-4).

4-4 EUROPEAN PHARMACOPOEIA BIOLOGICAL REFERENCE PREPARATIONS AND CHEMICAL REFERENCE SUBSTANCES FOR BIOLOGICALS

Most BRPs and some CRSs used for the testing of biological substances and preparations are established through the Biological Standardisation Programme, under the aegis of the Council of Europe and the European Commission. These reference standards are usually secondary standards calibrated against the corresponding WHO international standard. Where no international standard is available, they are primary standards with an assigned content/potency in European Pharmacopoeia Units or another suitable unit. They are established through interlaboratory studies where participating laboratories test the candidate material(s) and valid data are used to assign the official potency/content. Some of these studies are jointly organised with other organisations to establish a common material or batch of material as standard. In these cases, although the material constituting the European Pharmacopoeia reference standard may be identical to the international standard and its use may be validated through the same interlaboratory study, it is considered as a secondary standard for use as a working standard.

The study reports are endorsed by the study participants and approved by the relevant European Pharmacopoeia group of experts, where applicable, and by the Steering Committee of the Biological Standardisation Programme. The study results are then submitted to the European Pharmacopoeia Commission. Reference standards/materials established through the Biological Standardisation Programme are officially adopted by the European Pharmacopoeia Commission. The establishment reports are published in *Pharmeuropa Bio & Scientific Notes* (http://pharmeuropa.edqm.eu/PharmeuropaBioSN).

4-5 SECONDARY STANDARDS

A secondary standard should exhibit the same property or properties as the primary standard, relevant for the test(s) for which it is established. The extent of testing may not be as comprehensive as is required for the establishment of a primary standard. The secondary standard is established by comparison with the primary standard to which it is traceable. An official primary standard is used wherever possible for establishment of secondary standards.

5 MANUFACTURING, LABELLING, STORAGE AND DISTRIBUTION OF EUROPEAN PHARMACOPOEIA REFERENCE STANDARDS

5-1 MANUFACTURING

All operations are carried out according to the relevant best practices to ensure the traceability and integrity of the reference standard. The manufacturing record includes information regarding filling, labelling and storage. Reference standards are dispensed into containers under appropriate filling and closure conditions to ensure the integrity of the reference standard. The containers employed may be multi-use or single-use, but the latter is preferred to minimise the risk of decomposition, contamination or water uptake.

5-2 LABELLING

The label bears the name of the reference standard, the name and address of the supplier, the batch number and unit quantity (quantity per vial/ampoule).

An accompanying leaflet, considered as part of the labelling, is normally provided.

If used as an assay standard, the following information is also given:

- — the assigned percentage content;
- — or, the content in milligrams or millilitres of the chemical entity in the container;
- — or, the assigned potency (for biological assays or microbiological assays) in units either per milligram, per millilitre or per vial/ampoule.

For European Pharmacopoeia reference standards, no re-test or expiry date is given since the re-test programme (see section 6) monitors continued fitness for use. A batch validity statement (BVS) for each European Pharmacopoeia reference standard is available from the European Pharmacopoeia reference standards database (http://go.edqm.eu/RS).

5-3 STORAGE AND DISTRIBUTION

Reference standards are to be stored and distributed in conditions suitable to ensure optimal stability.

Most European Pharmacopoeia reference standards are stored in temperature-controlled rooms at 5 ± 3 °C. However, a number of reference standards are stored at −20 ± 5 °C and some (e.g. live virus preparations) are stored at − 80 ± 10 °C or at −196 °C to −170 °C under liquid nitrogen.

Appropriate packaging is used to minimise the risk of damage during transport, to keep the reference standard at the

appropriate temperature when necessary and to comply with the current transport regulations.

Reference standards that are stored at 5 ± 3 °C are normally transported without cooling when short-term excursions from the long-term storage temperature are not deleterious to the reference standard. Some of them may nevertheless be sent at + 5 °C, packed with cold packs in cases where an increase in temperature is detrimental to their stability. Reference standards stored at − 20 °C are packed with cold packs or on dry ice (solid carbon dioxide) and dispatched by express courier or air freight. Reference standards stored at − 80 °C or stored under liquid nitrogen are packed on solid carbon dioxide.

Delivery conditions, dispatch and storage temperatures are available in the Reference Standards Catalogue, the Terms of Supply and the European Pharmacopoeia reference standards database (http://go.edqm.eu/RS).

6 RE-TEST PROGRAMME OF EUROPEAN PHARMACOPOEIA STANDARDS

A system is established and implemented to ensure the continued fitness-for-use of the European Pharmacopoeia reference standards. Normally, a re-test programme is applied, taking account of the known physico-chemical properties and stability data for the reference standard. Reference standards are periodically tested for stability during storage. A monitoring programme is applied that is designed to detect at an early stage any sign of degradation using appropriate analytical techniques. The methods employed are typically chosen from amongst those performed during establishment of the reference standard so that baseline data is available.

The periodicity and extent of re-testing reference standards depends on a number of factors including:
- stability;
- container and closure system;
- storage conditions;
- hygroscopicity;
- physical form;
- intended use;
- presentation (single use/multiple use).

The re-test period may be lengthened with the support of sufficient data. The maximum permitted variation from the assigned content should be pre-defined, and if exceeded, the batch should be re-established or replaced.

N. Gene Transfer Medicinal Products for Human Use

(Ph. Eur. general texts 5.14)

This general chapter is published for information.

This general chapter contains a series of texts on gene transfer medicinal products for human use. The texts provide a framework of requirements applicable to the production and control of these products. For a specific medicinal product, application of these requirements and the need for any further texts is decided by the competent authority. The texts are designed to be applicable to approved products; the need for application of part or all of the texts to products used during the different phases of clinical trials is decided by the competent authority. The provisions of the chapter do not exclude the use of alternative production and control methods that are acceptable to the competent authority.

Further detailed recommendations on gene transfer medicinal products for human use are provided by the Note for Guidance on the Quality, Preclinical and Clinical Aspects of Gene Transfer Medicinal Products (CPMP/BWP/3088/99) and the Guideline on Development and Manufacture of Lentiviral Vectors (CHMP/BWP/2458/03) of the Committee for Medicinal Products for Human Use (including any subsequent revisions of these documents).

DEFINITION

For the purposes of this general chapter, gene transfer medicinal product (GTMP) shall mean a product obtained through a set of manufacturing processes aimed at the transfer, to be performed either *in vivo* or *ex vivo*, of a prophylactic, diagnostic or therapeutic gene (i.e. a piece of nucleic acid) to human/animal cells, and its subsequent expression *in vivo*. The gene transfer involves an expression system known as a vector, which can be of viral as well as non-viral origin. The vector can also be included in a human or animal cell.

Recombinant vectors, such as viral vectors and plasmids Recombinant vectors are either injected directly into the patient's body (*in vivo* gene transfer) or transferred into host cells before administration of these genetically modified cells to the patient (*ex vivo* gene transfer). Viral vectors are derived from various viruses (for example, adenoviruses, poxviruses, retroviruses, lentiviruses, adeno-associated-viruses, herpesviruses). These vectors can be replicative, non-replicative or conditionally replicative. Plasmid vectors include nucleic acids in a simple formulation (for example, naked DNA) or complexed to various molecules (synthetic vectors such as lipids or polymers). Genetic material transferred by GTMPs consists of nucleotide sequences, which may notably encode gene products, antisense transcripts or ribozymes. Chemically synthesised oligonucleotides are not within the scope of this general chapter. After transfer, the genetic material may remain either cytoplasmic or episomal, or may be integrated into the host cell genome, depending on the integrating or non-integrating status of the vector.

Genetically modified cells Genetically modified eukaryotic or bacterial cells are modified by vectors to express a product of interest.

PRODUCTION

Substances used in production

The raw materials used during the manufacturing process, including viral seed lot and cell bank establishment, where applicable, are qualified. Unless otherwise justified, all substances used are produced within a recognised quality management system using suitable production facilities. Suitable specifications are established to control notably their identity, potency (where applicable), purity and safety in terms of microbiological quality and bacterial endotoxin contamination. The quality of water used complies with the relevant corresponding monographs (*Purified water (0008)*, *Water for injections (0169)*). Where bovine serum is used, it complies with the monograph *Bovine serum (2262)*. The use of antibiotics is avoided wherever possible during production.

Viral safety

The requirements of chapter *5.1.7* apply.

Transmissible spongiform encephalopathies (*5.2.8*)

A risk assessment of the product with respect to transmissible spongiform encephalopathies is carried out and suitable measures are taken to minimise such risk.

RECOMBINANT VECTORS

PRODUCTION

GENERAL PROVISIONS

For viral vectors, production is based on a cell bank system and a virus seed-lot system, wherever possible.

For plasmid vectors, production is based on a bacterial cell bank system.

The production method shall have been shown to yield a vector of consistent quality. Unless otherwise justified and authorised, the vector in the final product shall have undergone no more passages or subcultures from the master seed lot than were used to prepare the vector shown in clinical trials to be satisfactory with respect to safety and efficacy.

SUBSTRATE FOR VECTOR PROPAGATION

The substrates used comply with relevant requirements of the European Pharmacopoeia (*5.2.2*, *5.2.3*, and the section Bacterial cells used for the manufacture of plasmid vectors for human use).

CHARACTERISATION OF THE VECTOR

Historical records of vector construction are documented, including the origin of the vector and its subsequent manipulation, notably deleted or modified regions.

The vector is characterised using suitable and validated methods.

The genetic stability of the vector at or beyond the maximum passage level or the maximum number of cell doublings of the cell line used for production is assessed by suitable methods.

PROPAGATION AND HARVEST

All processing of the cell banks and subsequent cell cultures is done in an area where no other cells or vectors are handled at the same time. Any material of human or animal origin used in the preparation of cell suspensions and culture media is qualified. The purity of the harvest is verified by suitable tests as defined in the corresponding specific sections.

PURIFIED HARVEST

The bulk of active substance is defined as a lot of purified recombinant vectors (viral vectors, or naked or complexed plasmids).

FINAL LOT

Unless otherwise justified and authorised, formulation and distribution of the final bulk is carried out under aseptic conditions using sterile containers (*3.2*).

The stability of the final lot is assessed using stability protocols including the duration, storage conditions, number of lots to be tested, test schedule and assays to be performed.

ASSAYS AND TESTS

The GTMPs comply with assays and tests described in the corresponding specific sections.

GENETICALLY MODIFIED CELLS

For cells to be modified with a recombinant vector, the data related to the recombinant vector are documented above, under Recombinant vectors.

PRODUCTION

CELL SUBSTRATE

For xenogeneic cell lines, including bacterial cells, a cell bank system comprising a master cell bank and working cell banks is established.

For autologous and allogeneic cells, a cell banking system comprising a master cell bank and working cell banks is established wherever possible.

TRANSFECTION / TRANSDUCTION

Cells are transfected or transduced using a recombinant vector (plasmid or viral vector) qualified as described under Recombinant vectors; the process is validated. They are handled under aseptic conditions in an area where no other cells or vectors are handled at the same time. All reagents used during cell manipulation steps are fully qualified. Antibiotics are avoided unless otherwise justified and authorised. Transfection or transduction is carried out under aseptic conditions.

FINAL LOT

In the case of frozen storage, the viability of genetically modified cells is assessed before freezing and after thawing.

If the cells are not used within a short period, stability is determined by verifying cell viability and expression of the genetic insert.

In the case of genetically modified cells encapsulated before implantation in man, any encapsulating component used is considered as part of the final product, and is therefore quality-controlled and fully characterised (for example, physical integrity, selective permeability, sterility).

ASSAYS AND TESTS

Controls of xenogeneic, allogeneic or autologous cells include the following:

— identity, counting and viability of cells;
— overall integrity, functionality, copies per cell, transfer and expression efficiency of the genetic insert;
— microbiological controls (*2.6.1* or *2.6.27*), endotoxin content, mycoplasma contamination (*2.6.7*), adventitious virus contamination and, where applicable, replicative vector generation.

The competent authority may approve a reduced testing programme where necessary because of limited availability of cells. Where necessary because of time constraints, the product may be released for use before the completion of certain tests.

PLASMID VECTORS FOR HUMAN USE

DEFINITION

Plasmid vectors for human use are double-stranded circular forms of bacterial DNA that carry a gene of interest or a nucleotide sequence encoding antisense sequences or ribozymes and its expression cassette; they are amplified in bacteria extrachromosomally. They are used to transfer genetic material into human somatic cells *in vivo* or to genetically modify autologous, allogeneic, xenogeneic or bacterial cells before administration to humans. Plasmid vectors may be presented as naked DNA or may be formulated with synthetic delivery systems such as lipids (lipoplexes), polymers (polyplexes) and/or peptide ligands that facilitate transfer across the cell membrane and delivery to the cell, or that target delivery via specific receptors.

Plasmids formulated with synthetic delivery systems are not within the scope of this section.

PRODUCTION

PLASMID CONSTRUCTION

A typical plasmid vector is composed of:

— the plasmid vector backbone that contains multiple restriction endonuclease recognition sites for insertion of the genetic insert and the bacterial elements necessary for

plasmid production, such as selectable genetic markers for the identification of cells that carry the recombinant vector;
- the required regulatory genetic elements to facilitate expression of the genetic insert;
- the genetic insert;
- a polyadenylation signal.

A complete description of the plasmid DNA, including its nucleotide sequence, is established with the identification, source, means of isolation and nucleotide sequence of the genetic insert. The source and function of component parts of the plasmid, such as the origin of replication, viral and eukaryotic promoters and genes encoding selection markers, are documented.

GENERAL PROVISIONS

Cell banks
Production of plasmid vectors is based on a bacterial cell-bank system with generation and characterisation of a master cell bank (MCB), working cell banks (WCBs) and end-of-production cells (EOPCs), which comply with the section Bacterial cells used for the manufacture of plasmid vectors for human use. The raw materials used during the manufacturing process, including cell bank establishment, are qualified.

Selection techniques
Unless otherwise justified and authorised, antibiotic-resistance genes used as selectable genetic markers, particularly for clinically useful antibiotics, are not included in the vector construct. Other selection techniques for the recombinant plasmid are preferred.

Reference standards
A suitable batch of the formulated plasmid, preferably one that has been clinically evaluated, is fully characterised and retained for use as a reference standard as necessary in routine control tests.

PROPAGATION AND HARVEST
Plasmid DNA is transferred to host strain bacterial cells and a single clone of transformed bacteria is expanded to create the MCB. The WCB is then derived from the MCB. The EOPCs are obtained from the WCB by fermentation in production conditions.

Plasmid DNA is isolated from harvested cells using an extraction step and is purified to obtain the bulk product.

Unless otherwise justified and authorised, caesium chloride-ethidium bromide density gradients are not used for production.

PURIFIED PLASMID
The production process is optimised to remove impurities consistently while retaining product activity. The requirement to test for a particular impurity depends on the following:
- the demonstrated capability of the manufacture and purification processes to remove or inactivate the impurity through process validation, using specific quantification methods;
- the potential toxicity associated with the impurity;
- the potential decrease of the efficacy of the genetic insert product associated with the impurity.

If selective resistance to specific antibiotics has been used for selection, data from validation studies of purification procedures are required to demonstrate the clearance capability for residual antibiotics.

Relevant in-process controls are performed to ensure that the process is continuously under control, for example, amount and form of plasmid after the extraction steps and amount of endotoxins after the extraction steps.

Only a batch of purified plasmid that complies with the following requirements may be used.

Identity and integrity of the purified plasmid
Identity and integrity of the purified plasmid are established by suitable methods such as sequencing or nucleic acid amplification techniques (NAT) (*2.6.21*); restriction enzyme analysis may be used where it is sufficient to detect potential critical modifications in the plasmid and confirm the plasmid identity.

Plasmid DNA
The following indications are given as examples.

DNA concentrations greater than 500 ng/mL may be determined using absorbance measurement at 260 nm. A 50 µg/mL double-stranded DNA solution has an absorbance of 1 (specific absorbance 200).

DNA concentrations less than 500 ng/mL are determined following incubation with fluorescent dyes that bind specifically to double-stranded DNA, using a reference standard of DNA to establish a calibration curve.

Liquid chromatography may also be used to determine the concentration of plasmid DNA using a reference standard. In some cases, capillary electrophoresis is also acceptable.

DNA forms
Plasmid DNA is characterised in terms of the proportions of supercoiled, multimeric, relaxed monomer and linear forms, using suitable analytical methods, examples of which are given below. For quantification of supercoiled forms, anion-exchange high performance liquid chromatography (HPLC) or capillary electrophoresis may be used. Capillary electrophoresis is also suitable for the quantification of other forms.

Residual host-cell DNA
The content of residual host-cell DNA is determined using a suitable method, unless the process has been validated to demonstrate suitable clearance. Quantitative PCR is recommended for its sensitivity and specificity, but other suitable techniques may also be used.

Residual RNA
The content of residual RNA is determined, unless the process has been validated to demonstrate suitable clearance. Reverse-phase HPLC (RP-HPLC) may be used, or quantitative reverse-transcriptase polymerase chain reaction (RT-PCR) (*2.6.21*) when a lower limit of detection is required.

Residual host-cell protein
The concentration of residual host-cell protein is determined using standard protein assays (*2.5.33*), SDS-PAGE followed by silver staining, or specific immuno-assays such as western blot or ELISA, unless the process has been validated to demonstrate suitable clearance.

Microbiological control
Depending on the preparation concerned, it complies with the test for sterility (*2.6.1*) or the bioburden is determined (*2.6.12*).

Bacterial endotoxins (*2.6.14*)
Less than the limit approved for the particular preparation.

FINAL BULK
Several purified harvests may be pooled during preparation of the final bulk. A stabiliser and other excipients may be added. The formulated product is filtered through a bacteria-retentive filter.

Only a final bulk that complies with the following requirement may be used in the preparation of the final lot.

Sterility (*2.6.1*)
It complies with the test for sterility.

FINAL LOT
Only a final lot that complies with each of the requirements given below under Identification, Tests and Assay may be released for use.

IDENTIFICATION
The plasmid vector is identified by restriction enzyme analysis or by sequencing. The test for biological activity also serves to identify the product.

TESTS
Tests carried out on the final lot include the following.

Appearance

pH (*2.2.3*)
Within the limits approved for the particular preparation.

Extractable volume (*2.9.17*)
It complies with the test for extractable volume.

Residual moisture (*2.5.12*)
Within the limits approved for the particular freeze-dried preparation.

DNA forms
The percentage of the specific monomeric supercoiled form is determined as described for the purified plasmid.

Sterility (*2.6.1*)
It complies with the test for sterility.

Bacterial endotoxins (*2.6.14*)
Less than the limit approved for the particular preparation.

ASSAY

Plasmid DNA
Not less than the quantity stated on the label, determined, for example, by 1 of the following methods.

DNA concentrations greater than 500 ng/mL may be determined using absorbance measurement at 260 nm. A 50 µg/mL double-stranded DNA solution has an absorbance of 1 (specific absorbance 200).

DNA concentrations less than 500 ng/mL are determined following incubation with fluorescent dyes that bind specifically to double-stranded DNA, using a reference standard of DNA to establish a calibration curve.

Liquid chromatography may also be used to determine the concentration of plasmid DNA using a reference standard. In some cases, capillary electrophoresis is also acceptable.

Biological activity
Wherever possible, biological activity is assessed through *in vitro* or *in vivo* bioassays. A well-defined, representative reference standard is required as a positive control for the assay. Bioassays employed to assay plasmid vectors generally involve transfection of a relevant cell line *in vitro*, followed by some functional measure of the expressed genetic insert. Such functional assays provide information about the activity of the product encoded by the genetic insert instead of the expression level of the genetic insert itself.

It may be necessary to supplement the bioassay with western-blot and ELISA assays to assess the integrity and quantity of the expressed product.

LABELLING
The label states:
— the plasmid DNA concentration;
— the recommended human dose;
— for freeze-dried preparations:
  — the name and volume of the liquid to be added;
  — the time within which the product is to be used after reconstitution.

BACTERIAL CELLS USED FOR THE MANUFACTURE OF PLASMID VECTORS FOR HUMAN USE

Production of plasmid vectors for human use is based on the use of a bacterial cell-bank system with generation and characterisation of a master cell bank (MCB), working cell banks (WCBs) and end-of-production cells (EOPCs).
A bacterial cell bank for the manufacture of plasmid vectors is a collection of vials containing bacterial cells stored under defined conditions, with uniform composition, and obtained from pooled cells derived from a single clone of a transformed host strain. The MCB has a known, documented history; it is preferably derived from a qualified repository source. The WCB is produced by expanding one or more vials of the MCB. Methods and reagents used to produce the bank and storage conditions are documented.

MCBs and WCBs are qualified by testing an aliquot of the banked material or testing a subculture of the cell bank.

The following table indicates the tests required at each stage of production.

Assay	Host strain	MCB	WCB	EOPCs*
Identity and purity				
Viability	+	+	+	+
Bacterial strain characterisation	+	+	–	+
Genotyping / phenotyping	+	+	–	+
Presence of the plasmid				
— Sequence of the DNA plasmid	–	+	–	+
— Copy number	–	+	+	+
— Restriction map	–	+	+	+
— Percentage of cells retaining the plasmid	–	+	+	+
Adventitious agents				
Purity by plating	+	+	+	+
Presence of bacteriophage	+	+	–	+

* EOPCs are cells with a passage number at least equivalent to that used for production. The analysis has to be done once to validate each new WCB, except for purity, which has to be tested for each fermentation.

IDENTITY AND PURITY TESTING

Viability
The number of viable cells is determined by plating a diluted aliquot of bacterial cells on an appropriate medium and counting individual colonies.

Biochemical and physiological bacterial strain characterisation
Depending on the bacterial strain used for production, relevant biochemical and physiological characterisation is performed to confirm cell identity at the species level.

Genotyping / phenotyping
The genotype of bacterial cells is verified by determination of the suitable specific phenotypic markers or by appropriate genetic analysis.

Presence of the plasmid
Sequencing The whole nucleotide sequence of the plasmid is verified.

Copy number The plasmid DNA is isolated and purified from a known number of bacteria and the copy number

determined by a suitable method such as quantitative PCR (*2.6.21*).

Restriction map Restriction endonuclease digestion is performed with sufficient resolution to verify that the structure of the plasmid is unaltered in bacterial cells.

Percentage of cells retaining the plasmid Bacterial elements present in the plasmid, such as selectable genetic markers, are used to define the percentage of bacteria retaining the plasmid.

ADVENTITIOUS AGENTS AND ENDOGENOUS VIRUSES

Purity by plating

Bacterial cells are streaked out onto suitable media and incubated in the required conditions in order to detect potential bacterial contaminants. In order to test for inhibition of the growth of contaminating organisms, additional tests in the presence of a definite amount of relevant positive control bacteria are carried out. A suitable number of colonies is examined; no contamination is detected.

Presence of bacteriophage

Bacterial cells are plated and incubated in a medium allowing proliferation of bacteriophages, to test for bacteriophage presence. The test is validated by the use of a reference bacteriophage strain and permissive cells as positive controls. A suitable number of colonies is examined; no contamination is detected.

ADENOVIRUS VECTORS FOR HUMAN USE

DEFINITION

Adenovirus vectors for human use are freeze-dried or liquid preparations of recombinant adenoviruses, genetically modified to transfer genetic material to human somatic cells *in vivo* or *ex vivo*.

PRODUCTION

VECTOR CONSTRUCTION

There are different approaches for the design and construction of an adenovirus vector. The purpose of clinical use determines which approach is optimal. A method is chosen that minimises the risk of generating replication-competent adenovirus vectors or that effectively eliminates helper viruses that might be used during production.

VECTOR PRODUCTION

The production method shall have been shown to yield a vector of consistent quality. Unless otherwise justified and authorised, the vector in the final product shall have undergone no more passages from the master seed lot than were used to prepare the vector shown in clinical trials to be satisfactory with respect to safety and efficacy.

The genetic and phenotypic stability of the vector at or beyond the maximum passage level used for production is assessed by suitable methods.

SUBSTRATE FOR VECTOR PROPAGATION

The vector is propagated in continuous cell lines (*5.2.3*) based on a cell bank system. The occurrence of replication-competent adenoviruses may be significant when large regions of homology exist between the viral genome and the genome of the complementation cells. This occurrence may be minimised by minimising the homology between both genomes. The use of cells with no sequence homology with the vector is recommended for production.

VECTOR SEED LOT

Production of the vector is based on a seed-lot system.

The strain of adenovirus used is identified by historical records that include information on its origin and its subsequent manipulation, notably deleted or modified regions. A detailed description of the genetic insert(s) and the flanking control regions is established, including the nucleotide sequence. The method by which the genetic insert is introduced into the vector is documented.

Only a seed lot that complies with the following requirements may be used for vector production.

Identification

The vector is identified in the master seed lot and each working seed lot by immunochemical methods (*2.7.1*), NAT (*2.6.21*) or restriction enzyme analysis.

Genetic and phenotypic characterisation

The following tests are carried out.

- The entire genome of the vector is sequenced at a passage level comparable to a production batch and the analytically determined sequence is compared to the theoretical sequence based on vector construction and available databases.
- Restriction enzyme analysis is performed on the vector DNA of the master seed lot, each working seed lot and a production batch. The viral DNA is extracted, purified and digested with sufficient resolution. The digested fragments are separated by gel electrophoresis or capillary electrophoresis and the observed restriction pattern is compared to the theoretical restriction pattern based on vector construction.
- A suitable number of isolated sub-clones are tested for expression of the genetic insert product(s) and biological activity at a passage level comparable to a production batch. Sub-clones giving lower levels of expression or biological activity need further characterisation.

Vector concentration

The titre of infectious vector or the concentration of vector particles in the master seed lot and each working seed lot are determined.

Extraneous agents (*2.6.16*)

The master seed lot and each working seed lot comply with the tests for extraneous agents.

Replication-competent adenoviruses

Replication-competent adenoviruses are generated by homologous recombination between the recombinant viral DNA and the adenovirus sequences integrated into the genome of the complementation cells.

Detection of replication-competent adenoviruses is performed by a suitable method approved by the competent authority. It is generally performed by an infectivity assay on sensitive detector cell lines, which are not able to complement for the genes deleted from the vector. Other indicators of viral replication may be used as appropriate.

When replication-competent adenoviruses are not supposed to be present in the test sample, considering vector construction and cell lines used, at least 2, but preferably 3 or 4 successive passages are performed on the detector cell line, where applicable. Detection of a cytopathic effect at the end of the passages reveals the presence of replication-competent adenoviruses in the preparation. Positive controls are included in each assay to monitor its sensitivity.

When replication-competent adenoviruses are expected to be present in the test sample, plaque-assays or limit dilution assays on a detector cell line may be performed.

PROPAGATION AND HARVEST

All processing of the cell bank and subsequent cell cultures is done in an area with a suitable containment level where no other cells or vectors are handled at the same time.

Any material of human or animal origin used in the preparation of cell suspensions and culture media is qualified.

The cell culture medium may contain a pH indicator such as phenol red and suitable antibiotics at the lowest effective concentration, but it is preferable to have a substrate free from antibiotics during production. Unless otherwise justified and authorised, at no stage during production is penicillin or streptomycin used. A portion of the production cell cultures is set aside as uninfected cell cultures (control cells).

Each single harvest that complies with the following requirements may be used in the preparation of the purified harvest.

Identification

The vector is identified by immunochemical methods (*2.7.1*), NAT (*2.6.21*) or restriction enzyme analysis.

Vector concentration

The titre of infectious vector and the concentration of vector particles in single harvests are determined.

Extraneous agents (*2.6.16*)

The single harvest complies with the tests for extraneous agents.

Control cells

Control cells comply with a test for identification (*5.2.3*) and a test for extraneous agents (*2.6.16*).

PURIFIED HARVEST

Several single harvests may be pooled before the purification process. The purification process is validated to demonstrate the satisfactory removal of impurities.

Purified harvests that comply with the following requirements may be used in the preparation of the final bulk.

Identification

The vector is identified by immunochemical methods (*2.7.1*), NAT (*2.6.21*) or restriction enzyme analysis.

Genomic integrity

Genomic integrity of the vector is verified by suitable methods such as restriction enzyme analysis.

Vector concentration

The titre of infectious vector and the concentration of vector particles in purified harvests are determined.

Residual host-cell protein

The concentration of residual host-cell protein is determined by a suitable immunochemical method (*2.7.1*), unless the process has been validated to demonstrate suitable clearance.

Residual host-cell DNA

The content of residual host-cell DNA is determined using a suitable method, unless the process has been validated to demonstrate suitable clearance. Quantitative polymerase chain reaction (PCR) is recommended for its sensitivity and specificity, but other suitable techniques may also be used.

Residual reagents

Where reagents are used during the production process, tests for these substances are carried out on the purified harvest, unless the process has been validated to demonstrate suitable clearance.

Residual antibiotics

Where antibiotics are used during the production process, their residual concentration is determined by a microbiological assay (adapted from general method *2.7.2*) or by other suitable methods (for example, liquid chromatography), unless the process has been validated to demonstrate suitable clearance.

FINAL BULK

Several purified harvests may be pooled during preparation of the final bulk. A stabiliser and other excipients may be added. The formulated product is filtered through a bacteria-retentive filter.

Only a final bulk that complies with the following requirement may be used in the preparation of the final lot.

Sterility (*2.6.1*)

It complies with the test for sterility.

FINAL LOT

Only a final lot that complies with each of the requirements given below under Identification, Tests and Assay may be released for use.

Provided that the tests for bovine serum albumin (when bovine serum is used to manufacture the vector) and replication-competent adenoviruses have been carried out with satisfactory results on the final bulk, they may be omitted on the final lot.

IDENTIFICATION

The vector is identified by immunochemical methods (*2.7.1*), NAT (*2.6.21*) or restriction enzyme analysis.

TESTS

Osmolality (*2.2.35*)

Within the limits approved for the particular preparation.

pH (*2.2.3*)

Within the limits approved for the particular preparation.

Extractable volume (*2.9.17*)

It complies with the test for extractable volume.

Residual moisture (*2.5.12*)

Within the limits approved for the particular freeze-dried preparation.

Bovine serum albumin

Not more than the limit approved for the particular preparation, determined by a suitable immunochemical method (*2.7.1*), where bovine serum has been used during production.

Replication-competent adenovirus concentration

Within the limits approved for the particular preparation.

Vector aggregates

Vector aggregates are determined by suitable methods (for example, light scattering).

Sterility (*2.6.1*)

It complies with the test for sterility.

Bacterial endotoxins (*2.6.14*)

Less than the limit approved for the particular preparation.

Thermal stability

Maintain samples of the vector final lot at a temperature and for a length of time that are adapted and authorised for the particular preparation. Determine the total infectious vector concentration after heating, as described below under Assay. Determine in parallel the vector concentration of a non-heated sample. The estimation of the difference between the total vector concentration without heating and after heating is within the limits approved for the particular preparation.

ASSAY

Vector particle concentration

Physical titration is performed by a suitable technique (for example, liquid chromatography, absorbance measurement or

NAT (*2.6.21*)). Use an appropriate vector reference standard to validate each assay.

The vector particle concentration of the preparation to be examined is not less than the concentration stated on the label.

Infectious vector titre

Titrate the preparation to be examined by inoculation into cell cultures. Titrate an appropriate vector reference standard to validate each assay.

The assay is invalid if:

— the confidence interval ($P = 0.95$) of the logarithm of the vector concentration is greater than a value authorised by the competent authority;
— the infectious vector titre of the reference standard is outside limit values defined by a control chart.

Ratio of vector particle concentration to infectious vector titre

Within the limits approved for the particular preparation.

Expression of the genetic insert product

The expression of the genetic insert product(s) is determined wherever possible, following inoculation of cell cultures with the particular preparation at a predetermined multiplicity of infection, by suitable immunochemical (*2.7.1*) or biochemical assays or by flow cytometry (*2.7.24*).

Biological activity

Unless otherwise justified and authorised, biological activity is determined by a suitable *in vitro* or *in vivo* test.

LABELLING

The label states:

— the content of active substance;
— the recommended human dose, expressed in vector particle concentration;
— for freeze-dried preparations:
  — the name or composition and the volume of the reconstituting liquid to be added;
  — the time within which the product is to be used after reconstitution.

POXVIRUS VECTORS FOR HUMAN USE

DEFINITION

Poxvirus vectors for human use are freeze-dried or liquid preparations of recombinant poxviruses, genetically modified to transfer genetic material to human somatic cells *in vivo* or *ex vivo*.

PRODUCTION

VECTOR CONSTRUCTION

The general design of a poxvirus vector is currently as follows: the genetic insert is inserted downstream of a poxvirus promoter. This expression cassette is inserted into the poxvirus genome in such a manner that it interrupts a viral gene non-essential for replication or is positioned between 2 virus open reading frames.

In most strategies used so far for the construction of the vector, the expression cassette is first inserted within the target site of a virus DNA fragment cloned into a bacterial plasmid. The plasmid is then introduced into host cells, cultured *in vitro*, which are simultaneously infected with the parental poxvirus. DNA recombination occurs within the infected cells, between homologous sequences in the viral genome and viral sequences in the plasmid so as to transfer the genetic insert into the targeted site of the viral genome. The correct targeting of the inserted DNA is checked by restriction-enzyme mapping, NAT (*2.6.21*) and sequencing.

Successive plaque-cloning steps are performed to purify the recombinant poxvirus from the mixture of parental and recombinant poxviruses. A variety of methods (for example, foreign marker genes, DNA hybridisation, immunological detection, phenotypic changes in the virus) are employed to facilitate recognition and/or selection of the recombinant poxvirus from the background of parental virus. Where foreign marker genes have been transiently employed, they are removed by appropriate methods from the final recombinant poxvirus.

An alternative strategy for creating poxvirus vectors begins with the *in vitro* construction of a full-length virus genome harbouring the expression cassette within a chosen target site. This recombinant genome is then introduced into host cells simultaneously infected with a helper poxvirus that is unable to multiply. The helper virus may be a poxvirus of the same species whose ability to multiply has been inactivated, or another poxvirus species that does not multiply in the host cells.

The construction of non-replicative poxvirus vectors relies on specific host cell lines or primary cells that are naturally permissive, or on host cell lines that have been modified to express an essential poxvirus gene. These cells fulfill the general requirements for the production of medicinal products (*5.2.3*) and do not allow the generation of replicative vectors.

VECTOR PRODUCTION

The production method shall have been shown to yield a vector of consistent quality. Unless otherwise justified and authorised, the vector in the final product shall have undergone no more passages from the master seed lot than were used to prepare the vector shown in clinical trials to be satisfactory with respect to safety and efficacy.

The genetic and phenotypic stability of the vector at or beyond the maximum passage level used for production is assessed by suitable methods.

SUBSTRATE FOR VECTOR PROPAGATION

The vector is propagated under aseptic conditions in human diploid cells (*5.2.3*), in continuous cell lines (*5.2.3*) or in cultures of chick-embryo cells derived from a chicken flock free from specified pathogens (*5.2.2*). When the vector is propagated in a continuous cell line or in human diploid cells, a cell-bank system is established.

VECTOR SEED LOT

Production of the vector is based on a seed-lot system.

The strain of poxvirus used is identified by historical records that include information on its origin and its subsequent manipulation, notably deleted or modified regions. A detailed description of the genetic insert(s) and the flanking control regions is established, including the nucleotide sequence. The method by which the genetic insert is introduced into the vector is documented.

Only a seed lot that complies with the following requirements may be used for vector production.

Identification

The vector is identified in the master seed lot and each working seed lot by immunochemical methods (*2.7.1*) or NAT (*2.6.21*).

Genetic and phenotypic characterisation

The following tests are carried out.

— The entire genome of the vector is sequenced at a passage level comparable to a production batch and the analytically determined sequence is compared to the

theoretical sequence based on vector construction and available databases.
- Restriction enzyme analysis is performed on the vector DNA of the master seed lot, each working seed lot and a production batch. The viral DNA is extracted, purified and digested with sufficient resolution. The digested fragments are separated by gel electrophoresis or capillary electrophoresis and the observed restriction pattern is compared to the theoretical restriction pattern based on vector construction.
- A suitable number of isolated sub-clones are tested for expression of the genetic insert product(s) and biological activity at a passage level comparable to a production batch. Sub-clones giving lower levels of expression or biological activity need further characterisation.
- The host range is verified by determining the replication properties of the vector and comparing them with that of the parental virus, at a passage level comparable to a production batch.

Infectious vector titre
The titre of infectious vector in the master seed lot and each working seed lot is determined.

Extraneous agents (*2.6.16*)
The master seed lot and each working seed lot comply with the tests for extraneous agents, except where cytopathic strains cannot be neutralised and the vector causes interference. Where a test cannot be performed, carry out a suitable validated alternative.

PROPAGATION AND HARVEST
All processing of the cell bank and subsequent cell cultures is done under aseptic conditions in an area with a suitable containment level where no other cells or vectors are handled at the same time. Any material of human or animal origin used in the preparation of cell suspensions and culture media is qualified. The cell culture medium may contain a pH indicator such as phenol red and suitable antibiotics at the lowest effective concentration, but it is preferable to have a substrate free from antibiotics during production. Unless otherwise justified and authorised, at no stage during production is penicillin or streptomycin used. A portion of the production cell culture is set aside as uninfected cell cultures (control cells).

Each single harvest that complies with the following requirements may be used in the preparation of the purified harvest.

Identification
The vector is identified by immunochemical methods (*2.7.1*) or NAT (*2.6.21*).

Infectious vector titre
The titre of infectious vector in single harvests is determined.

Extraneous agents (*2.6.16*)
The single harvest complies with the tests for extraneous agents, except where cytopathic strains cannot be neutralised and the vector causes interference. Where a test cannot be performed, carry out a suitable validated alternative.

Control cells
If human diploid cells or a continuous cell line are used for production, the control cells comply with a test for identification (*5.2.3*). They comply with the tests for extraneous agents (*2.6.16*).

PURIFIED HARVEST
Processing is carried out under aseptic conditions. Several single harvests may be pooled before the purification process. The harvest is first clarified to remove cells and then, where applicable, purified by validated methods.

Purified harvests that comply with the following requirements may be used in the preparation of the final bulk.

Identification
The vector is identified by immunochemical methods (*2.7.1*) or NAT (*2.6.21*).

Genomic integrity
Genomic integrity of the vector is verified by suitable methods such as restriction enzyme analysis.

Infectious vector titre
The titre of infectious vector in purified harvests is determined.

Ratio of infectious vector titre to total protein concentration
The total protein concentration is determined by a suitable method (*2.5.33*). The ratio between infectious vector titre and total protein concentration is calculated.

Residual host-cell protein
The concentration of residual host-cell protein is determined by a suitable immunochemical method (*2.7.1*), unless the process has been validated to demonstrate suitable clearance.

Residual host-cell DNA
The content of residual host-cell DNA is determined using a suitable method, unless the process has been validated to demonstrate suitable clearance. Quantitative PCR is recommended for its sensitivity and specificity, but other suitable techniques may also be used.

Residual reagents
Where reagents are used during the production process, tests for these substances are carried out on the purified harvest, unless the process has been validated to demonstrate suitable clearance.

Residual antibiotics
Where antibiotics are used during the production process, their residual concentration is determined by a microbiological assay (adapted from general method *2.7.2*) or by other suitable methods (for example, liquid chromatography), unless the process has been validated to demonstrate suitable clearance.

FINAL BULK
Several purified harvests may be pooled during preparation of the final bulk. A stabiliser and other excipients may be added.

Only a final bulk that complies with the following requirement may be used in the preparation of the final lot.

Sterility (*2.6.1*)
It complies with the test for sterility.

FINAL LOT
Only a final lot that complies with each of the requirements given below under Identification, Tests and Assay may be released for use.

Provided that the test for bovine serum albumin (when bovine serum is used to manufacture the vector) has been carried out with satisfactory results on the final bulk, it may be omitted on the final lot.

IDENTIFICATION
The vector is identified by immunochemical methods (*2.7.1*) or NAT (*2.6.21*).

TESTS
Osmolality (*2.2.35*)
Within the limits approved for the particular preparation.

pH (*2.2.3*)
Within the limits approved for the particular preparation.

Extractable volume (*2.9.17*)
It complies with the test for extractable volume.

Residual moisture (*2.5.12*)
Within the limits approved for the particular freeze-dried preparation.

Bovine serum albumin
Not more than the limit approved for the particular preparation, determined by a suitable immunochemical method (*2.7.1*), where bovine serum has been used during production.

Sterility (*2.6.1*)
It complies with the test for sterility.

Bacterial endotoxins (*2.6.14*)
Less than the limit approved for the particular preparation.

Thermal stability
Maintain samples of the vector final lot at a temperature and for a length of time that are adapted and authorised for the particular preparation. Determine the total infectious vector concentration after heating, as described below under Assay. Determine in parallel the vector concentration of a non-heated sample. The estimation of the difference between the total vector concentration without heating and after heating is within the limits approved for the particular preparation.

ASSAY

Infectious vector titre
Titrate at least 3 vials of the preparation to be examined by inoculation into cell cultures. Titrate a vial of an appropriate vector reference standard to validate each assay.

The vector titre of the preparation to be examined is not less than the minimum titre stated on the label.

The assay is invalid if:
- the confidence interval ($P = 0.95$) of the logarithm of the vector concentration is greater than a value authorised by the competent authority;
- the infectious vector titre of the reference standard is outside limit values defined by a control chart.

Expression of the genetic insert product
The expression of the genetic insert product(s) is determined, wherever possible, following inoculation of cell cultures with the particular preparation at a predetermined multiplicity of infection, by suitable immunochemical (*2.7.1*) or biochemical assays or by flow cytometry (*2.7.24*).

Biological activity
Unless otherwise justifed and authorised, biological activity is determined by a suitable *in vitro* or *in vivo* test.

LABELLING

The label states:
- the minimum vector titre per human dose;
- the recommended human dose;
- for freeze-dried preparations:
  - the name or composition and the volume of the reconstituting liquid to be added;
  - the time within which the product is to be used after reconstitution.

RETROVIRIDAE-DERIVED VECTORS FOR HUMAN USE

DEFINITION

Retroviridae-derived vectors for human use are liquid or freeze-dried preparations of recombinant retroviruses, lentiviruses or spumaviruses, genetically modified to render them replication-incompetent, which are used to transfer genetic material to human somatic cells *in vivo* or *ex vivo*. This section applies to non-replicative vectors.

PRODUCTION

VECTOR CONSTRUCTION

A typical vector is composed of:
- the minimal genome from parental viruses containing the structural genetic elements shown to be indispensable for vector production;
- the required regulatory genetic elements for expression of the genetic insert (for example, long terminal repeats (LTRs));
- the genetic insert.

The vector construction is designed to prevent the generation of replication-competent viruses.

VECTOR PRODUCTION

The production method shall have been shown to yield a vector of consistent quality. Unless otherwise justified and authorised, the packaging or producer cells shall have undergone no more cell doublings from the master cell bank (MCB) than were used to prepare the vector shown in clinical trials to be satisfactory with respect to safety and efficacy.

The genetic and phenotypic stability of the packaging or producer cells at or beyond the maximum number of cell doublings used for production is assessed by suitable methods.

Vectors are produced in continuous cell lines (*5.2.3*) using a cell-bank system. Production may involve either stably or transiently transfected cells.

DEFINITIONS

Packaging cells A source cell line stably transfected with plasmids containing the viral genes necessary for production of empty vector particles: *gag*, *pol*, *env*.

Producer cells Contain the viral genes and expression cassette necessary for vector production.
- In stable production systems, the producer cells are generated by stable transfection of the packaging cell line by a transfer plasmid containing the sequence of interest.
- In transient production systems, the producer cells are generated at the time of manufacture by simultaneous transfection of the source cell line with both the viral genes and the transgene expression plasmid, or by transient transfection of the packaging cell line by a transfer plasmid containing the sequence of interest.

PRODUCTION INTERMEDIATES

Packaging cells

Copy number
The genomic DNA is isolated and purified from a known number of cells and the *gag*, *pol* and *env* genes copy number is determined by a suitable method such as quantitative PCR (*2.6.21*).

Sequence integrity of the viral genes
Complete nucleotide sequencing of the inserted viral genes and their regulatory elements is performed.

Genetic stability
Genetic stability of the packaging cells is verified at or beyond the maximum number of cell doublings used for production.

Plasmids

Production of the vector requires the use of plasmid intermediates. For each plasmid DNA used during

production, a complete description is established, including identification, source, means of isolation and nucleotide sequence. The source and function of component parts of these plasmids, such as the origin of replication, viral and eukaryotic promoters and genes encoding selection markers, are documented.

Production of plasmid intermediates is based on a bacterial cell-bank system. The MCB complies with the requirements of the section Bacterial cells used for the manufacture of plasmid vectors for human use. Plasmids are purified by suitable techniques.

Only plasmid batches that comply with the following requirements may be used for the production of the vector.

Identification
Plasmids are identified by restriction enzyme analysis, sequencing or NAT (*2.6.21*).

Genomic integrity
Genomic integrity of the plasmid is verified by suitable methods such as restriction enzyme analysis of the viral genes, the genetic insert and their respective regulation elements.

Plasmid DNA
The following indications are given as examples.

DNA concentrations greater than 500 ng/mL may be determined using absorbance measurement at 260 nm. A 50 µg/mL double-stranded DNA solution has an absorbance of 1 (specific absorbance 200).

DNA concentrations less than 500 ng/mL are determined following incubation with fluorescent dyes that bind specifically to double-stranded DNA, using a reference standard of DNA to establish a calibration curve.

Liquid chromatography may also be used to determine the concentration of plasmid DNA using a reference standard. In some cases, capillary electrophoresis is also acceptable.

Residual host-cell DNA
The content of residual host-cell DNA is determined using a suitable method, unless the production process has been validated to demonstrate suitable clearance. Quantitative PCR is recommended for its sensitivity and specificity, but other suitable techniques may also be used.

Bacterial endotoxins (*2.6.14*)
Less than the limit approved for the particular preparation.

Sterility (*2.6.1*)
It complies with the test for sterility.

Producer cells used in a stable production system

Copy number
The copy number of the integrated viral genes and expression cassette is determined by a suitable method.

Genetic stability
Genetic stability of the producer cells at or beyond the maximum number of cell doublings used for production is confirmed.

Sequence integrity of the viral genes and expression cassette
Complete nucleotide sequencing of the inserted viral genes, the expression cassette and their respective regulation elements (for example, LTRs, promoters, psi sequence, polyadenylation signal) is performed.

Replication-competent viruses
The detection of replication-competent viruses is performed by suitable methods. Detection may be based on a co-cultivation for several cell doublings of the producer cells with a permissive cell line, followed by detection (either by observation of a cytopathic or haemadsorbing effect on indicator cells like PG4 S+L-, by detection using indicator cell lines by NAT (*2.6.21*) or by marker-rescue assay). Positive controls are included in each assay to monitor its sensitivity. No replication competent viruses are found.

PRODUCTION AND HARVEST
All processing of the cell bank and subsequent cell cultures is done in an area with a suitable containment level where no other cells or vectors are handled at the same time.
Any material of human or animal origin used in the preparation of cell suspensions and culture media must be qualified. It is preferable to have a substrate free from antibiotics during production. Unless otherwise justified and authorised, at no stage during production is penicillin or streptomycin used.

Each single harvest that complies with the following requirements may be used in the preparation of the purified harvest.

Identification
The vector is identified by immunochemical methods (*2.7.1*), NAT (*2.6.21*) or restriction enzyme analysis.

Vector concentration
The titre of infectious vector and/or the concentration of vector particles in single harvests is determined.

Extraneous agents
Each single harvest complies with the tests for extraneous agents (*2.6.16*).

Control cells
Where a transient production process is used, control cells comply with a test for identification (*5.2.3*) and a test for extraneous agents (*2.6.16*).

PURIFIED HARVEST
Several single harvests may be pooled before purification. Purified harvests that comply with the following requirements may be used in the preparation of the final bulk.

Identification
The vector is identified by immunochemical methods (*2.7.1*), NAT (*2.6.21*) or restriction enzyme analysis.

Genomic integrity
Genomic integrity of the vector is verified by a suitable method.

Vector concentration
The infectious particle titre is determined by a suitable method, for example infection of permissive cells followed by quantitative NAT (for example, quantitative PCR), Southern blot or protein expression. For lentivirus vectors, the physical titre is measured, for example by ELISA (p24).

Replication-competent viruses
Detection of replication-competent viruses is performed by suitable methods. It is generally performed by amplification on permissive cells followed by NAT (*2.6.21*), by detection of a viral antigen (for example, p24 by ELISA) or by marker-rescue assay. Positive controls are included in each assay to monitor its sensitivity.

Detection of replication-competent viruses is performed on the purified harvest or on the final lot. No replication-competent viruses are found.

Residual host-cell protein
The concentration of residual host-cell protein is determined by a suitable immunochemical method (*2.7.1*), unless the process has been validated to demonstrate suitable clearance.

Residual host-cell DNA
The content of residual host-cell DNA is determined using a suitable method, unless the process has been validated to demonstrate suitable clearance. Quantitative PCR is recommended for its sensitivity and specificity, but other suitable techniques may also be used.

Residual reagents
Where reagents are used during production, tests for these substances are carried out on the purified harvest, unless the process has been validated to demonstrate suitable clearance.

Residual antibiotics
Where antibiotics are used during the production process, their residual concentration is determined by a microbiological assay (adapted from general method *2.7.2*) or by other suitable methods (for example, liquid chromatography), unless the process has been validated to demonstrate suitable clearance.

Residual plasmids
Where a transient production process is used, the concentration of residual contaminating plasmids must be quantified.

FINAL BULK
Several purified harvests may be pooled during preparation of the final bulk. A stabiliser and other excipients may be added. The formulated product is filtered through a bacteria-retentive filter.

Only a final bulk that complies with the following requirement may be used in the preparation of the final lot.

Sterility (*2.6.1*)
It complies with the test for sterility.

FINAL LOT
Only a final lot that complies with each of the requirements given below under Identification, Tests and Assay may be released for use.

Provided that the tests for bovine serum albumin (when bovine serum is used to manufacture the vector) and replication-competent viruses have been carried out with satisfactory results on the purified harvest, they may be omitted on the final lot.

IDENTIFICATION
Retroviridae-derived vectors are identified by NAT (*2.6.21*), immunochemical methods (*2.7.1*) or restriction enzyme analysis.

TESTS
Osmolality (*2.2.35*)
Within the limits approved for the particular preparation.

pH (*2.2.3*)
Within the limits approved for the particular preparation.

Extractable volume (*2.9.17*)
It complies with the test for extractable volume.

Residual moisture (*2.5.12*)
Within the limits approved for the particular freeze-dried preparation.

Bovine serum albumin
Where bovine serum has been used during production, not more than the limit approved for the particular preparation, determined by a suitable immunochemical method (*2.7.1*).

Replication-competent viruses
Detection of replication-competent viruses is performed by suitable methods. It is generally performed by amplification on permissive cells followed by NAT (*2.6.21*), detection of a viral antigen (for example, p24 by ELISA) or marker-rescue assay. Positive controls are included in each assay to monitor its sensitivity.

Detection of replication-competent viruses is performed on the purified harvest or on the final lot. No replication-competent viruses are found.

Sterility (*2.6.1*)
It complies with the test for sterility.

Bacterial endotoxins (*2.6.14*)
Less than the limit approved for the particular preparation.

ASSAY
Vector-particle concentration
Physical titration is performed by a suitable technique (for example, immunochemical methods (*2.7.1*) or NAT (*2.6.21*)). Use an appropriate vector reference standard to validate each assay.

Infectious vector titre
Titrate the preparation to be examined by inoculation into cell cultures. Titrate an appropriate vector reference standard to validate each assay.

The infectious vector titre of the preparation to be examined is not less than the minimum titre stated on the label.

The assay is invalid if:
- the confidence interval ($P = 0.95$) of the logarithm of the vector concentration is greater than a value authorised by the competent authority;
- the infectious vector titre of the reference standard is outside limit values defined by a control chart.

Ratio of vector-particle concentration to infectious vector titre
Within the limits approved for the particular product, where applicable.

Expression of the genetic insert product
The expression of the genetic insert product(s) is determined wherever possible, following inoculation of cell cultures with the product at a predetermined multiplicity of infection, by suitable immunochemical (*2.7.1*) or biochemical assays or by flow cytometry (*2.7.24*).

Biological activity
Unless otherwise justified and authorised, biological activity is determined by a suitable *in vitro* or *in vivo* test.

LABELLING
The label states:
- the minimum vector titre per human dose;
- the recommended human dose;
- for freeze-dried preparations:
  - the name or composition and the volume of the reconstituting liquid to be added;
  - the time within which the product is to be used after reconstitution.

ADENO-ASSOCIATED-VIRUS VECTORS FOR HUMAN USE

DEFINITION
Adeno-associated-virus (AAV) vectors for human use are freeze-dried or liquid preparations of recombinant AAV (rAAV), genetically modified to transfer genetic material to human somatic cells *in vivo* or *ex vivo*.

PRODUCTION
VECTOR CONSTRUCTION
RAAV vectors are developed by replacement of the *rep* and *cap* genes with the genetic insert of interest. The inverted terminal repeat (ITR) sequences are retained in the rAAV

vector since these are the only AAV sequences absolutely required in *cis* to function as the origin of replication. The *rep* and *cap* genes are required in *trans* and function for replication and packaging respectively. In summary, the rAAV vector contains the ITRs and the genetic insert.

Wild-type AAV normally replicate only in the presence of helper functions, provided by a coinfecting adenovirus or herpes virus. Therefore, there are different approaches to the manufacture of an AAV vector. The manufacturing strategy chosen is designed to minimise the risk for the generation of replication-competent AAV vectors and effectively eliminate helper viruses that might be used during production.

VECTOR PRODUCTION

The production method shall have been shown to yield a vector of consistent quality and stability.

To produce AAV vectors, several strategies are currently used, for example:

— transient co-transfection of a cell line with plasmids containing the ITRs and the genetic insert, *rep* and *cap* genes and helper functions;
— infection with a replication-deficient helper virus of a producer cell line harbouring *rep* and *cap* genes, the ITRs and the genetic insert;
— infection of a permissive cell line with 1 or several production viruses encoding *rep* and/or *cap* and/or the genetic insert and the ITRs, and that may or may not provide helper functions (helper viruses and baculoviruses, respectively).

Depending on the strategy used to produce AAV vectors, different production intermediates are required (plasmids, viruses used for production, packaging cells).

The occurrence of replication-competent AAV may be significant when regions of homology exist between the genomes of the production intermediates and the rAAV vector. This occurrence may be minimised by reducing the homology between these genomes to a minimum. The use of production intermediates with no sequence homology is recommended for production.

The genetic and phenotypic stability of the vector at or beyond the maximum number of passage levels used for production is assessed by suitable methods.

PRODUCTION INTERMEDIATES

Viruses used for production and the rAAV vector are produced in continuous cell lines (*5.2.3*) using a seed lot and a cell-bank system.

Packaging and producer cells

Copy number

The genomic DNA is isolated and purified from a known number of cells and the copy number of the inserted viral genes and of the expression cassette is determined by a suitable method such as quantitative PCR (*2.6.21*).

Sequence integrity of the viral genes and expression cassette

Complete nucleotide sequencing of the inserted viral genes, of their regulatory elements and where applicable, of the expression cassette is performed.

Genetic stability

Genetic stability of the cells is verified at or beyond the maximum number of cell doublings used for production.

Wild-type AAV

The absence of wild-type AAV is verified using NAT (*2.6.21*).

Plasmids

Production of the AAV vector by transient co-transfection requires the use of plasmid intermediates. For each plasmid DNA used during production, a complete description is established, including identification, source, means of isolation and nucleotide sequence. The source and function of component parts of these plasmids, such as the origin of replication, viral and eukaryotic promoters and genes encoding selection markers, are documented.

Production of plasmid intermediates is based on a bacterial cell-bank system. The master cell bank complies with the requirements of the section Bacterial cells used for the manufacture of plasmid vectors for human use. Plasmids are purified by suitable techniques.

Only plasmid batches that comply with the following requirements may be used for the production of the AAV vector.

Identification

Plasmids are identified by restriction enzyme analysis, sequencing or NAT (*2.6.21*).

Genomic integrity

Genomic integrity of the plasmid is verified by suitable methods such as restriction enzyme analysis of the region corresponding to *rep*, *cap* and the expression cassette.

Plasmid DNA

The following indications are given as examples.

DNA concentrations greater than 500 ng/mL may be determined using absorbance measurement at 260 nm. A 50 µg/mL double-stranded DNA solution has an absorbance of 1 (specific absorbance 200).

DNA concentrations less than 500 ng/mL are determined following incubation with fluorescent dyes that bind specifically to double-stranded DNA, using a reference standard of DNA to establish a calibration curve.

Liquid chromatography may also be used to determine the concentration of plasmid DNA using a reference standard. In some cases, capillary electrophoresis is also acceptable.

Residual host-cell DNA

The content of residual host-cell DNA is determined using a suitable method, unless the production process has been validated to demonstrate suitable clearance. Quantitative PCR is recommended for its sensitivity and specificity, but other suitable techniques may also be used.

Bacterial endotoxins (*2.6.14*)

Less than the limit approved for the particular preparation.

Sterility (*2.6.1*)

It complies with the test for sterility.

Viruses used for production

Their production is based on a seed lot and a cell-bank system or, where applicable (for example, for baculoviruses), on a transient system. The strain of virus used is identified by historical records that include information on its origin and its subsequent manipulation, notably deleted or modified regions. The nucleotide sequence of the viruses is documented.

Only a virus used for production that complies with the following requirements may be used.

Identification

Viruses used for production are identified by immunochemical methods (*2.7.1*), NAT (*2.6.21*) or restriction enzyme analysis.

Genomic integrity
Genomic integrity of the virus used for production is verified by suitable methods such as restriction enzyme analysis. Where viruses are modified to express *rep* or *cap* genes or the expression cassette, genomic integrity is assessed by sequencing or by quantitative PCR of these regions.

Genetic stability
Where a stable production system is used, genetic stability is verified at or beyond the maximum number of cell doublings used for production.

Virus titration
The infectious titre is determined by a suitable assay.

Wild-type AAV
Where applicable, the absence of wild-type AAV in helper virus seed lots is verified using NAT (*2.6.21*).

Replication-competent viruses
Detection of replication-competent viruses is performed by suitable methods. No replication-competent viruses are found.

Extraneous agents (*2.6.16*)
It complies with the test for extraneous agents. In addition, detection of potential contamination with specific insect viruses is required where applicable.

PRODUCTION AND HARVEST
All processing of the cell bank and subsequent cell cultures is done in an area with a suitable classified space with appropriate containment level where no other cells, viruses or vectors are handled at the same time. Any material of human or animal origin used in the preparation of cell suspensions and culture media is qualified. The cell culture medium may contain a pH indicator such as phenol red and suitable antibiotics at the lowest effective concentration. It is preferable to have a substrate free from antibiotics during production, and at no stage during production is penicillin or streptomycin used. A portion of the production cell cultures is set aside as uninfected cell cultures (control cells).

Each single harvest that complies with the following requirements may be used in the preparation of the purified harvest.

Identification
The vector is identified by immunochemical methods (*2.7.1*), NAT (*2.6.21*) or restriction enzyme analysis.

Vector concentration
The titre of infectious vector and the concentration of vector particles in single harvests are determined.

Extraneous agents (*2.6.16*)
The single harvest complies with the tests for extraneous agents.

Control cells
Control cells comply with a test for identification (*5.2.3*) and a test for extraneous agents (*2.6.16*) and specific insect viruses, where insect cell lines are used for production.

PURIFIED HARVEST
Several single harvests may be pooled before the purification process. The purification process is validated to demonstrate satisfactory removal of impurities.

Purified harvests that comply with the following requirements may be used in the preparation of the final bulk.

Identification
The vector is identified by immunochemical methods (*2.7.1*), NAT (*2.6.21*) or restriction enzyme analysis.

Genetic characterisation
The following tests are carried out.
- The entire genome of the vector is sequenced for a suitable number of production runs at the level of the purified harvest or final bulk and the analytically determined sequence is compared to the theoretical sequence based on vector construction and available databases.
- Genomic integrity is checked on the vector DNA. PCR analysis may be used.

Vector concentration
The titre of infectious vector and the concentration of vector particles are determined.

Residual viruses used for production
Residual viruses used for production are assessed by plaque assays or tissue culture infective dose 50 ($TCID_{50}$) on permissive cell lines or by quantitative PCR, according to the production system used.

Residual proteins
The concentrations of residual host-cell and/or viral proteins are determined by a suitable immunochemical method (*2.7.1*), unless the process has been validated to demonstrate suitable clearance.

Residual DNA
The content of residual producer-cell DNA and of residual DNA from intermediates such as plasmids and production viruses where applicable, is determined using a suitable method, unless the process has been validated to demonstrate suitable clearance. Quantitative PCR is recommended for its sensitivity and specificity, but other suitable techniques may also be used.

Residual reagents
Where reagents are used during production, tests for these substances are carried out on the purified harvest, unless the process has been validated to demonstrate suitable clearance.

Residual antibiotics
Where antibiotics are used during the production process, their residual concentration is determined by a microbiological assay (adapted from general method *2.7.2*) or by other suitable methods (for example, liquid chromatography), unless the process has been validated to demonstrate suitable clearance.

FINAL BULK
Several purified harvests may be pooled during preparation of the final bulk. A stabiliser and other excipients may be added. The formulated product is filtered through a bacteria-retentive filter.

Only a final bulk that complies with the following requirement may be used in the preparation of the final lot.

Sterility (*2.6.1*)
It complies with the test for sterility.

FINAL LOT
Only a final lot that complies with each of the requirements given below under Identification, Tests and Assay may be released for use.

Provided that the tests for bovine serum albumin (when bovine serum is used to manufacture the vector), replication-competent AAV and residual viruses used for production have been carried out with satisfactory results on the final bulk, they may be omitted on the final lot.

IDENTIFICATION
The vector is identified by immunochemical methods (*2.7.1*), NAT (*2.6.21*) or restriction enzyme analysis.

TESTS

Osmolality (*2.2.35*)
Within the limits approved for the particular preparation.

pH (*2.2.3*)
Within the limits approved for the particular preparation.

Extractable volume (*2.9.17*)
It complies with the test for extractable volume.

Residual moisture (*2.5.12*)
Within the limits approved for the particular freeze-dried product.

Bovine serum albumin
Where bovine serum has been used during production, not more than the limit approved for the particular preparation, determined by a suitable immunochemical method (*2.7.1*).

Replication-competent AAV concentration
Within the limits approved by the competent authority.

Detection of replication-competent AAV is performed by a replication assay on a permissive cell line previously infected with a helper virus and analysis of the replicative forms by Southern blot on low-molecular-weight DNA, or by detection of the *rep* gene by quantitative PCR.

Vector aggregates
Vector aggregates are determined by suitable methods (for example, light scattering).

Sterility (*2.6.1*)
It complies with the test for sterility.

Bacterial endotoxins (*2.6.14*)
Less than the limit approved for the particular preparation.

ASSAY

Vector-particle concentration
Vector-particle concentration is determined using a suitable method such as quantitative PCR by comparison with a standard curve obtained using the recombinant AAV plasmid or an AAV reference standard. This concentration is within the limits approved for the particular product.

Infectious vector titre
Titrate the preparation to be examined by inoculation into cell cultures. Titrate an appropriate vector reference standard to validate each assay.

The infectious vector titre of the preparation to be examined is not less than the minimum amount stated on the label.

The assay is invalid if:
- the confidence interval ($P = 0.95$) of the logarithm of the vector concentration is greater than a value authorised by the competent authority;
- the infectious vector titre of the reference standard is outside limit values defined by a control chart.

Ratio of vector-particle concentration to infectious vector titre
Within the limits approved for the particular product.

Expression of the genetic insert product
The expression of the genetic insert product is determined wherever possible, following inoculation of cell cultures with the product at a predetermined multiplicity of infection, by suitable immunochemical (*2.7.1*) or biochemical assays or by flow cytometry (*2.7.24*).

Biological activity
Unless otherwise justified and authorised, biological activity is determined by a suitable *in vitro* or *in vivo* test.

LABELLING

The label states:
- the content of active substance;
- the recommended human dose;
- for freeze-dried preparations:
  - the name or composition and the volume of the reconstituting liquid to be added;
  - the time within which the product is to be used after reconstitution.

O. Functionality-related Characteristics of Excipients

(Ph. Eur. general texts 5.15)

This chapter and the Functionality-related characteristics (FRCs) sections in individual monographs are not mandatory and are published for information and guidance.

PREAMBLE

Excipients that have previously been evaluated for safety are used in the formulation of pharmaceutical preparations to bring functionality to the formulation. The intended function of an excipient is to guarantee the required physico-chemical and biopharmaceutical properties of the pharmaceutical preparation.

The functionality of an excipient is determined by its physical and chemical attributes and, in some cases, also by its content of by-products or of additives used to improve the intended functionality. In addition, the functionality may depend on complex interactions between the constituents of the formulation and stresses related to the process. Excipient functionality can therefore be evaluated only in the context of a particular formulation and manufacturing process, frequently by the use of a number of analytical methods.

Pharmaceutical development should be science-based and ensure a quality medicinal product. It is defined in the current ICH guideline Q8 Pharmaceutical Development. Its purpose is to provide justification for the dosage form selected, the qualitative and quantitative composition of the medicinal product, the manufacturing process, the critical quality attributes (CQAs) of the constituents and medicinal product, and the critical process parameters (CPPs). The 'quality by design' (QbD) concept described in the ICH Q8 guideline requires a comprehensive understanding of the chemical and physical nature of the individual active substance(s) and excipients, and of the way their attributes interact in the formulation and with the manufacturing process.

Pharmaceutical development includes identification of which excipient quality attributes are critical.

A CQA is a physical, chemical, biological or microbiological property or characteristic that must be within appropriate limits to ensure the desired medicinal product quality.

Monographs of the European Pharmacopoeia on excipients are designed to ensure acceptable quality for users. Information on the characters of the excipient, and requirements concerning identity, chemical and microbiological purity and physical characteristics associated with the chemical structure, such as optical rotation, are given in individual monographs and in the general monograph *Substances for pharmaceutical use (2034)*.

Certain excipient attributes, such as the particle size of an excipient intended for a solid dosage form or the molecular mass of a polymeric material used as a viscosity-increasing agent, may however relate to functionality in a more general

sense. These attributes are called functionality-related characteristics (FRCs). When the pharmaceutical development work of a specific product has identified the existence of one or more critical FRCs for an excipient, they are considered to be CQAs for that excipient and should be controlled accordingly.

Knowledge of FRCs may facilitate the application of process analytical technology (PAT).

FRCs are included in excipient monographs to aid excipient manufacturers and users in establishing specifications based on standard analytical methods. They provide a common language to support the supply of excipients with specified properties. FRCs may be stated (in the certificate of analysis, for example) by the excipient manufacturer with a reference to the Pharmacopoeia monograph, thus indicating the method used to test a particular characteristic. The FRCs section in individual monographs contains FRCs that are known to have an impact on the functionality of the excipient for the stated uses. The uses and the FRCs listed are not exhaustive due to the multiple uses of many excipients and the development of new uses.

REGULATORY GUIDANCE

According to current regulatory guidelines, for example the ICH Q8 guideline, the marketing authorisation application should discuss the excipients chosen, their concentration, and the attributes that can influence the medicinal product quality or manufacturability relative to the respective function of each excipient. The ability of excipients to provide their intended functionality and to perform throughout the intended medicinal product shelf life should also be demonstrated. The information on excipient performance can be used as appropriate to justify the choice and quality attributes of the excipient.

During pharmaceutical development, the attributes that are critical to the manufacturing process and quality of the medicinal product are identified. Having identified the critical attributes of the excipients, preferably by a risk-based approach, pharmaceutical development may establish the acceptable range of the critical material attributes including both the physical and the chemical property variation. The FRCs concerned may not be properties controlled by the excipient manufacturer and could therefore be variable. The design of a robust manufacturing process for the medicinal product that limits the effect of the normal excipient variability is preferable.

Evaluation of the physical and chemical grades and, where appropriate, the setting of a specification for the critical attributes and thus the critical FRCs, is part of the pharmaceutical development irrespective of the non-mandatory character of FRCs. This evaluation should be seen in light of regulatory guidance on pharmaceutical development and the appropriate acceptance criteria should be based on an understanding of the extent to which the variation of the critical FRCs can have an impact on the quality of the medicinal drug product. An acceptable range of FRCs may be established within the limits of the design space. The design space may be described as a space in which the quality attributes of the constituents and the process parameters may vary without modification of the quality of the medicinal product.

PHYSICAL GRADES

Excipients that are particulate solids can be available in a variety of physical grades, for example with regard to particle-size distribution, which is usually controlled by the excipient supplier. However, FRCs for these excipients may concern a wide range of properties, resulting from solid-state properties and properties of the particulate solid, which may not be controlled by the excipient supplier.

Properties of particulate solids include for example particle-size distribution, specific surface area, bulk density, flowability, wettability and water sorption. Depending on the size range, the particle-size distribution can be determined by sieve analysis (see general chapter *2.9.38. Particle-size distribution estimation by analytical sieving*) or instrumental methods, for example *2.9.31. Particle size analysis by laser light diffraction*. The method described in general chapter *2.9.26. Specific surface area by gas adsorption* is based on the Brunauer-Emmett-Teller (BET) technique. Methods to characterise flowability and bulk density of powders are described in general chapters *2.9.36. Powder flow* and *2.9.34. Bulk density and tapped density*. Solid-state properties may have an impact on the wettability (see general chapter *2.9.45. Wettability of porous solids including powders*) and water-solid interactions (see general chapter *2.9.39. Water-solid interactions: determination of sorption-desorption isotherms and of water activity)* of particulate solids.

Examples of solid-state properties to be considered in the development of solid dosage forms include polymorphism, pseudopolymorphism, crystallinity and density. Techniques to study them are given in general chapters *5.9. Polymorphism*, *5.16. Crystallinity* and *2.2.42. Density of solids*.

CHEMICAL GRADES

Excipients that are available in different chemical grades are of natural, semi-synthetic or synthetic origin. Individual monographs usually control the chemical composition of excipients that are composed of a mixture of related compounds, for example the composition of fatty acids in vegetable oils or surfactants. There are, however, individual monographs in the Pharmacopoeia each describing a class of polymeric materials that may vary in their composition with regard to the structure of homopolymers, block polymers and copolymers, the degree of polymerisation, the molecular mass and mass distribution, the degree of substitution and in some cases even different substituents on the polymer backbone. This variation may, however, have a profound effect on the functionality of the excipient and should be subject to investigations during the pharmaceutical development, preferably to establish the acceptable range of each attribute being critical to the manufacturing process and quality of the medicinal product.

FUNCTIONALITY-RELATED CHARACTERISTICS SECTION IN MONOGRAPHS

Monographs on excipients may have a section entitled 'Functionality-related characteristics'. This section is included for information for the user and is not a mandatory part of the monograph. The section gives a statement of characteristics that are known to be relevant for certain uses of the excipient. The use for which the characteristic is relevant is stated. For other uses, the characteristic may be irrelevant. For this reason, the section should not be seen simply as a supplement to the monograph. It is the responsibility of the manufacturer of the medicinal product to decide how the information on FRCs will be applied in the manufacturing process in light of the use of the excipient and data from pharmaceutical development.

The information on the FRCs may be given in different ways:

— name of the FRC;

— name of the FRC and a recommended method for its determination, referring wherever possible to a general chapter of the Pharmacopoeia;
— name of the FRC with a recommended method for its determination and typical values, which may be in the form of tolerances from the nominal value.

A given characteristic may be the subject of a mandatory requirement in the monograph. If it is relevant for certain uses, it is also referred to in the FRCs section as a relevant characteristic that the manufacturer of the medicinal product may choose to specify for the grade used of a particular pharmaceutical preparation.

The section on FRCs is intended to reflect current knowledge related to the major uses of an excipient. In view of the multiple uses of some excipients and the continuous development of new uses, the section may not be complete. In addition, the methods cited for the determination of a particular characteristic are given as recommendations for methods that are known to be satisfactory for the purpose, and the use of other methods is not excluded.

PHARMACOPOEIAL HARMONISATION

A number of excipient monographs are subject to pharmacopoeial harmonisation among the European, Japanese and United States pharmacopoeias (see general chapter *5.8. Pharmacopoeial harmonisation*). Introduction of the FRCs section in the monographs of the European Pharmacopoeia means that the presentation of harmonised monographs differs. Tests for physical and chemical attributes regarded as both quality-related and functionality-related in the European Pharmacopoeia are, in the 2 other pharmacopoeias, included only in the body of the monograph. The different format has no implications on the specification of excipient characteristics for the manufacturer of the medicinal product. Current regulatory guidance recommends the identification and specification of only such critical attributes that impact the manufacturing process and the quality of the medicinal product. The different legal environments of the 3 pharmacopoeias allow for different formats of the monographs without affecting the pharmacopoeial harmonisation status.

GLOSSARY

Critical characteristic or critical quality attribute (CQA)
A physical, chemical, biological or microbiological property or characteristic that must be within appropriate limits or an appropriate range or distribution to ensure the desired product quality.

Critical process parameter (CPP)
A process parameter whose variability has an impact on a critical quality attribute and therefore should be monitored or controlled to ensure the process produces the desired quality.

Design space
The multidimensional combination and interaction of input variables (e.g. material attributes) and process parameters that have been demonstrated to provide assurance of quality.

Functionality-related characteristic (FRC)
A controllable physical or chemical characteristic of an excipient that is shown to impact on its functionality.

Process analytical technology (PAT)
A system for designing, analysing and controlling manufacturing through timely measurements (i.e. during processing) of critical quality and performance attributes of raw and in-process materials and processes with the goal of ensuring final product quality.

Quality by design (QbD)
A systematic approach to development that begins with predefined objectives and emphasises product and process understanding and process control, based on sound science and quality risk management.

P. Guidelines for Using the Test for Sterility

(Ph. Eur. general texts 5.1.9)

The purpose of the test for sterility (*2.6.1*), as that of all pharmacopoeial tests, is to provide an independent control analyst with the means of verifying that a particular material meets the requirements of the European Pharmacopoeia. A manufacturer is neither obliged to carry out such tests nor precluded from using modifications of, or alternatives to, the stated method, provided he is satisfied that, if tested by the official method, the material in question would comply with the requirements of the European Pharmacopoeia.

PRECAUTIONS AGAINST MICROBIAL CONTAMINATION

Aseptic conditions for performance of the test can be achieved using, for example, a class A laminar-air-flow cabinet located within a class B clean room, or an isolator

GUIDANCE TO MANUFACTURERS

The level of assurance provided by a satisfactory result of a test for sterility (the absence of contaminated units in the sample) as applied to the quality of the batch is a function of the homogeneity of the batch, the conditions of manufacture and the efficiency of the adopted sampling plan. Hence for the purpose of this text a batch is defined as a homogeneous collection of sealed containers prepared in such a manner that the risk of contamination is the same for each of the units contained therein.

In the case of terminally sterilised products, physical proofs, biologically based and automatically documented, showing correct treatment throughout the batch during sterilisation are of greater assurance than the sterility test.
The circumstances in which parametric release may be considered appropriate are described under *5.1.1. Methods of preparation of sterile products*. The method of media-fill runs may be used to evaluate the process of aseptic production. Apart from that, the sterility test is the only analytical method available for products prepared under aseptic conditions and furthermore it is, in all cases, the only analytical method available to the authorities who have to examine a specimen of a product for sterility.

The probability of detecting micro-organisms by the test for sterility increases with their number present in the sample tested and varies according to the readiness of growth of micro-organism present. The probability of detecting very low levels of contamination even when it is homogenous throughout the batch is very low. The interpretation of the results of the test for sterility rests on the assumption that the contents of every container in the batch, had they been tested, would have given the same result. Since it is manifest that every container cannot be tested, an appropriate sampling plan should be adopted. In the case of aseptic production, it is recommended to include samples filled at the beginning and at the end of the batch and after significant intervention.

OBSERVATION AND INTERPRETATION OF RESULTS

Conventional microbiological/biochemical techniques are generally satisfactory for identification of micro-organisms recovered from a sterility test. However, if a manufacturer wishes to use condition (d) as the sole criterion for invalidating a sterility test, it may be necessary to employ sensitive typing techniques to demonstrate that a micro-organism isolated from the product test is identical to a micro-organism isolated from the test materials and/or the testing environment. While routine microbiological/biochemical identification techniques can demonstrate that 2 isolates are not identical, these methods may not be sufficiently sensitive or reliable enough to provide unequivocal evidence that 2 isolates are from the same source. More sensitive tests, for example molecular typing with RNA/DNA homology, may be necessary to determine that micro-organisms are clonally related and have a common origin.

Q. Elemental Impurities

(Ph. Eur. general text 5.20)

The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) has adopted the ICH Q3D guideline for elemental impurities in medicinal products.

The European Pharmacopoeia (Ph. Eur.) applies this guideline to medicinal products (referred to hereunder and in the ICH Q3D guideline as 'drug products'), with the exception of products for veterinary use, unlicensed preparations and products excluded from the scope of the guideline. The guideline prescribes a permitted daily exposure (PDE) according to the route of administration for elements of toxicological concern which may be present in medicinal products. The guideline also describes some acceptable approaches to establishing concentrations of elemental impurities in medicinal products or components that would ensure that the PDEs for these impurities are not exceeded.

Parts of the introduction and the scope of the guideline are reproduced hereunder, and the full text is available from the ICH website.

The drug products containing purified proteins and polypeptides (including proteins and polypeptides produced from recombinant or non-recombinant origins), their derivatives, and products of which they are components (e.g., conjugates) are within the scope of the guideline, as are drug products containing synthetically produced polypeptides, polynucleotides, and oligosaccharides.

The guideline does not apply to herbal products, radiopharmaceuticals, vaccines, cell metabolites, DNA products, allergenic extracts, cells, whole blood, cellular blood components or blood derivatives including plasma and plasma derivatives, dialysate solutions not intended for systemic circulation, and elements that are intentionally included in the drug product for therapeutic benefit. The guideline does not apply to products based on genes (gene therapy), cells (cell therapy) and tissue (tissue engineering). In some regions, these products are known as advanced therapy medicinal products.

The guideline does not apply to drug products used during clinical research stages of development. As the commercial process is developed, the principles contained in the guideline can be useful in evaluating elemental impurities that may be present in a new drug product.

Elemental impurities in drug products may arise from several sources; they may be residual catalysts that were added intentionally in synthesis or they may be present as impurities (e.g., through interactions with processing equipment or container/closure systems or by being present in components of the drug product). Because elemental impurities do not provide any therapeutic benefit to the patient, their levels in the drug product should be controlled within acceptable limits.

An applicant is not expected to tighten the limits based on process capability, provided that the elemental impurities in drug products do not exceed the PDEs. The PDEs established in the guideline are considered to be protective of public health for all patient populations. In some cases, lower levels of elemental impurities may be warranted when levels below toxicity thresholds have been shown to have an impact on other quality attributes of the drug product (e.g., element catalysed degradation of drug substances).

R. Chemometric Methods Applied to Analytical Data

(Ph. Eur. general text 5.21)

The following chapter is published for information only. It is an introduction to the use of chemometric techniques for processing analytical data sets. The objective is to provide indications on good chemometric practice and requirements.

1 GENERAL ASPECTS

1-1 INTRODUCTION

1-1-1 Scope of the chapter

This chapter is an introduction to the use of chemometric techniques for the processing of analytical data sets, which is an area of interest for research, quality control and manufacturing in the pharmaceutical industry. The objective is to provide information on the requirements for good chemometric practice and to also present a selection of established chemometric methods, but not an exhaustive review of these techniques, as refinements and innovations are constantly being introduced. The principles of the proposed methods will be briefly described along with their critical aspects and limitations. Mathematical details and algorithms are mostly omitted and a glossary is provided at the end of the chapter.

1-1-2 Definition

The actual definition of chemometrics is "the chemical discipline that uses mathematical and statistical methods, (a) to design or select optimal measurement procedures and experiments, and (b) to provide maximum chemical information by analysing chemical data".

From a more general point of view, chemometrics is not limited to chemical data and can contribute greatly to system understanding by analysing data, when limited knowledge and theory do not sufficiently explain observations and behaviour. Chemometric methods consist mainly of multivariate data-driven modelling techniques that result in empirical mathematical models that are subsequently used for the indirect prediction of properties of interest.

1-1-3 Background

Applications of chemometrics can be qualitative or quantitative, and it can help the analyst to structure the data set and to recognise hidden variable relationships within the system. However, it should be stressed that although such

data-driven methods may be powerful, they would not replace a verified or established theory if available.

Chemometric methods have revolutionised near-infrared spectroscopy (NIR) and such techniques are now integral components of process analytical technology (PAT) and quality by design (QbD) for use in improved process monitoring and quality control in a variety of fields. Chemometric methods can be found throughout the scientific and technological community, with a principal but non-exclusive focus on life and health sciences such as agriculture, food, pharmacy, chemistry, biochemistry and genomics, but also other industries such as petrochemicals, textiles, sensors and cosmetics, with the potential to expand even further into other domains.

The associated mathematical principles have been understood since the early twentieth century, but chemometrics came of age with the development of digital technology and the related progress in the elaboration of mathematical algorithms. Many techniques and methods are based on geometric data representations, transformations and modelling. Later, mathematical and theoretical developments were also consolidated.

1-1-4 Introducing chemometrics

In chemometrics, a property of interest is evaluated solely in terms of the information contained in the measured samples. Algorithms are applied directly to the data set, and information of interest is extracted with models (modelling or calibration step). Chemometrics is associated with multivariate data analysis, which usually depends less on assumptions about the distribution of the data than many other statistical methods since it rarely involves hypothesis testing. During modelling the most sensitive changes in properties of interest can be amplified, while the less relevant changes in disturbing factors, whatever their origin, i.e. physical, chemical, experimental or instrumental variation, are minimised to the level of noise.

A model in chemometrics is a prediction method and not a formal or simplified representation of a phenomenon from physics, chemistry, etc. The ability of a model to predict properties has to be assessed with regard to its performance. The best model or calibration will provide the best estimations of properties of interest. A useful model is one that can be trusted and used for decision-making, for example. Adoption of a model in decision-making must be based on acceptable, reliable, and well-understood assessment procedures.

In univariate analysis, identified variables in a system are analysed individually. However, in reality, systems tend to be more complex, where interactions and combination effects occur between sample variables and cannot be separated. Multivariate data analysis handles many variables simultaneously and the relationship within or between data sets (typically matrices) has to be rearranged to reveal the relevant information. In multivariate methods, the original data is often combined linearly to account as much as possible for the explainable part of the data and ideally, only noise will remain unmodelled. The model, when properly validated, can be used in place of costly and time-consuming measurements in order to predict new values.

Generally, projection techniques such as principal components analysis (PCA), principal components regression (PCR) or partial least squares regression (PLS) are recommended. However, the approach will be different depending on whether the data has been generated using experimental design (i.e. designed data) or has been collected at random from a given population (i.e. non-designed data). With designed data matrices, the variables are orthogonal by construction and traditional multilinear statistical methods are therefore well suited to describing the data within. However, in non-designed data matrices, the variables are seldom orthogonal, but are more or less collinear, which favours the use of multivariate data analysis.

1-1-5 Qualitative and quantitative data analysis

Qualitative data analysis can be divided into exploration, an unsupervised analysis where data from a new system is to be analysed, and classification, a supervised analysis where class-labels are predicted.

Unsupervised analysis

In exploratory data analysis, multivariate tools are used to gather an overview of the data in order to build hypotheses, select suitable analytical methods and sampling schemes, and to determine how multivariate analysis of current and future data of similar type can be performed. When the first exploratory treatment is finalised, classification can be subsequently carried out in the form of a secondary treatment, where samples are organised into specific groups or classes.

Supervised analysis

Classification is the process of determining whether or not samples belong to the same class as those used to build the model. If an unknown sample fits a particular model well, it is said to be a member of that class. Many analytical tasks fall into this category, e.g. materials may be sorted according to quality, physical grade and so on. Identity testing is a special situation where unknown samples are compared with suitable reference materials, either by direct comparison or indirect estimation, e.g. using a chemometric model.

Quantitative data analysis, on the other hand, mainly consists of calibration, followed by direct application to new and unknown samples. Calibration consists of predicting the mathematical relationship between the property to be evaluated (e.g. concentration) and the variables measured.

1-2 GOOD CHEMOMETRIC PRACTICE

The following notation will be used in the chapter:

X, Y	data sets
X	independent variable
Y	dependent variable
$\mathbf{X}$, $\mathbf{Y}$	matrices
$\mathbf{x}$, $\mathbf{y}$	vectors
x, y	scalar values
i, j	indices, points
x_i	i^{th} value of vector $\mathbf{x}$
$x_{i,j}$	i^{th} and j^{th} value of matrix $\mathbf{X}$
$\mathbf{X}^T$	transpose of matrix $\mathbf{X}$
$\mathbf{X}^{-1}$	inverse (if it exists) of matrix $\mathbf{X}$
$\hat{\mathbf{X}}$	estimate of matrix $\mathbf{X}$
$\lvert\mathbf{X}\rvert$	determinant of (square) matrix $\mathbf{X}$
$\lVert\mathbf{x}\rVert$	norm of vector $\mathbf{x}$
b	regression equation coefficient
e	residuals of X
f	residuals of Y

1-2-1 Figures of merits for regression

In quantitative analysis, building a regression model involves fitting a mathematical relationship to the corresponding independent data (X) and dependent data (Y).
The independent data may represent a collection of signals, i.e. responses from a number of calibration samples, while the dependent data may correspond to the values of an attribute, i.e. the property of interest in the calibration samples. It is advisable to test the regression model with internal and external test sets. The internal test set consists of

samples that are used to build the model (or achieve calibration) by applying resampling within the calibration data and samples that are initially left out of the calibration in order to validate the model. Use of the internal test set is part of model optimisation and model selection. The external independent test set represents data that normally is available after the model has been fixed, thus the external test set challenges the model and tests its robustness for the analysis of future data.

1-2-1-1 Root mean square error of prediction

The link between X and Y is explored through a common set of samples (calibration set) from which both x and y-values have been collected and are clearly known. For a second set of samples (validation set) the predicted y-values are then compared to the reference y-values, resulting in a prediction residual that can be used to compute a validation residual variance, i.e. a measure of the uncertainty of future predictions, which is referred to as root mean square error of prediction (*RMSEP*). This value estimates the average uncertainty that can be expected when predicting y-values for new samples. Since no assumptions concerning statistical error distribution are made during modelling, prediction error cannot be used to report a valuable statistical interval for the predicted values. Nevertheless, *RMSEP* is a good error estimate in cases where both calibration and validation sample sets are representative of future samples.

A confidence interval for predicted y-values would be $\pm\ n \times RMSEP$, with n fixed by the operator. A common choice is $n = 2$. This choice should be dependent on the requirements of the specific analytical method.

Chemometric models can end up with better precision than the reference methods used to acquire calibration and testing data. This is typically observed for water content determinations by NIR and PLS where semi-micro determination titration (*2.5.12*) is the reference method.

1-2-1-2 Standard error of calibration and coefficient of determination

Figures of merit can be calculated to help assess how well the calibration fits the data. Two examples of such statistical expressions are the standard error of calibration (*SEC*) and the coefficient of determination (R^2).

SEC has the same units as the dependent variables and reflects the degree of modelling error, but cannot be used to estimate future prediction errors. It is an indication of whether the calculation using the calibration equation will be sufficiently accurate for its intended purpose. In practice *SEC* has to be compared with the error of the reference method (*SEL, Standard Error of Laboratory*, see Glossary). Usually *SEC* is larger than *SEL*, in particular if modelling does not account for all interferences in the samples or if other physical phenomena are present.

The coefficient of determination (R^2) is a dimensionless measure of how well the calibration fits the data. R^2 can have values between 0 and 1. A value close to 0 indicates that the calibration fails to relate the data to the reference values and as the coefficient of determination increases, the X-data becomes an increasingly more accurate predictor of the reference values. Where there is more than 1 independent variable, adjusted R^2 should be used rather than R^2, since the number of independent variables in the model inflates the latter even if the fraction of variance explained by the model is not increased.

1-2-2 Implementation steps

The implementation of chemometric methods varies case by case depending on the specific requirements of the system to be analysed. The following generic approach can be followed when analysing non-designed data sets:

- in formulating the study problem, define the precise objective of data collection and the expected analysis results;
- investigate the origin and availability of the data. The data set should cover the variation of the explored variable(s) or attribute(s);
- if the available data does not cover the expected variation, prepare and measure samples that fill the gap;
- variable selection: sometimes selecting the right variables can give more robustness and also enhance model accuracy;
- raw data may have to be transformed and mathematical pre-treatments performed;
- elaborate the model through calibration and validation;
- challenge the model and check its performance on new samples or data;
- validate the method according to current pharmaceutical usage and requirements.

1-2-3 Data considerations

1-2-3-1 Sample quality

Careful sample selection increases the likelihood of extracting useful information from the analytical data. Whenever it is possible to actively adjust selected variables or parameters according to an experimental design, the quality of the results is increased. Experimental design (also referred to as design of experiments, DoE) can be used to introduce systematic and controlled changes between samples, not only for analytes, but also for interferences. When modelling, common considerations include the determination of which variables are necessary to adequately describe the samples, which samples are similar to each other and whether the data set contains related sub-groups.

1-2-3-2 Data tables, geometrical representations

Sample responses result in a group of numerical values relating to signal intensities (X-data), i.e. the independent variables. However, it should be recognised that these variables are not necessarily linearly independent (i.e. orthogonal) according to mathematical definitions. These values are best represented in data tables and by convention each sample is associated with a specific row of data. A collection of such rows constitutes a matrix, where the columns are the variables. Samples can then be associated with certain features reflecting their characteristics, i.e. the value of a physical or chemical property or attribute and these data are usually referred to as the Y-data, i.e. the dependent variables. It is possible to add this column of values to the sample response matrix, thereby combining both the response and the attribute of each sample.

When n objects are described by m variables the data table corresponds to an $n \times m$ matrix. Each of the m variables represents a vector containing n data values corresponding to the objects. Each object therefore appears as a point in an m dimensional space described by its m coordinate values (1 value for each variable in the m axes).

1-2-3-3 First assessment of data

Before performing multivariate data analysis, the quality of the sample response can be optionally assessed using statistical tools. Graphical tools are recommended for the 1st visual assessment of the data, e.g. histograms and/or boxplots for variables for evaluation of the data distribution, and scatter plots for detection of correlations. Descriptive statistics are useful for obtaining a rapid evaluation of each variable, taken separately, before starting multivariate

analysis. For example, mean, standard deviation, variance, median, minimum, maximum and lower/upper quartile can be used to assess the data and detect out-of-range values and outliers, abnormal spread or asymmetry. These statistics reveal anomalies in a data table and indicate whether a transformation might be useful or not. Two-way statistics, e.g. correlation, show how variations in 2 variables may correlate in a data table. Verification of these statistics is also useful when reducing the size of the data table, as they help in avoiding redundancies.

1-2-3-4 Outliers

An outlier is a sample that is not well described by the model. Outliers can be *X* or *Y* in origin. They reflect unexpected interference in the original data or measurement error. The predicted data that is very different from the expected value calls into question the suitability of the modelling procedure and the range spanned by the original data. In prediction mode, outliers can be caused by changes in the interaction between the instrument and samples or if samples are outside the model's scope. If this new source of variability is confirmed and is relevant, the corresponding data constitutes a valuable source of information. Investigation is recommended to decide whether the existing calibration requires strengthening (updating) or whether the outliers should be ignored as uncritical or unrelated to the process (i.e. operator error).

In the case of classification an outlier test should be performed on each class separately.

1-2-3-5 Data error

Types of data error include random error in the reference values of the attributes, random error in the collected response data and systematic error in the relationship between the two. Sources of calibration error are problem specific, for example, reference method errors and errors due to either sample non-homogeneity or the presence of non-representative samples in the calibration set. Model selection during calibration usually accounts for only a fraction of the variance or error attributable to the modelled analytical technique. However, it is difficult to assess if this error is more significant than the reference method error or vice versa.

1-2-3-6 Pre-processing and variable selection

The raw data may not be optimal for analysis and are generally pre-processed before performing chemometric calculations to improve the extraction of physical and chemical information.

Interferences, for example background effects, baseline shifts and measurements in different conditions, can impede the extraction of information when using multivariate methods. It is therefore important to minimise the noise introduced by such effects by carrying out pre-processing operations.

A wide range of transformations (scaling, smoothing, normalisation, derivatives, etc.) can be applied to *X*-data as well as *Y*-data for pre-processing prior to multivariate data analysis in order to enhance the modelling. The main purpose of these transformations is focussing the data analysis on the pertinent variability within the data set. For example, pre-processing may involve mean centering of variables so that the mean does not influence the model and thus reduce the model rank.

The selection of the pre-processing is mostly driven by parameters such as type of data, instrument or sample, the purpose of the model and user experience. Pre-processing methods can be combined, for example standard normal variate (SNV) with 1st derivative, as an empirical choice.

1-2-4 Maintenance of chemometric models

Chemometric methods should be reassessed regularly to demonstrate a consistent level of acceptable performance. In addition to this periodical task, an assessment should be carried out for critical parameters when changes are made to application conditions of the chemometric model (process, sample sources, measurement conditions, analytical equipment, software, etc.).

The aim of maintaining chemometric models up-to-date is to provide applications that are reliable over a longer period of use. The extent of the validation required, including the choice of the necessary parameters, should be based on risk analysis, taking into account the analytical method used and the chemometric model.

1-3 ASSESSMENT AND VALIDATION OF CHEMOMETRIC METHODS

1-3-1 Introduction

Current use of the term 'validation' refers to the regulatory context as applied to analytical methods, but the term is also used to characterise a typical computation step in chemometrics. Assessment of a chemometric model consists of evaluating the performance of the selected model in order to design the best model possible with a given set of data and prerequisites. Provided sufficient data are available, a distribution into 3 subsets should always be considered: 1) a learning set to elaborate models, 2) a validation set to select the best model, i.e. the model that enables the best predictions to be made, 3) an independent test set to estimate objectively the performance of the selected final model. Introducing a 3rd set for objective model performance evaluation is necessary to estimate the model error, among other performance indicators. An outline is given below on how to perform a typical assessment of a chemometric model, starting with the initial validation, followed by an independent test validation and finally association/correlation with regulatory requirements.

1-3-2 Assessment of chemometric models

1-3-2-1 Validation during modelling

Typically, algorithms are iterative and perform self-optimisation during modelling through an on-going evaluation of performance criteria and figures of merit. This step is called validation. The performance criteria are specific to the chemometric technique used and to the nature of the analytical data, as well as the purpose of the overall method which includes both the analytical side and the chemometric model. The objective of the validation is to evaluate the model and provide help to select the best performing model. Selected samples are either specifically assigned for this purpose or are selected dynamically through reselection/re-use of data from a previous data set (sometimes called resampling – for clarification, see Glossary). A typical example of data reselection is cross-validation with specific segments, for example 'leave-one-out' cross-validation when samples are only a few, or 'leave-subset-out' cross-validation (Figure 5.21.-1). Another type of resampling is bootstrapping.

1-3-2-2 Assessment of the model

Once the model matches the optimisation requirements, fitness for purpose is assessed. Independent samples not used for modelling or model optimisation are introduced at this stage as an independent test-set in order to evaluate the performance of the model. Ideally, when sufficient data are available, the sample set can be split into 3 subsets comprising 1 learning set for model computation, 1 validation set for optimisation of the model, and 1 test set for

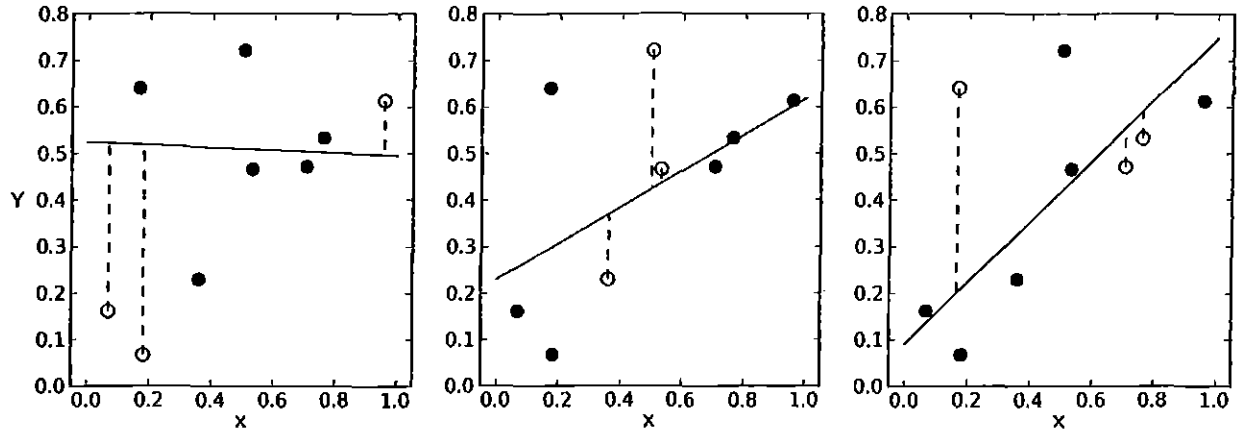


Figure 5.21.-1. – *Cross-validation with leave subset of 3 out applied to linear regression. Regression model data = •. Subset used for test = o. The errors of fit (interrupted lines) are collected to form the cumulated cross-validation error.*

evaluation of the prediction ability, i.e. whether the model is fit for purpose. The 3 subsets are treated independently and their separation should be performed in such a way that model computation is not biased. The aim is to obtain a representative distribution of the samples within the 3 subsets with regard to their properties and expected values.

1-3-2-3 Size and partitioning of data sets

The size of the data set needed for building the calibration is dependent on the number of analytes and interfering properties that needs to be handled in the model. The size of the learning data set for calibration usually needs to be larger when the interfering variations are acquired randomly than when all major interferences are known and they can be varied according to a statistical experimental design.

The lowest possible number of samples needed to cover the calibration range can be estimated from the corresponding design. The size of the independent test set should be in the order of 20-40 per cent of the samples used for the calibration model. However, when representative samples are abundant, the larger the test data set (above 40 per cent), the more reliably the prediction performance can be estimated.

It is common practice to mix learning and model validation sets and as a result, the definitive assessment of the model relies on the separate test set.

1-3-3 Validation according to the regulatory framework

Validation principles and considerations are described in established international guidelines and apply to the validation of analytical methods. However, due to the special nature of data treatment and evaluation, as carried out in most chemometric methods, additional aspects have to be taken into account when validating analytical procedures.

In this context, validation comprises both the assessment of the analytical method performance and the evaluation of the model. In some special cases, it might only be necessary to validate the chemometric model (see section 1-2-4).

1-3-3-1 Qualitative models

For validation of qualitative models, the most critical parameters are specificity and robustness. When not applicable, scientific justification is required.

Specificity

During validation it has to be shown that the model possesses sufficient discriminatory capability. Therefore, a suitable set of materials that pose a risk of mix-up must be defined and justified. If, in addition to chemical identification, other parameters (such as polymorphism, particle size, moisture content, etc.) are relevant, a justification for these parameters should also be included. The selection of materials to be included when validating specificity should be based on logistic considerations (e.g. materials handled close to the process under review, especially those with similar appearance), chemical considerations (e.g. materials with similar structure) and also physical considerations where relevant (e.g. materials with different physical properties). After definition of this set of materials, the discriminatory ability of the chemometric method to reject them must be proven. Therefore, for each material a representative set of samples covering typical variance within the material has to be analysed and evaluated. If the specificity of the chemometric model is insufficient, the parameters of the model should be optimised accordingly and the method revalidated.

Whenever new elements that may potentially affect identification are introduced, e.g. new materials that are handled at the same site and represent a risk of mix-up, a revalidation of specificity should be carried out. This revalidation can be limited to the new element and does not necessarily need to encompass the complete set of elements, whose constituents may not all be affected by the change.

If properties of materials change over time (e.g. batches of materials with lower or higher particle size, lower or higher moisture content etc.) and these changes become relevant, they should also be included as part of the validation. This can be achieved for example, by an amendment to the validation protocol and does not necessarily require a complete revalidation of the chemometric model.

To assess specificity, the number of false-positive and false-negative errors can be evaluated by classification of the test set.

Robustness

For validation of robustness, a comprehensive set of critical parameters (e.g. process parameters such as temperature, humidity, instrumental performance of the analytical equipment) should be considered. The reliability of the analytical method should be challenged by variation of these parameters. It can be advantageous to use experimental design (DoE) to evaluate the method.

To assess robustness the number of correct classifications, correct rejections, false-positive and false-negative errors can be evaluated by classification of samples under robustness conditions.

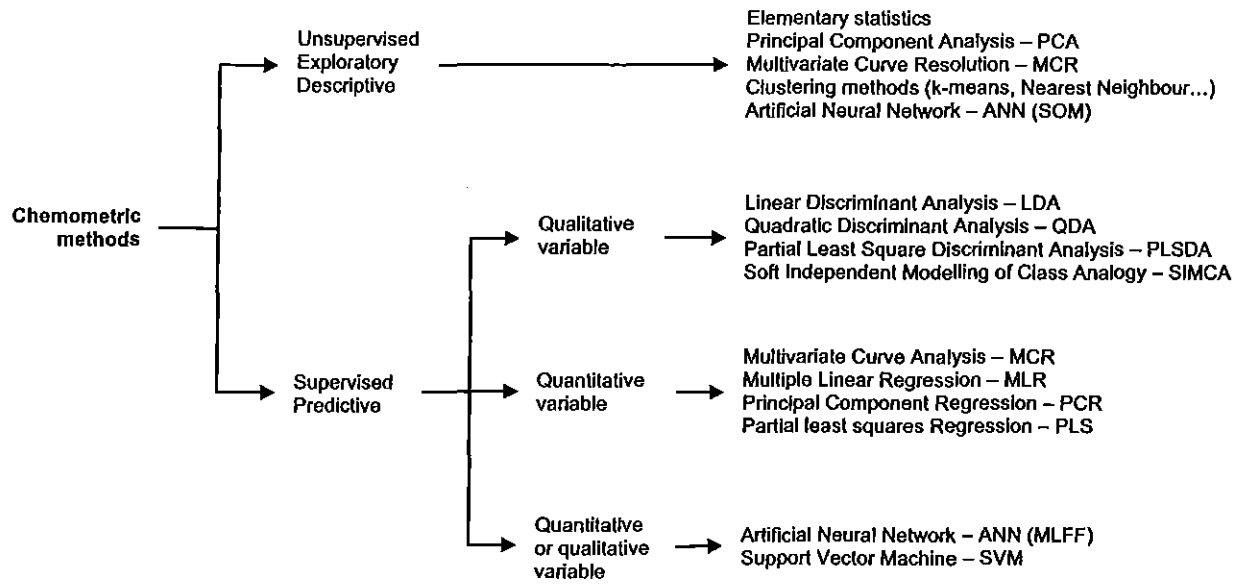


Figure 5.21.-2. – *Map of chemometric methods discussed in the chapter*

1-3-3-2 Quantitative models

The following parameters should be addressed unless otherwise justified: specificity, linearity, range, accuracy, precision and robustness.

Specificity

It is important to detect that the sample that is quantified is not an outlier with respect to the calibration space. This can be done using the correlation coefficient between the sample and the calibration mean, as well as Hotelling T^2 among others.

Linearity

Linearity should be validated by correlating results from the chemometric model with those from an analytical reference method. It should cover the entire range of the method and should involve a specifically selected set of samples that is not part of the calibration set. For orientation purposes, a 'leave-subset-out' cross-validation based on the calibration set may be sufficient, but should not replace assessment using an independent test set. Linearity can be evaluated through the correlation coefficient, slope and intercept of predicted values versus reference values.

Range

The range of analyte reference values defines the range of the chemometric model, and its lower limit determines the limits of detection and quantification of the analytical method. Controls must be in place to ensure that results outside this range are recognised as such and identified. Within the range of the model, acceptance criteria for accuracy and precision have to be fulfilled.

Accuracy

The accuracy of the chemometric model can be determined by comparison of analytical results obtained from the chemometric model with those obtained using a reference method. The evaluation of accuracy should be carried out over the defined range of the chemometric model using an independent test set. It may also be helpful to assess the accuracy of the model using a 'leave-subset-out' cross-validation, although, this should not replace assessment using an independent test set. Accuracy can be evaluated through the slope and intercept of predicted values versus reference values.

Precision

The precision of the analytical method should be validated by assessing the standard deviation of the measurements performed through the chemometric model. Precision covers repeatability (replicate measurements of the same sample by the same person on the same day) and intermediate precision (replicate measurements of the same sample by another person on different days). Precision should be assessed at different analyte values covering the range of the chemometric model, or at least at a target value.

Robustness

For validation of robustness, the same principles as described for qualitative methods apply. Extra care should be taken to investigate the effects of any parameters relevant for robustness on the accuracy and precision of the chemometric model. It can be an advantage to evaluate these parameters using experimental design.

The chemometric model can also be investigated using challenge samples, which may be samples with analyte concentrations outside the range of the method or samples of different identity. During the validation, it must be shown that these samples are clearly recognised as outliers.

2 CHEMOMETRIC TECHNIQUES

A non-exhaustive selection of chemometric methods are discussed below. A map of the selected methods is given in Figure 5.21.-2.

2-1 PRINCIPAL COMPONENTS ANALYSIS

2-1-1 Introduction

The complexity of large data sets or tables makes human interpretation difficult without supplementary methods to aid in the process. Principal components analysis (PCA) is a projection method used to visualise the main variation in the data. PCA can show in what respect 1 sample differs from another, which variables contribute most to this difference and whether these variables contribute in the same way and are correlated or are independent of each other. It also reveals sample set patterns or groupings within the data set. In addition, PCA can be used to estimate the amount of useful information contained in the data table, as opposed to noise or meaningless variations.

2-1-2 Principle

PCA is a linear data projection method that compresses data by decomposing it to so-called latent variables.

The procedure yields columns of orthogonal vectors (scores), and rows of orthonormal vectors (loadings). The principal components (PCs), or latent variables, are a linear combination of the original variable axes. Individual latent variables can be interpreted via their connection to the original variables. In essence, the same data is shown but in a new coordinate system. The relationships between samples are revealed by their projections (scores) on the PCs. Similar samples group together in respect to PCs. The distance between samples is a measure of similarity/dissimilarity.

The original data table is transformed into a new, rearranged matrix whose structure reveals the relationships between rows and columns that may be hidden in the original matrix (Figure 5.21.-3). The new structure constitutes the explained part of the original data. The procedure models the original data down to a residual error, which is considered the unexplained part of the data and is minimised during the decomposition step.

The underlying idea is to replace a complex data table with a simpler counterpart version having fewer dimensions, but still fitting the original data closely enough to be considered a good approximation (Figure 5.21.-4). Extraction of information from a data table consists of exploring variations between samples, i.e. finding out what makes a sample different from or similar to another. Two samples can be described as similar if they have similar values for most variables. From a geometric perspective, the combination of measurements for 1 sample defines a point in a multidimensional space with as many dimensions as there are variables. In the case of close coordinates the 2 points are located in the same area or volume. With PCA, the number of dimensions can be reduced while keeping similar samples close to each other and dissimilar samples further apart in the same way as in the multidimensional space, but compressed into an alternate lower dimensional coordinate system.

The principle of PCA is to find the directions in the data space that describe the largest variation of the data set, i.e. where the data points are furthest apart. Each direction is a linear combination of the initial variables that contribute most to the actual variation between samples.

By construction, principal components (PCs) are orthogonal to each other and are also ranked so that each carries more information than any of those that follow. Priority is therefore given to the interpretation of these PCs, starting with the 1st, which incorporates the greatest variation and thereby constitutes an alternative less complex system that is more suitable for interpreting the data structure. Normally, only the 1st PCs contain pertinent information, with later PCs being more likely to describe noise. In practice, a specific criterion is used to ensure that noise is not mistaken for information and this criterion should be used in conjunction with a method such as cross-validation or evaluation of loadings in order to determine the number of PCs to be used for the analysis. The relationships between samples can then be subsequently viewed in 1 or a series of score plots.

Residuals $\hat{E}$ keep the variation that is not included in the model, as a measure of how well samples or variables fit that model. If all PCs were retained, there would be no approximation at all and the gain in simplicity would consist only of ordering the variation of the PCs themselves by size. Deciding on the number of components to retain in a PCA model is a compromise between simplicity, robustness and goodness of fit/performance.

2-1-3 Assessment of model

Total explained variance R^2 is a measure of how much of the original variation in the data is described by the model. It expresses the proportion of structure found in the data by the model. Total residual and explained variances show how well the model fits the data. Models with small total residual variance (close to 0 per cent) or large total explained variance (close to 100 per cent) can explain most of the variation in the data. With simple models consisting of only a few components, residual variance falls to 0; otherwise, it usually means that the data contains a large amount of noise. Alternatively, it can also mean that the data structure is too complex to be explained using only a few components. Variables with small residual variance and large explained variance for a particular component are well defined by the model. Variables with large residual variance for all or the 1st components have a small or moderate relationship with other variables. If some variables have much larger residual variance than others for all or the 1st components, they may be excluded in a new calculation and this may produce a model that is more suitable for its purpose. Independent test set variance is determined by testing the model using data that was not used in the actual building of the model itself.

2-1-4 Critical aspects

PCA catches the main variation within a data set. Thus comparatively smaller variations may not be distinguished.

2-1-5 Potential use

PCA is an unsupervised method, making it a useful tool for exploratory data analysis. It can be used for visualisation, data compression, checking groups and trends in the data, detecting outliers, etc.

For exploratory data analysis, PCA modelling can be applied to the entire data table once. However, for a more detailed overview of where a new variation occurs, evolving factor analysis (EFA) can be used and, in this case, PCA is applied in an expanding or fixed window, where it is possible to identify, for example, the manifestation of a new component from a series of consecutive samples.

PCA also forms the basis for classification techniques such as SIMCA and regression methods such as PCR. The property of PCA to capture the largest variations in the 1st principal components allows subsequent regression to be based on fewer latent variables. Examples of utilising components as independent data in regression are PCR, MCR, and ANN.

PCA is used in multivariate statistical process control (MSPC) to combine all available data into a single trace and to apply a signature for each unit operation or even an entire manufacturing process based on, for example, Hotelling T^2 statistics, PCA model residuals or individual scores.

In addition to univariate control charts, 1 significant advantage with PCA is that it can be used to detect multivariate outliers, i.e. process conditions or process output that has a different correlation structure than the one present in the previously modelled data.

2-2 MEASURES BETWEEN OBJECTS

The primary use of the following algorithms is to measure the degree of similarity between an object and a group or the centre of the data.

2-2-1 Similarity measures

Calculation of the correlation is the simplest statistical tool used to compare data and to determine the degree of similarity, provided the data sets have the same dimension, e.g. spectral data. It is a measure of the linear association between a pair of vectors. A correlation score between -1 and +1 is calculated for the match, based on the system below,

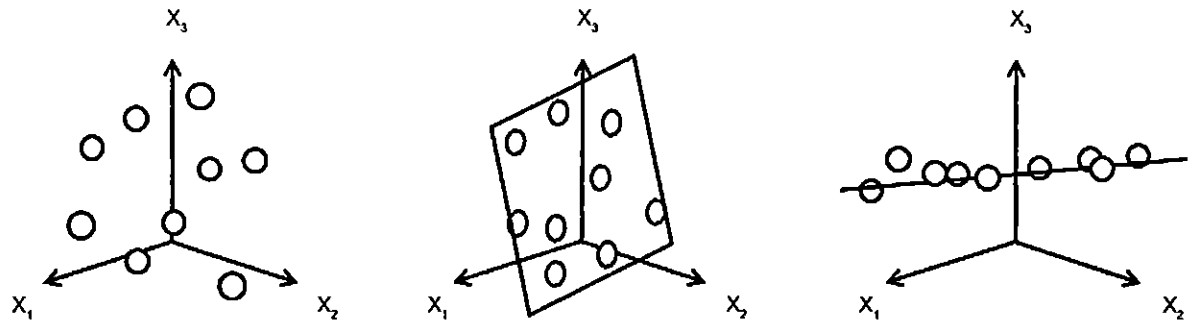
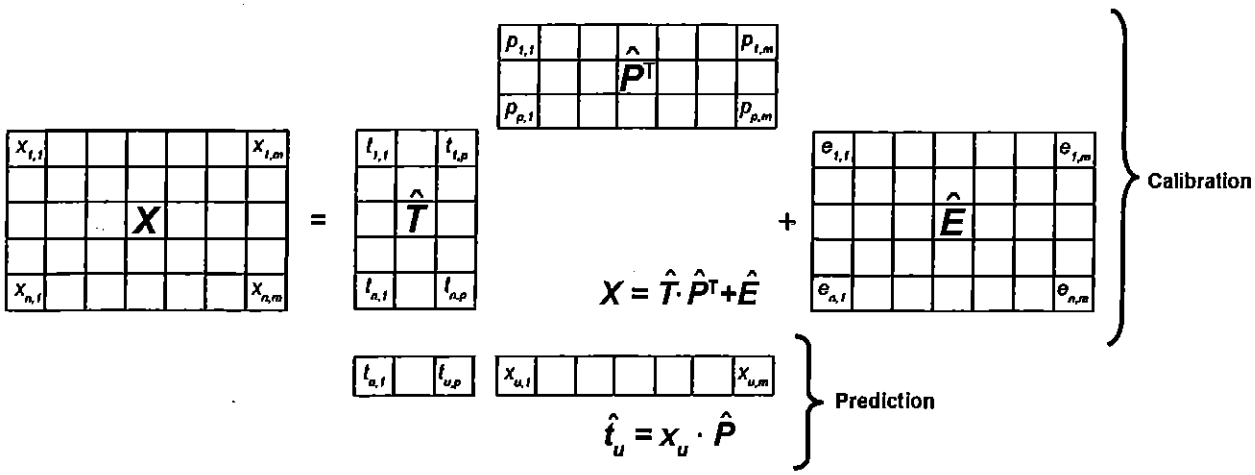


Figure 5.21.-3. – *Geometrical representation of 3 different X-data sets. On the left, objects are plotted in the multivariate space, and the following examples reveal a hidden structure, i.e. a plane and a line respectively*



$\hat{X}$ = original data matrix of n rows and m columns
$\hat{T}$ = score matrix with n rows and p columns
$\hat{P}^T$ = loadings matrix with p rows and m columns
$\hat{E}$ = residual matrix (same size as matrix X)
m = number of data points (variables)
n = number of measurements (samples)
p = number of factors
x_u = data of unknown sample
$\hat{t}_u$ = score values for unknown sample

Figure 5.21.-4. – *Decomposition of the X-matrix for principal components analysis (PCA)*

where a perfect match (mirror image) would have a score of +1 and 2 lines that are complete opposites would have a score of -1 (Figure 5.21.-5).

Correlation is used to compare data sets in any of the following ways:

— comparison of 2 selected samples;
— comparison of 1 or more selected samples represented by vectors with a reference data library (from a group or class).

The reference data can be the average of a group of typical characteristics.

Correlation r between 2 mean centred vectors x and y of the same dimension can be calculated using the following equation:

$$r = \frac{\Sigma_i x_i y_i}{\sqrt{\Sigma_i x_i^2 \Sigma_i y_i^2}}$$

2-2-2 Distance measures

In the object space, a collection of objects will be seen as points that are more or less close to each other and will gather into groups or clusters. Measuring the distance between points will express the degree of similarity between objects. In the same way, measuring the distance of a point to the centre of a group will give information about the group membership of this object. The following algorithms are given to illustrate the way objects can be compared.

2-2-2-1 Euclidean distance

The Euclidean distance $ed_{i,j}$ between 2 points i and j can be calculated as:

$$ed_{i,j} = \sqrt{\sum_{k=1}^{m} (x_{i,k} - x_{j,k})^2}$$

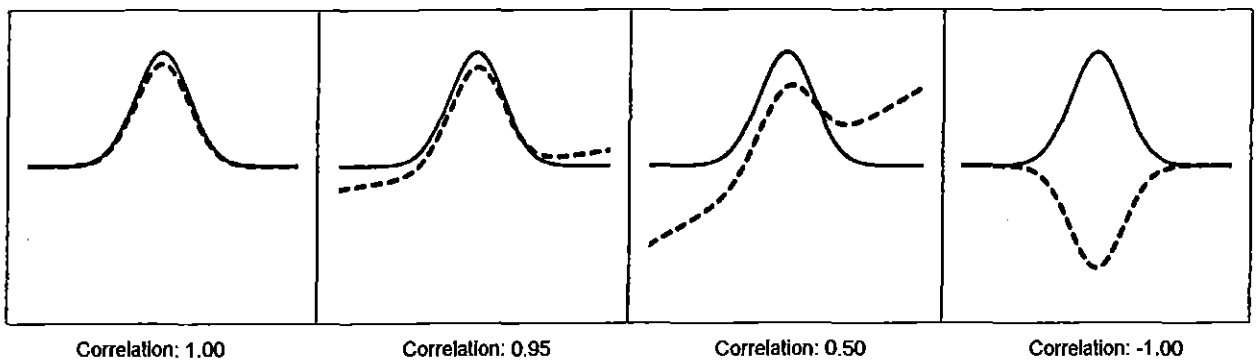


Figure 5.21.-5. – *Examples of correlation scores illustrated by matching of the shapes*

Similarly, the Euclidean distance $ed_{i,c}$ between the point i and the centre c of the data can be calculated as the square root of the sum of the squared differences of the coordinates of point i to the mean value of the x-coordinates for each of the m axes, which can be expressed by the following matrix notation:

$$ed_{i,c} = \sqrt{(x_i - \bar{x})(x_i - \bar{x})^T}$$

where x_i denotes the m values of coordinates describing the point i and $\bar{x}$ denotes the mean coordinates calculated for the m variables. The superscript T indicates that the 2nd term of the equation is transposed.

2-2-2-2 Mahalanobis distance

The Mahalanobis distance (*md*) takes into account the correlation between variables by using the inverse of the variance-covariance matrix.

The variance-covariance matrix C_x is calculated using the following equation:

$$C_x = (1/(n-1))X_C^T X_C$$

where X_c is the $n \times m$ data matrix centred over the mean of each column. Thus C_x is a square matrix that contains the variance of each variable over its diagonal and the covariance between variables on both sides of the diagonal.

The Mahalanobis distance of point i to the centre c of the data is given by the following equation:

$$md_{i,c} = \sqrt{(x_i - \bar{x})C_x{}^{-1}(x_i - \bar{x})^T}$$

The C_x^{-1} matrix is the inverse of the variance-covariance matrix and $C_x C_x^{-1} = I$, where I is the identity matrix.

The number of variables or principal components involved in calculating the distance is designated p, and n is the number of objects in the group or in the data set. Under the assumption that the data is normally distributed, the random variable $(n\text{-}1)^2/n \times md^2$ is beta distributed with degrees of freedom $u = p/2$ and $v = (n\text{-}p\text{-}1)/2$. Thus, if for a point x_i this expression exceeds the (1 - α)-quantile of the beta distribution then the point can be classified as an outlier with the significance level α (i.e. α is the probability of the type-I error classifying the point as an outlier although it is not).

In the same way, the leverage effect (h) of a data point located at the extremity of the X-space on the regression parameters of a multivariate model can be calculated using the following equation:

$$h = \frac{1}{n} + \frac{(md_{i,c})^2}{(n-1)}$$

Data points with high leverage have a large influence on the model.

2-2-2-3 Critical aspects

Euclidean distances only express the similarities or differences between data points when the variables are strictly uncorrelated. If correlations between variables exist they contain at least partially the same information and the dimensionality of data space is in fact smaller than the number of variables. Mahalanobis distances allow for the correction of correlations but their calculation supposes the variance-covariance matrix to be invertible. In some instances where there is high collinearity in the data set, this matrix is singular and cannot be inverted. This is especially the case with spectroscopic data where the high resolution of spectrometers introduces redundancy by essentially describing the same signal through measurements at several consecutive wavelengths. Another constraint in variance-covariance matrix inversion is that the number of variables has to be smaller than the number of objects ($n > m$).

Distances can be computed in the PC space, thus providing the benefits of reduced dimensionality, orthogonality between PCs and also PC ordering. As the 1st PCs carry the maximum amount of information, data reduction without loss of information can be achieved by eliminating the later insignificant PCs.

If a sufficient number of PCs are used to closely model the data, the Euclidean distance of data points to the centre of the data set will be identical when calculated from PC scores and from the coordinates on the original variables. This can be understood by considering that PCA calculation does not transform data but only extracts latent variables to describe the data space without distorting it. The same applies when using Mahalanobis distances, where the values are identical whether the original data space or that of the PCs is used. The only difference is the simplification of the calculation of Mahalanobis distances. Due to the orthogonality between PCs, Mahalanobis distances can be computed as the Euclidean distances calculated over the range of the normalised scores using the multiplication factor $\sqrt{n-1}$.

2-2-3 Linear and quadratic discriminant analysis

2-2-3-1 Principle

In Linear Discriminant Analysis and Quadratic Discriminant Analysis (LDA, QDA), the assignment of a test object x_i to

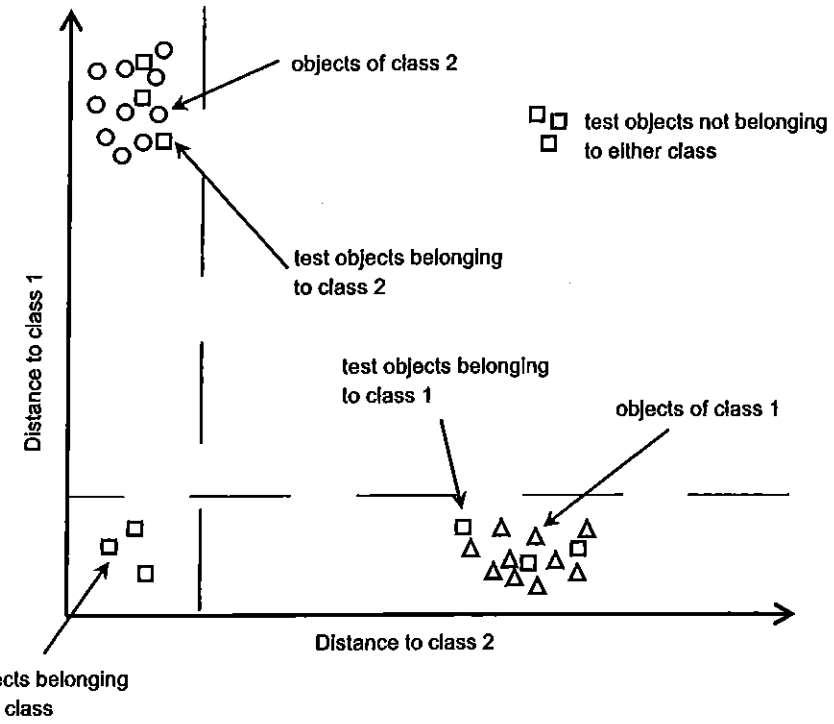


Figure 5.21.-6. – *Plot representing the 4 possible classifications of test objects in a two-class SIMCA analysis (□ = unknown sample to classify, Δ = class 1 sample, ○ = class 2 sample)*

one of K predefined groups (or classes) identified in the data set is determined by the classification score, which is given in the following equation for LDA:

$$cf(x_{i,K}) = (x_i - \bar{x}_K)^T C^{-1}(x_i - \bar{x}_K) + \ln|C| - 2\ln(\pi_K)$$

where π_K is the prior probability of group K and is equal to the number of objects contained in group K divided by the total number of objects in the training set. C is the variance-covariance matrix and $|C|$ is its determinant.

2-2-3-2 Critical aspects

LDA assumes that the variance-covariance matrix for all classes is identical, while QDA estimates a variance-covariance matrix for each class. Hence in QDA far more parameters need to be estimated which should only be done if sufficient data are available.

2-2-3-3 Potential use

It can be used in the case of straightforward classification schemes.

2-3 SOFT INDEPENDENT MODELLING OF CLASS ANALOGY

2-3-1 Introduction

Soft independent modelling of class analogy (SIMCA) is a method for supervised classification of data. The method requires a training set, which consists of samples with known attributes that have been pre-assigned to different classes. SIMCA classes can be overlapping and share common elements. Therefore, a sample can belong to 1, multiple or none of the classes.

2-3-2 Principle

PCA models are 1st established for individual classes. The samples of the training set have to be analysed by PCA (see the section on PCA) and for each class a distinct principal components model is generated. The number of relevant principal components can be adjusted for each class of objects separately. According to this procedure the data sets of each class can be reduced to the relevant principal components models.

New objects are then classified based on the individual PCA models. A new object is projected into each of these models and assigned to a certain class when its residual distance from this model is below the limit for this class (Figure 5.21.-6). Distances of objects to the respective classes can be calculated by procedures such as either Euclidean or Mahalanobis distance. Consequently an object may belong to either 1 or multiple classes if the corresponding distances are within the required threshold. If the distance of an object to all of the SIMCA classes is above the threshold, then it will be classified as an outlier.

2-3-3 Critical aspects

Since SIMCA is mainly based on PCA principles, the validation of the method should follow that of PCA.

In addition to this, the overlap of different classes must also be taken into account. For example, a molecule can have several chemical groups that appear in its spectroscopic profile. Thus, grouping such data into chemical subgroups results in overlap since separation is not possible.

2-3-4 Potential use

SIMCA is often used for the classification of analytical data from techniques such as near-infrared (NIR) or mass spectroscopy, and other analytical techniques such as chromatography and chemical imaging. SIMCA is more suitable than PCA for discriminating between classes that are difficult to separate.

2-4 CLUSTERING

2-4-1 Introduction

A cluster consists of a group of objects or data points similar to each other. Clustering tools can be used to visualise how

data points 'self-organise' into distinct groups or to highlight the degree of similarity between data objects. Data points from a particular cluster share some common characteristics that differentiate them from those gathered in other clusters. Clusters are characterised using 3 main properties; size, shape and distance to the nearest cluster. Clustering is an unsupervised method of data analysis and is used either for explanatory or confirmatory analysis. It differs from discriminant analysis, which is a supervised classification technique, where an unlabelled object is assigned to a group of pre-classified objects.

2-4-2 Principle

Numerous data clustering approaches are available and are typically classed as either hierarchical or non-hierarchical. Hierarchical clustering leads to the classical dendrogram graphical representation of the data, whereas non-hierarchical clustering finds clusters without imposing a hierarchical structure. Numerous algorithms are described in the literature, where data is partitioned either in a specific way or by optimising a particular clustering criterion. This simple and exclusive distinction is incomplete since mixed algorithms have similarities to both approaches. Hierarchical clustering recursively finds clusters either in agglomerative (bottom-up) or divisive (top-down) mode to form a tree shaped structure. Agglomerative mode starts with defining each data point as its own cluster and then merging similar clusters in pairs before repeating this step until the complete data set is classified (Figure 5.21.-7). Divisive mode starts by considering the entire data set as a single cluster, which is then recursively divided until only clusters containing a unique data point are obtained. Algorithms differ in the way they calculate the similarity between clusters. Complete link and single link algorithms calculate the distance between all pairs of objects that belong to different clusters in order to evaluate the similarity between them. In the single link method, this distance corresponds to the minimum distance separating 2 objects originating from 2 different clusters whereas in complete link algorithm this distance corresponds to the largest distance between 2 objects from 2 different clusters. Ward's algorithm, also called the minimum variance algorithm, calculates the similarity between clusters by means of decreasing cluster variance when the 2 most similar clusters are merged.

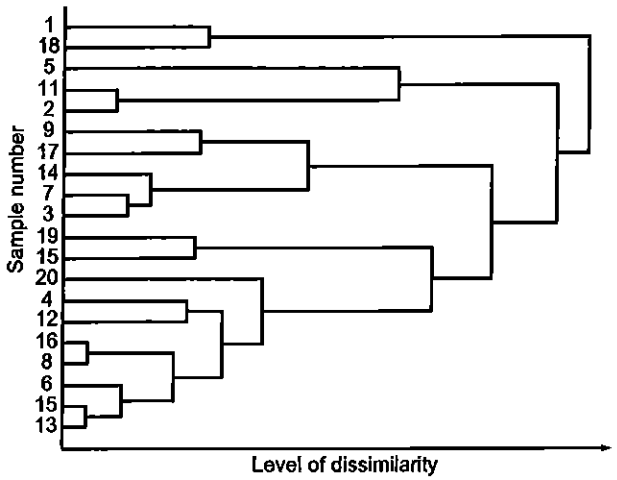


Figure 5.21.-7. – *Dendrogram for agglomerative hierarchical clustering until clusters containing a unique data point are obtained*

Non-hierarchical clustering cannot be described and categorised as easily as hierarchical clustering. Different algorithms exist, which give rise to different classification schemes. An overview of the different categories of algorithms is given below ranging from simple distance based methods such as the minimum spanning tree and the nearest neighbour algorithms, to more sophisticated methods such as the *K*-means algorithm (often cited as a classical partition method), the expectation-maximisation algorithm (for 'model-based' methods) and DBSCAN for 'density-based' algorithms and, also, the 'grid-based' methods which are exemplified by the statistical information grid (STING) algorithm.

Minimum spanning tree clustering, such as Kruskal's algorithm, is similar to the graph theory algorithm as all the data points are first of all connected by drawing a line between the closest points. When all data points are linked, the lines of largest length are broken, leaving clusters of closely connected points. For nearest neighbour clustering, an iterative procedure is used to assign a data point to a cluster when the distance between this point and its immediate neighbour (that belongs to a cluster) is below a pre-defined threshold value.

The *K*-means algorithm is one of the most popular and as with partition algorithms, the number of clusters must be chosen *a priori*, together with the initial position of the cluster centres. A squared error criterion measures the sum of the squared distance between each object and the centroid of its corresponding cluster. The *K*-means algorithm starts with a random initial partition and progresses by reassigning objects to clusters until the desired criteria reach a minimum. Some variants of the *K*-means algorithm allow the splitting or merging of clusters in order to find the optimum number of clusters, even when starting from an arbitrary initial clustering. Model-based clustering attempts to find the best fit for the data using a preconceived model. An example of this is the EM or expectation-maximisation algorithm, which assigns each object to a particular cluster according to the probability of membership for that object. In the EM algorithm, the probability function is a multivariate Gaussian distribution and that is iteratively adjusted to data by use of the maximum-likelihood estimation. The EM algorithm is considered as an extension of the *K*-means algorithm since the residual sum of squares used for *K*-means convergence is similar to the maximum-likelihood criterion.

Density-based (DB) clustering, such as the DBSCAN algorithm, assimilates clusters to regions of high density separated by regions of low or no density.
The neighbourhood of each object is examined to determine the number of other objects that fit within a specified radius and a cluster is defined when a sufficient number of objects inhabit this neighbourhood.

Grid-based algorithms, such as STING, divide the data space into a finite number of cells. The distribution of objects within each cell is then computed in terms of mean, variance, minimum, maximum and type of distribution. There are several levels of cells, providing different levels of resolution and each cell of a particular level corresponds to the union of 4 child cells from the lower level.

2-4-3 Critical aspects

Algorithms are sensitive to the starting conditions used to initialise the clustering of data. For example, *K*-means needs a pre-set number of clusters and the resultant partitioning will vary according to the chosen number of clusters.
The metrics used in distance calculation will also influence

data clustering. For Euclidean distances, the *K*-means algorithm will define spherical clusters whereas they could be ellipsoidal when using Mahalanobis distances. The cluster shape can be modified by data pre-treatments prior to cluster analysis. DB algorithms can deal with arbitrarily shaped clusters, but their weakness is their limitation in handling high-dimensional data, where objects are sparsely distributed among dimensions.

When an object is considered to belong to a cluster with a certain probability, algorithms such as density based clustering, allow a soft or fuzzy clustering. In this case, the border region of 2 adjacent clusters can house some objects belonging to both clusters.

2-4-4 Potential use

Clustering is an exploratory method of analysis that helps in the understanding of data structure by grouping objects that share the same characteristics and in addition, hierarchical clustering allows for classification within data objects. Clustering is used in a vast variety of fields, in particular for information retrieval from large databases. For the latter, the term 'data mining' is frequently used, where the objective is to extract hidden and unexploited information from a large volume of raw data in search of associations, trends and relationships between variables.

2-5 MULTIVARIATE CURVE RESOLUTION

2-5-1 Introduction

Multivariate curve resolution (MCR) is related to principal components analysis (PCA) but, where PCA looks for directions that represent maximum variance and are mutually orthogonal, MCR strives to find contribution profiles (i.e. MCR scores) and pure component profiles (i.e. MCR loadings). MCR is also known as self-modelling curve resolution (SMCR) or end-member extraction. When optimising MCR parameters the alternating least squares (ALS) algorithm is commonly used.

2-5-2 Principle

MCR-ALS estimates the contribution profiles C and the pure component profiles S from the data matrix X, i.e. $X = C \cdot S^T + E$ just as in classical least squares (CLS). The difference between CLS and ALS is that ALS is an iterative procedure that can incorporate information that is known about the physico-chemical system studied and use this information to constrain the components/factors. For example, neither contribution nor absorbance can be negative by definition. This fact can be used to extract pure component profiles and contributions from a well-behaved data set. There are also other types of constraints that may be used, such as equality, unimodality, closure and mass balance.

It is often possible to obtain an accurate estimation of the pure component spectra or the contribution profiles and these estimates can then be used as initial values in the constrained ALS optimisation. New estimates of the profile matrix S and of the contribution profile C are obtained during each iteration. In addition, the physical and chemical knowledge of the system can be used to verify the result, and the resolved pure component contribution profiles should be explainable using existing knowledge. If the MCR results do not match the known system information, then other constraints may be needed.

2-5-3 Critical aspects

Selection of the correct number of components for the ALS calculations is important for a robust solution and a good estimate can be obtained using for example, evolving factor analysis (EFA) or fixed-size moving window EFA.

Furthermore, the constraints can be set as either 'hard' or 'soft', where hard constraints are strictly enforced while soft constraints leave room for deviations from the restricted value. Generally, due to inherent ambiguities in the solution obtained, the MCR scores will need to be translated into, for example, the concentration of the active pharmaceutical ingredient, using a simple linear regression step. This means that the actual content must be known for at least 1 sample. When variations of 2 or more chemical entities are in some way correlated, rank deficiency occurs, for example 1 entity is formed while the other is consumed, or 2 entities are consumed at the same rate to yield a third. As a result, the variation of the individual substance is essentially masked and in such cases, simultaneous analysis of data from independent experiments using varied conditions or combined measurements from 2 measurement techniques generally results in better strategies than analysing the experiments separately one by one.

2-5-4 Potential use

MCR can be applied when the analytical method produces multivariate data for which the response is either linear or can be linearised. This has the advantage that only 1 standard is needed per analyte, which is particularly beneficial when the measurements are at least partly selective between analytes. When linearity and selectivity is an issue, more standards per analyte may be required for calibration. When there is no pure analytical response for an analyte, it is also possible to estimate starting vectors by applying PCA to analyte mixtures together alongside varimax rotation of the PCA coordinate system. ALS implementations of MCR may also allow analyte profiles that are freely varied by the algorithm, which can then be used to model a profile that is difficult to estimate separately, for example a baseline.

2-6 MULTIPLE LINEAR REGRESSION

2-6-1 Introduction

Multiple Linear Regression (MLR) is a classical multivariate method that uses a combined set of x-vectors (X-data matrix) in linear combinations that are fitted as closely as possible to the corresponding single y-vector.

MLR extends linear regression to more than 1 selected variable in order to perform a calibration using least squares fit.

2-6-2 Principle

In MLR, a direct least squares regression is performed between the X- and the Y-data. For the sake of simplicity, the regression of only 1 column vector y will be addressed here, but the method can be readily extended to a Y-matrix, as is common when MLR is applied to data from experimental design (DoE), with multiple responses. In this case, single independent MLR models for each y-variable can be applied to the same X-matrix.

The following MLR model equation is an extension of the normal univariate straight line equation; it may also contain cross and square terms:

$$y = b_0 + b_1 x_1 + b_2 x_2 + \ldots + b_k x_k + f$$

This can be compressed into the convenient matrix form:

$$y = Xb + f$$

The objective is to find the vector of regression coefficients b that best minimises the error term f. This is where the least squares criterion is applied to the squared error terms, i.e. to find b-values so that y-residuals f are minimised.

MLR estimates the model coefficients using the following equation:

$$b = (X^T X)^{-1} X^T y$$

This operation involves the matrix inversion of the matrix $(X^T X)^{-1}$. If any of the X-variables show any collinearity with each other i.e. if the variables are not linearly independent, then the MLR solution will not be robust or a solution may not even be possible.

2-6-3 Critical aspects

MLR requires independent variables in order to adequately explain the data set, but as pharmaceutical samples are comprised of a complex matrix in which components interact to various degrees, the selection of appropriate variables is not straightforward. For example, in ultra-violet spectroscopy, observed absorbance values are linked because they may describe related behaviours in the spectroscopic data set. When observing the spectra of mixtures, collinearity is commonly found among the wavelengths, and consequently, MLR will struggle to perform a usable linear calibration.

The ability to vary the x-variables independently of each other is a crucial requirement when using variables as predictors with this method. This is why in DoE the initial design matrix is generated in such a way as to establish this independence (i.e. orthogonality) from the start. MLR has the following constraints and characteristics:

— the number of X-variables must be smaller than the number of samples ($n > m$), otherwise the matrix cannot be inverted;
— in case of collinearity among X-variables, the b-coefficients are not reliable and the model may be unstable;
— MLR tends to over-fit.

To avoid overfitting MLR is often used with variable selection.

The selection of the optimal number of X-variables can be based on their residual variance, but also on the prediction error.

2-6-4 Potential use

MLR is typically suited to simple matrices/data sets, where there is a high degree of specificity and full rank. As matrices become more complex, more suitable methods such as PLS may be required to provide more accurate and/or robust calibration. In these cases, MLR may be used as a screening technique prior to the application of more advanced calibration methodologies.

2-7 PRINCIPAL COMPONENTS REGRESSION

2-7-1 Introduction

Principal components regression (PCR) is an expansion of principal components analysis (PCA) for use in quantitative applications. It is a two-step procedure whereby the calibration matrix X is first of all transformed by PCA into the scores and loadings matrices $\hat{T}$ and $\hat{P}$ respectively. In the following step, the score matrix for the principal components is used as the input for an MLR model to establish the relationship between the X- and the Y-data.

2-7-2 Principle

As in PCA, the calibration matrix is decomposed into scores and loadings matrices in such a way as to minimise the residual matrix E that ideally consists only of random errors, i.e. noise. For quantitative calibration, an additional matrix Y with the reference analytical data of the calibration samples is necessary. As the concentration information is contained in the orthogonal score vectors of the $\hat{T}$-matrix it can be optimally correlated by multiple linear regression using the actual concentrations in the Y-matrix via the matrix $\hat{Q}$ (Figure 5.21.-8), while minimising the entries in the residual matrix F.

2-7-3 Critical aspects

A crucial point in the development of a model is the selection of the optimal number of principal components. In this respect, the plot of the number of principal components versus the residual Y-variance is an extremely useful diagnostic tool when defining the optimal number of PCs, i.e. when the minimum of the residual Y-variance observed during model assessment has been reached. In most cases, additional PCs beyond this point do not improve the prediction performance but the calibration model falls into overfitting.

Despite its value as an important tool when dealing with collinear X-data, the weakness of PCR lies in its independent decomposition of the X and Y matrices. This approach may take into account variations of the X-data that are not necessarily relevant for an optimal regression with the Y-data. Also, Y-correlated information may even get lost in higher order principal components that are neglected in the above-mentioned selection process of the optimal number of PCs.

A stepwise principal component selection (e.g. selection of PC2 instead of PC1) may be useful to improve the performance of the calibration model.

2-7-4 Potential use

PCR is a multivariate technique with many diagnostic tools for the optimisation of the quantitative calibration models and the detection of erroneous measurements.

In spectroscopy for example, PCR provides stable solutions when dealing with the calibration data of either complete spectra or large spectral regions. However, it generally requires more principal components than PLS and in view of the limitations and disadvantages discussed above, PLS regression has become the preferred alternative for quantitative modelling of spectroscopic data.

2-8 PARTIAL LEAST SQUARES REGRESSION

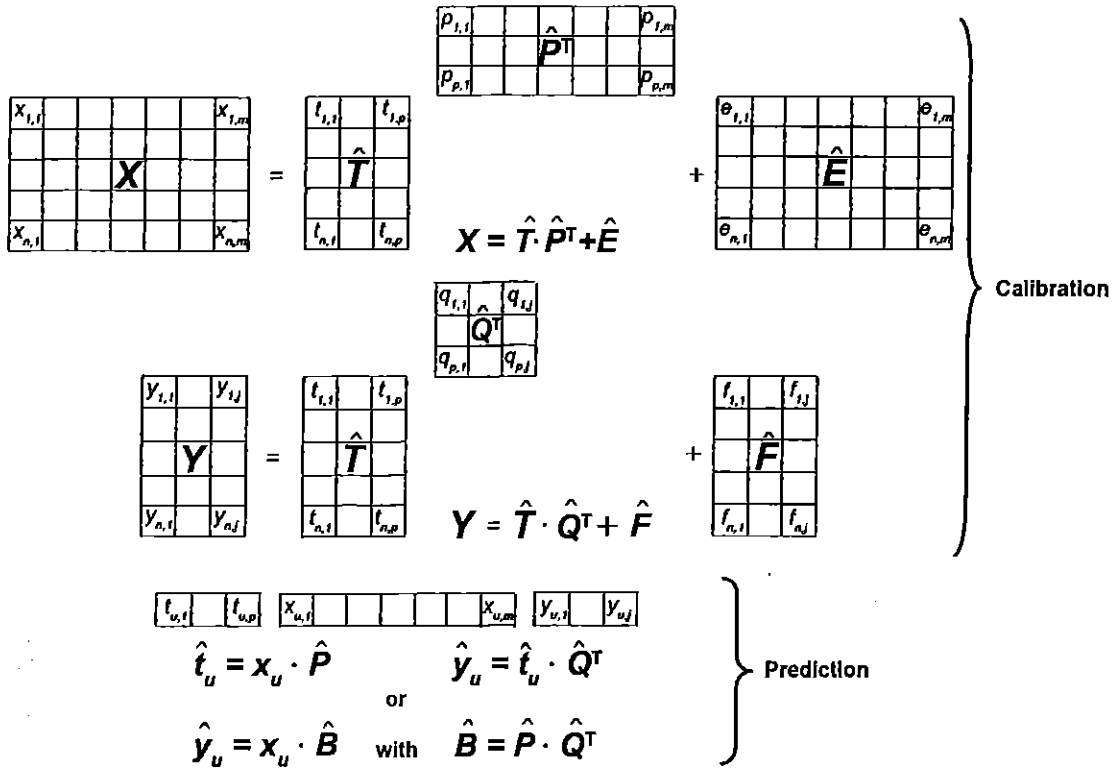
2-8-1 Introduction

Partial least squares regression (PLSR, generally known as PLS and alternatively named projection on latent structures) has developed into the most popular algorithm for multivariate regression.

PLS relates 2 data sets (X and Y) irrespective of collinearity. PLS finds latent variables from X and Y data blocks simultaneously, while maximising the covariance structure between these blocks. In a simple approximation PLS can be viewed as 2 simultaneous PCA analyses applied to the X and Y-data in such a way that the structure of the Y-data is used for the search of the principal components in the X-data. The amount of variance modelled, i.e. the explained part of the data, is maximised for each component. The non-explained part of the data set is made up of residuals, which function as a measure of the modelling quality.

2-8-2 Principle

The major difference between PCR and PLS regression is that the latter is based on the simultaneous decomposition of the X and Y-matrices for the derivation of the components (preferably denoted as PLS factors, factors, or latent-variables). Consequently, for the important factors, the information that describes a maximum variation in X, while correlating as much as possible with Y, is collected. This is



X	=	original data matrix of n rows and m columns	m	=	number of data points (variables)
$\hat{T}$	=	scores matrix with n rows and p columns	n	=	number of measurements (samples)
$\hat{P}^T$	=	loadings matrix with p rows and m columns	j	=	number of property values per sample
$\hat{E}$	=	residual matrix (same size as X-matrix)	p	=	number of principal components (factors)
Y	=	property matrix of n rows and j columns	x_u	=	data of unknown sample
$\hat{Q}^T$	=	correlation matrix of p rows and j columns	$\hat{y}_u$	=	predicted property values of unkown sample
$\hat{F}$	=	residual matrix (same size as Y-matrix)	$\hat{t}_u$	=	score values for unknown sample
$\hat{B}$	=	matrix of regression coefficients			

Figure 5.21.-8. – *Decomposition of the matrices for principal components regression (PCR)*

precisely the information that is most relevant for the prediction of the *Y*-values of unknown samples. In practice PLS can be applied to either 1 *Y*-variable only (PLS1), or to the simultaneous calibration of several *Y*-variables (PLS2 model).

As the detailed PLS algorithms are beyond the scope of this chapter, a simplified overview is instead given (Figure 5.21.-9). Arrows have been included between the $\hat{T}$ and $\hat{U}$ scores matrices in order to symbolise the interaction of their elements in the process of this iteration. While the *Y*-matrix is decomposed into the loadings and scores matrices $\hat{Q}$ and $\hat{U}$ respectively, the decomposition of the *X*-matrix produces not only the loadings and scores matrices $\hat{P}$ and $\hat{T}$, but also a loading weights matrix $\hat{W}$, which represents the relationship between the *X* and *Y*-data.

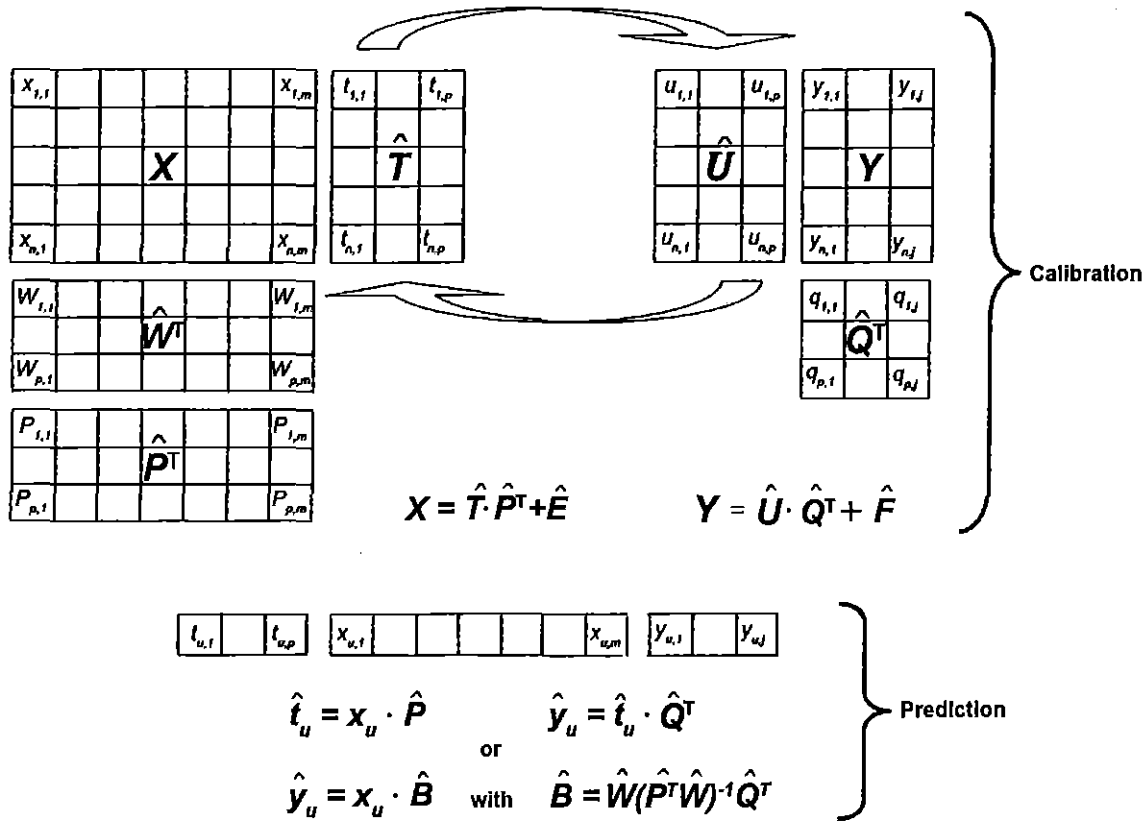
To connect the *Y*-matrix with the *X*-matrix decomposition for the first estimation of the $\hat{T}$ score values, the *Y*-data are used as a guide for the decomposition of the *X*-matrix. By interchanging the score values of the $\hat{U}$ and $\hat{T}$ matrices, an interdependent modelling of the *X* and *Y* data is achieved, thereby reducing the influence of large *X*-variations that do not correlate with *Y*. Furthermore, simpler calibration models with fewer PLS-factors can also be developed where, as is the case for PCR, residual variances are used during validation to determine the optimal number of factors that model useful information and consequently, avoid overfitting.

2-8-3 Critical aspects

A critical step in PLS is the selection of the number of factors. Selecting too few factors will inadequately explain variability in the training data set, while too many factors will cause overfitting and instability in the resulting calibration (Figure 5.21.-10). The optimal number of factors is estimated during validation of the calibration.

Figure 5.21.-10 shows the changes in the calibration error (A) of a model and 2 cases of prediction errors (B, C) according to the number of factors used in the model. The calibration error decreases continuously as the number of factors increases. In case B prediction error reveals that no minimum can be observed; however, a minimum is observed in case C. In the absence of a minimum, the number of components can be chosen based on where no significant decreasing of error is observed.

As far as the decision between PLS1 or PLS2 models is concerned PLS1 modelling is chosen if there is only 1 *Y*-variable of interest. In cases where there is more than 1 *Y*-variable of interest, either one PLS2 model or individual



X = original data matrix of n rows and m columns
Y = property matrix of n rows and j columns
$\hat{T}$ = scores matrix with n rows and p columns
$\hat{P}^T$ = loadings matrix with p rows and m columns
$\hat{W}^T$ = loading weights matrix with p rows and m columns
$\hat{E}$ = residual matrix (same size as X-matrix)
$\hat{Q}^T$ = loadings matrix of Y-data
$\hat{U}$ = scores matrix of Y-data
$\hat{F}$ = residual matrix (same size as Y-matrix)
$\hat{B}$ = matrix of regression coefficients
m = number of data points (variables)
n = number of measurements (samples)
j = number of property values per sample
p = number of factors
x_u = data of unknown sample
$\hat{y}_u$ = predicted property values of unknown sample
$\hat{t}_u$ = score values for unknown sample.

Figure 5.21.-9. – *Decomposition of the data matrices for PLS regression*

PLS1 models for each Y-variable can be calculated. In general, PLS2 is the preferred approach for screening purposes and in cases of highly correlated Y-variables of interest; otherwise separate PLS1 models for the different Y-variables will yield more satisfactory prediction results.

2-8-4 Potential use

PLS has emerged as a preferable alternative to PCR for quantitative calibration because it incorporates the intervention of the Y-data structure for the decomposition of the calibration X-matrix. Consequently, information from the most important factors is collected and is capable of describing maximal variation in the X-data, while also correlating as closely as possible with the Y-data. In general, this yields simpler models with fewer factors compared to PCR and also provides superior interpretation possibilities and visualisation diagnostics for the optimisation of the calibration performance. In addition PLS can handle presence of noise in both X- and Y-data.

PLS discriminant analysis (PLS-DA) is a special case of PLS where X-matrix is regressed into a dummy Y-matrix consisting of ones and zeroes. Ones and zeroes indicate the class to which samples are belonging or not. PLS-DA is used as a semi-quantitative method in, for example, chemical imaging estimating the pixel components.

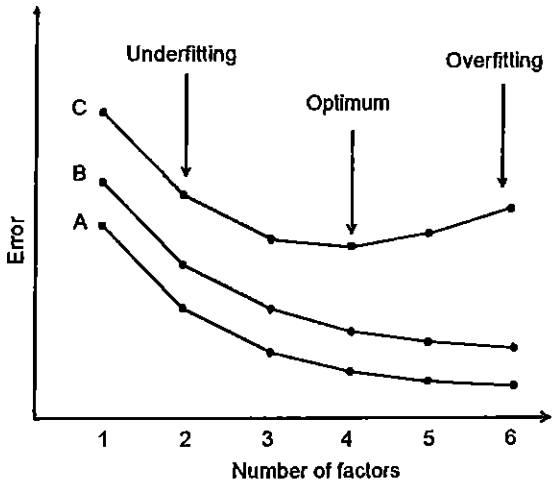


Figure 5.21.-10. – *Effects of adding factors to a model; the calibration becomes more accurate i.e. the residual error diminishes, while the predictive performance of the model could deteriorate as for prediction C. The optimum number of factors reflects a compromise.*

2-9 SUPPORT VECTOR MACHINES

2-9-1 Introduction

To achieve classification, multivariate techniques reduce the dimensionality and complexity of the data set. Kernel methods project data into higher dimensional feature spaces.

2-9-2 Principle

Support vector machines (SVMs) project *X*-data of the training set into a feature space of usually much higher dimension than the original data space. In the feature space a hyperplane (also called decision plane) is computed that separates individual points of known group membership (Figure 5.21.-11). The best discriminating separation is achieved by maximising the margin between groups. The margin is defined by 2 parallel hyperplanes at an equal distance from the decision plane. The optimum position of the decision plane is obtained if the margin is maximal. Points in the feature space that define the margin are called support vectors.

For each training point the distance to the decision plane is computed. In the case of a two-class separation for example, the sign of the distance gives the group membership and the value corresponds to the certainty of classification. During modelling the distance between the training points and the hyperplane contributes to the weight attributed to the point. Very distant points will have a lesser weight, and to avoid overfitting, distances smaller than a trade-off parameter will not be considered.

For non-separable object groups overlapping is allowed to a certain extent. So-called slack variables are added to objects, with value 0 if correctly classified and a positive value otherwise. The optimal hyperplane is found by maximising the margin at the same time allowing for a minimum number of training points to be misclassified (Figure 5.21.-12). The proportion of misclassified points becomes a control parameter during margin maximisation.

In practice SVM computation is extremely complex and would be infeasible without simplifying of the optimisation problem. To project *X*-data into the feature space the original data is expanded by a set of basis functions. Selecting particular basis functions makes it possible to reformulate the whole optimisation procedure. Only products of the expanded variables remain part of the optimisation procedure and they can be advantageously replaced by a Kernel function.

2-9-3 Critical aspects

Numerous algorithms and different types of software can be used to compute SVMs, which may lead to differing results. Optimisation will vary depending on the algorithm used. Control criteria may differ, thus leading either to divergence during the iterations, or to unstable computations sensitive to redundant and uninformative data.

During SVM computation training points that are well inside their class boundaries have little or even no effect on the position of the decision plane. The latter focusses mainly on points that are difficult to separate and not on objects that are clearly distinct. Thus, SVMs are sensitive to redundant values and atypical points like outliers, for example. As a consequence it may be pertinent to select or screen out specific variables prior to performing SVM. The data should be normalised and standardised to avoid having input data of different scales, which may lead to poor conditions for the boundary optimisation.

The best performing model must be adequately validated and test data that is completely untouched during iterations is required for this purpose. It should be ensured that this data is well balanced in the sense that both easy and difficult samples are equally represented in the training and validation sets.

2-9-4 Potential use

SVMs are mainly used for binary supervised classification. They can be generalised to multiclass classification or extended to regression problems, though these applications are not considered within the scope of this chapter. Objects that are difficult to classify rather than those that are clearly distinct drive the optimisation process in SVMs. SVMs can be used for the separation of classes of objects, but not for the identification of these objects. They operate well on large data sets that are obtained, for example, by NIR spectroscopy, magnetic resonance, chemical imaging or process data mining, where PCA and related methods fail. Their strength mainly lies in separation of samples featuring highly correlated signals, i.e. polymorphs, excipients, tracing of adulterated substances, counterfeits etc.

2-10 ARTIFICIAL NEURAL NETWORKS

2-10-1 Introduction

Artificial neural networks (ANNs) are general computational tools, whose initial development was inspired by the need for further understanding of biological neural networks and which have since been widely used in various areas that require data processing with computers or machines. The methods for building ANN models and their subsequent applications can be dramatically different depending on the architecture of the neural networks themselves. In the field of chemometrics, ANNs are generally used for multivariate calibration and unsupervised classification, which is achieved by using multi-layer feed-forward (MLFF) neural networks, or self-organising maps (SOM) respectively. As a multivariate calibration tool, ANNs are more generally associated with the mapping of non-linear relationships.

2-10-2 Principle

2-10-2-1 General

The basic data processing element in an artificial neural network is the artificial neuron, which can be understood as a mathematical function that uses the sum of a weighted vector and a bias as the input. The vector is the 'input' of the neuron and is obtained either directly from a sample in the

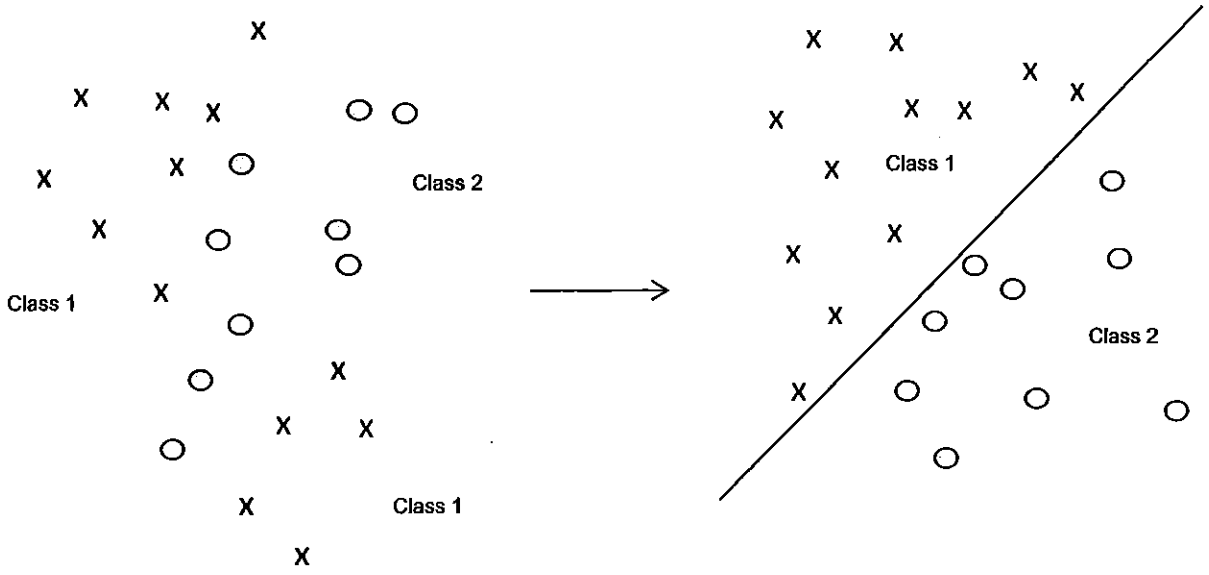


Figure 5.21.-11. – *The object space, where separation of the 2 classes is not possible, is mapped into a feature space where separation is possible*

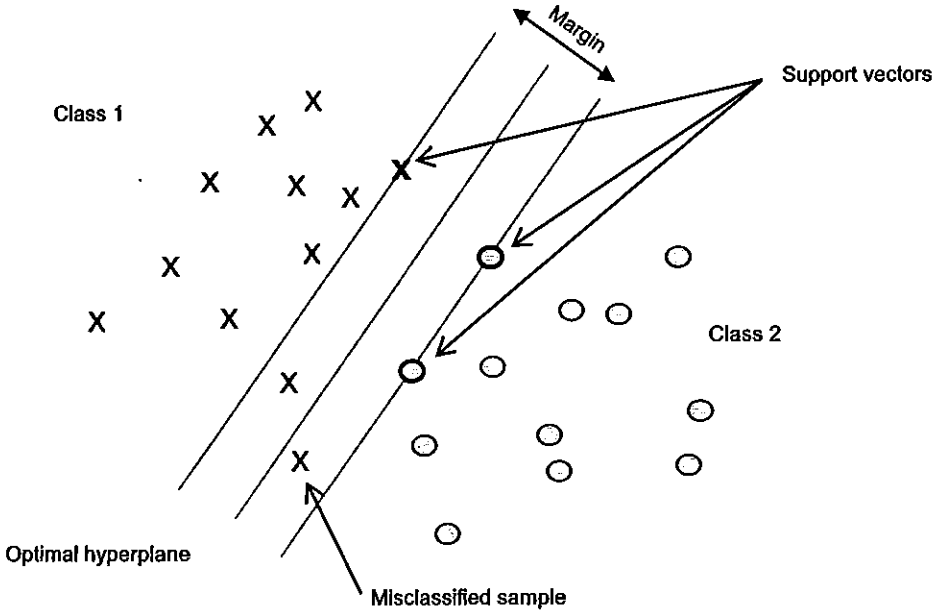


Figure 5.21.-12. – *In the feature space, the separation of classes 1 and 2 was achieved with toleration of certain misclassified samples*

data set or calculated from previous neurons. The user chooses the form of the function (called transfer function). The weights and bias are the coefficients of the ANN model and are determined through a learning process using known examples. An ANN often contains many neurons arranged in layers, where the neurons in each layer are arranged in parallel. They are connected to neurons in the preceding layer from which they receive inputs and also to neurons in the following layer where the outputs are sent (Figure 5.21.-13). The output of 1 neuron is therefore used as the input for neurons in the following layer. The input layer is a special layer that receives data directly from the user and sends this information directly to the next layer without applying a transfer function. The output layer is similar in that its output is also directly used as the model output without any additional processing. The unlimited possibilities when connecting different numbers and layers of neurons, is often called an ANN architecture, and provides the potential for ANNs to meet any complicated data modelling requirements.

2-10-2-2 Multi-layer feed-forward artificial neural network

A multi-layer feed-forward network (MLFF ANN) contains an input layer, an output layer and 1 or more layers of neurons in-between called hidden layers. Even though there is no limit on how many hidden layers may be included, an MLFF ANN with only 1 hidden layer is sufficiently capable of handling most multivariate calibration tasks in chemometrics. In an MLFF ANN, each neuron is fully connected to all the neurons in the neighbouring layers. A hyperbolic tangent sigmoid transfer function is usually used in MLFF ANN, but other transfer functions, including linear functions, can also be used.

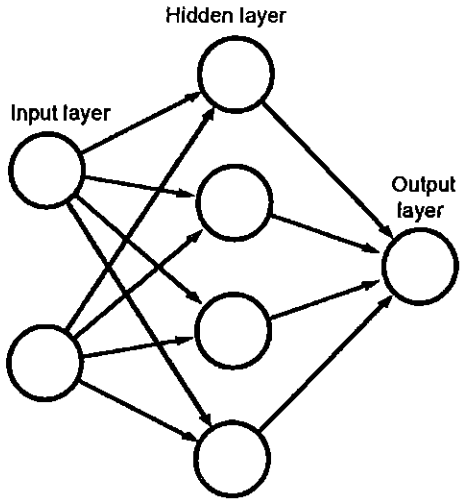


Figure 5.21.-13. - *Typical arrangements of neuron layers and their inter-connections*

The initial weights and biases can be set as small random numbers, but can also be initialised using other algorithms. The most popular training algorithm for determining the final weights and biases is the back-propagation (BP) algorithm or its related variants. In the BP algorithm, the prediction error, calculated as the difference between the ANN output and the actual value, is propagated backward to calculate the changes needed to adjust the weights and biases in order to minimise the prediction error.

An MLFF ANN must be optimised in order to achieve acceptable performance. This often involves a number of considerations including the number of layers, the number of neurons in each layer, transfer functions for each layer or neuron, initialisation of weights, learning rate, etc.

2-10-2-3 Self-organising map

The aim of the self-organising map (SOM) is to create a map where observations that are close to each other have more similar properties than more distant observations.

The neurons in the output layer are usually arranged in a two-dimensional map, where each neuron can be represented as a square or a hexagon. SOMs are trained using competitive learning that is different from the above described method using BP. The final trained SOM is represented as a two-dimensional map of the observations.

2-10-3 Critical aspects

The 2 most common pitfalls of using ANNs are over-training and under-training. Over-training means that an ANN model can predict the training set very well but ultimately fails to make good predictions. Under-training means that the ANN training ended too soon and therefore the resultant ANN model underperforms when making predictions. Both of these pitfalls should be avoided when using ANNs for calibration. A representative data set with a proper size, i.e. more observations or samples than variables, is required before a good ANN model can be trained. Generally, since the models are non-linear, more observations are needed than for a comparable data set subjected to linear modelling. As for other multivariate calibration methods, the input may need pre-processing to balance the relative influence of variables. One advantage of pre-processing is the reduction in the number of degrees of freedom of input to the ANN, for example by compression of the *X*-data to scores by PCA and then using the resulting scores for the observations as input.

2-10-4 Potential use

The advantage of MLFF ANN in multivariate calibration lies in its ability to model non-linear relationships. Since the neurons are fully connected, all the interactions between variables are automatically considered. It has been proven that a MLFF ANN with sufficient hidden neurons can map any complicated relationship between the inputs and outputs.

SOMs can be used to visualise high-dimensional data while preserving the topology in the original data. They are based on unsupervised learning, and are mainly useful as tools to explore features in data sets where no prior knowledge of the patterns and relationships of the samples exists.

The ANNs often have a large number of coefficients (weights and biases) that give the ANN the potential to model any complicated relationships in the data set but as a result, can also make the interpretation of the coefficients more difficult. However, when linear modelling methods are not flexible enough to provide the required prediction or classification accuracy, ANNs may be a good alternative.

3 GLOSSARY

β-distribution

Continuous probability distribution with density function

$$f(x;u,v) = \frac{1}{B(u,v)} x^{u-1}(1-x)^{v-1}$$

where $u > 0$, $v > 0$ are shape parameters (degrees of freedom) and B is the beta function,

$$B(u,v) = \int_0^1 t^{u-1}(1-t)^{v-1}dt$$

The γ-quantile of the $\beta(u,v)$-distribution is denoted by $\beta_{u,v,\gamma}$ and it is the value q such that the value of the distribution function F is γ:

$$F(x;u,v) = \int_0^q f(x;u,v)dx = \gamma$$

Bootstrapping

A number of sample sets of size n that is produced from an original sample set of the same size n by means of a random selection of samples with replacement.

Centring

A data set is mean centred by calculating the mean value of each variable and subtracting the variable mean values from each column of variables, in order to make the comparison and interpretation of the data easier.

Collinear/non-collinear

A family of vectors is collinear if at least 1 of the vectors can be represented as a linear combination of the other vectors. Hence a family of vectors is non-collinear if none of the vectors can be represented as a linear combination of the others.

Component (or factor, latent variable)

In chemometrics: underlying, non-observed, non-measured, hypothetical variable that contributes to the variance of a collection of measured variables. The variables are linear combinations of the factors and these factors are assumed to be uncorrelated with each other.

Data mining

Process of exploration, extraction and modelling of large collections of data in order to discover *a priori* unknown relationships or patterns.

Dependent variable
Also a response, regressand: a variable that is related by a formal (explicit) or empirical mathematical relationship to 1 or more other variables (typically the *Y*-data).

Empirical model
A data-driven model established without assuming an explicit mathematical relationship, or without a description of the behaviour of a system based on accepted laws of physics.

Exploratory data analysis
The process for uncovering unexpected or latent patterns in order to build future hypotheses.

Factor
See component.

Hotelling T^2 statistics
Multivariate version of the t-statistic. In general, this statistic can be used to test if the mean vector of a multivariate data set has a certain value or to compare the means of the variables. The T^2 statistic is also used for detection of outliers in multivariate data sets. A multivariate statistical test using the Hotelling T^2 statistic can be done. A confidence ellipse can be included in score plots to reveal points outside the ellipse as potential outliers.

Independent variable
Input variable on which other variables are dependent through a mathematical relationship (typically the *X*-data).

Indirect prediction
Process for estimating the value of a response on the basis of a multivariate model and observed data.

Interference
Effect of substances, physical phenomena or instrument artefacts, separate from the target analyte, that can be measured by the chosen analytical method. Then there is a risk of confusion between the analyte and interference if the interference is not varied independently or at least randomly in relation to the analyte.

Latent variable
See component.

Leave-one-out
In a 'leave-one-out' procedure only 1 sample at a time is removed from the data set in order to create a new data set.

Leave-subset-out
In a 'leave-subset-out' procedure a subset of samples is removed from the data set in order to create a new data set.

Leverage
A measure of how extreme a data point or a variable is compared to the majority. Points or variables with a high leverage are likely to have a large influence on the model.

Loadings
Loadings are estimated when information carried by several variables is focussed onto a few components. Each variable has a loading alongside each model component. The loadings show how well a variable is taken into account by the model components.

Orthogonal
2 vectors are orthogonal if their scalar product is 0.

Orthonormal vectors
Orthogonal and normalised (unit-length) vectors.

Outlier
For a numerical data set, it relates to a value statistically different from the rest. Also refers to the sample associated with that value. Specific statistical testing for outliers may be used.

Overfitting
For a model, overfitting is a tendency to describe too much of the variation in the data, so that in addition to the consistent underlying structure, some noise or non-informative variation is also taken into account and unreliable predictions will be obtained.

Property
See variable.

Resampling
The process of impartial rearrangement and sub-sampling of the original data set. This occurs during optimisation/validation procedures that repeatedly calculate a property and the error associated with it. Typical examples are cross-validation and bootstrapping, which create successive evaluation data sets by repeated sub-sampling.

Reselection
Reuse of samples (see resampling).

Residuals
A measure of the variation that is not taken into account by the model or a deviation between predicted and reference values.

Root mean square error of prediction
A function of the predictive residual sum of squares to estimate the accuracy:

$$RMSEP = \sqrt{\frac{\sum_{i=1}^{n}(y_i - \hat{y}_i)^2}{n}}$$

where $\hat{y}_i$ is the predicted response for the i^{th} sample of the test data set and y_i the observed response of the i^{th} sample, and n is the number of samples.

Sample
Object, observation, or individual from which data values are collected.

Sample attribute
Qualitative or quantitative property of the sample.

Sample selection
The process of drawing a subset or a collection from a population in order to estimate the properties of the population.

Scores or factor score coefficients
Coordinates of the samples in the new coordinate system defined by the principal components. Scores represent how samples are related to each other's given the measurement variables.

Score (normalised)
j^{th} score value $t_{i,j}$ of the i^{th} sample divided by the norm of the scores matrix:

$$|t_{i,j}| = \frac{t_{i,j}}{\sqrt{\sum_{j=1}^{p} t_{i,j}^2}}$$

where p is the number of parameters in the model.

Standard error of calibration
A function of the predictive residual sum of squares to estimate the accuracy considering the number of parameters:

$$SEC = \sqrt{\frac{\sum_{i=1}^{n}(y_i - \hat{y}_i)^2}{n - p}}$$

where n is the number of samples of the learning set, p the number of parameters in the model to be estimated by using the sample data, $\hat{y}_i$ the i^{th} fitted value in the calibration, and y_i the i^{th} reference value. In multiple regression with m variables $p = m + 1$ (1 coefficient for each of the m variables and 1 intercept).

Standard error of laboratory
Concerns to the intermediate precision or reproducibility, whichever is applicable.

Supervised
Refers to modelling data labelled by classes or values.

Unsupervised (non-supervised)
Refers to exploring data without prior assumptions.

Underfitting
The reverse of overfitting.

Variable
Property of a sample that can be assessed (attribute, descriptor, feature, property, characteristics).

Varimax rotation
Orthogonal analytical rotation of factors that maximises the variance of squared factor loadings, thereby increasing the large factor loadings and large eigenvalues and decreasing the small ones in each factor.

4 ABBREVIATIONS

ALS	alternating least squares
ANN	artificial neural network
BP	back-propagation
CLS	classical least squares
DB	density-based
DBSCAN	density-based spatial clustering of applications with noise
DoE	design of experiments
EFA	evolving factor analysis
EM	expectation maximisation
LDA	linear discriminant analysis
MCR	multivariate curve resolution
MLFF	multi-layer feed-forward
MLR	multiple linear regression
MSPC	multivariate statistical process control
NIR	near-infrared
PAT	process analytical technology
PC	principle component
PCA	principal components analysis
PCR	principal components regression
PLS	partial least squares regression
PLS-DA	partial least squares discriminant analysis
PLSR	partial least squares regression
QbD	quality by design
QDA	quadratic discriminant analysis
RMSEP	root mean square error of prediction
SEC	standard error of calibration
SEL	standard error of laboratory
SIMCA	soft independent modelling of class analogy
SMCR	self-modelling curve resolution
SNV	standard normal variate
SOM	self-organising map
STING	statistical information grid
SVM	support vector machine

S. Raw Materials of Biological Origin for the Production of Cell-based and Gene Therapy Medicinal Products

(Ph. Eur. general texts 5.2.12)

This general chapter is published for information.

It contains sections on the quality requirements of raw materials used for the production of cell-based and gene therapy medicinal products for human use. The provisions of the chapter do not exclude the use of different production and control methods. It is the responsibility of the manufacturer of a raw material to qualify (prove to be suitable for the intended use) the raw material in accordance with the requirements given in this general chapter.

However, it is ultimately the responsibility of the user of a raw material to ensure it is of suitable quality for the specific use.

The quality of the raw materials may be considered according to the stage of development of the cell-based or gene therapy medicinal product, thereby acknowledging the inherent evolution of the quality profile of the product during its pharmaceutical and clinical development. Nevertheless, patient safety needs to be ensured in early phase clinical development. The aim is to have an appropriate qualification strategy for the raw materials when used for the production of cell-based/gene therapy medicinal products. It should be noted that changes in raw materials during the lifecycle of the cell-based/gene therapy medicinal product may affect the quality of the medicinal product and thus require additional studies to demonstrate comparability.

The impact of the raw material on the quality, safety and efficacy of the cell-based/gene therapy medicinal product is evaluated using a risk-based approach. Raw materials are used in order to consistently yield an active substance or medicinal product of a specified quality in terms of, for example, biological activity, purity/impurity profile, the risk of adventitious agents (bacteria, viruses, etc.) and stability.

From a risk perspective, the use of raw materials free from human or animal substances is preferred.

The biological nature of a raw material used for the production of cell-based/gene therapy medicinal products places special requirements on its quality. Examples of the critical quality attributes specific to each class of raw material are given in this general chapter.

1 SCOPE

This general chapter applies to raw materials of biological origin used for the production of cell-based/gene therapy medicinal products. The raw materials used in the manufacture of active substances are not intended to form part of the active substance. The raw materials can be extracted from various biological sources or produced by recombinant DNA technology.

This general chapter applies to the following classes of raw materials:

- sera and serum replacements;
- proteins produced by recombinant DNA technology such as growth factors, cytokines, hormones, enzymes and monoclonal antibodies;
- proteins extracted from biological material such as enzymes and polyclonal antibodies;
- vectors.

The principles of this general chapter may also be applied to other classes of biological raw materials where appropriate.

Medical devices, plastics and chemically synthesised raw materials, such as basal media (purely composed of chemicals), synthetic peptides or synthetic polynucleotides, are not within the scope of this general chapter.

2 RISK ASSESSMENT

Evaluation of the impact of the raw material on the quality, safety and efficacy of cell-based/gene therapy medicinal products must be performed by the user of the raw material. No single measure or combination of measures can guarantee the quality, functionality and safety of a raw material for its intended use. Therefore, a risk assessment must consider the biological origin and traceability of the raw material, the production steps applied to it and the ability of the drug product manufacturing process to control or remove the raw material from the final medicinal product.

Any risk factor must be evaluated in relation to the clinical benefit/risk of the cell-based or gene therapy medicinal product. When evaluating the risk posed by the raw material to the final medicinal product, the exposure of a patient to residual amounts of raw material with potential harmful effects (e.g. adverse immune reactions) should be considered in relation to the clinical benefit/risk of the cell-based or gene therapy medicinal product.

3 GENERAL REQUIREMENTS

3-1 ORIGIN

The origin of the raw material and if relevant any biological substances used for the production of the raw material must be known. Special attention must be paid to risks related to the sourcing (including pooling) of the substances used for the production of the raw material. Depending on the source of the raw material and the substances used in its production, raw materials can be divided into 3 categories:

1) raw materials of human or animal origin;

2) raw materials produced using substances of human or animal origin;

3) raw materials free from substances of human or animal origin.

Traceability of all raw materials is required, with particular attention to those materials with an inherent safety concern i.e. those of human or animal origin.

Due to the inherent risk of transmitting adventitious agents, it is recommended to minimise, wherever possible, the use of raw materials of human or animal origin. If such raw materials are required for the production of cell-based/gene therapy medicinal products, appropriate measures are taken to minimise the risks of transmitting adventitious agents such as viruses, prions, bacteria and protozoa.

For human blood and tissue-derived materials, only carefully evaluated donors who have been adequately tested for infectious transmissible agents may be used. These materials comply with appropriate EU and/or national legislation applicable to transplantation and transfusion. Traceability measures enable each donation to be followed from the donation to the raw material and to the final product, and vice-versa.

When raw materials of animal origin are used, these animals fulfil specific health requirements and should be fit for human consumption and reared under controlled conditions, when applicable. If the origin of the animals is not fully traceable (e.g. animals collected from the wild), information on their geographic location at the time of sourcing should be considered.

When vectors or proteins produced by recombinant DNA technology are used as raw materials, traceability to the master cell bank/virus seed lot is required.

For all raw materials of human or animal origin, or raw materials produced using substances of human or animal origin, a viral risk assessment is performed according to the requirements of general chapter *5.1.7. Viral safety*. The extent of viral safety testing is dependent on the results of the initial risk assessment. In addition, a risk assessment with respect to transmissible spongiform encephalopathies is carried out and suitable measures are taken to minimise such risks as described in general chapter *5.2.8. Minimising the risk of transmitting animal spongiform encephalopathy agents via human and veterinary medicinal products*.

3-2 PRODUCTION

All raw materials are produced within a suitable quality management system and production facilities.

Suitable in-process controls are in place to ensure that the production process is under control and consistently produces raw materials of defined quality.

Quality attributes for raw materials include identity, purity and biological activity where applicable, and they are to be demonstrated using appropriate, qualified control methods. Relevant specifications in terms of identity, purity/impurity profile and assays are to be established.

The production process is optimised to consistently minimise and/or remove adventitious agents and harmful impurities, whilst retaining the quality of the raw material. This can be achieved using one or a combination of the following measures:

- using validated inactivation/removal procedures such as gamma sterilisation or low pH during chromatography, where possible;
- demonstrating the ability of a production process to minimise, remove or inactivate adventitious agents or harmful impurities;
- testing for adventitious agents or harmful impurities.

A raw material is sterile and produced under aseptic conditions and/or subject to terminal sterilisation, unless otherwise justified. If the raw material is not sterile, the level of microbial contamination must be known.

Additives, such as stabilisers, may be added to the raw material. In cases where antibiotics and stabilisers of biological origin are used in the production of the raw material, their presence is justified and careful consideration is given to their selection, use, quality and concentration in the raw material, as well as their impact on the actual raw material itself.

3-3 GENERAL QUALITY REQUIREMENTS

Raw materials must meet pre-defined quality requirements for identity, purity and biological activity. In order to ensure the function of the raw material, it is subject to testing using appropriately qualified methods. The identity test must reflect the uniqueness of the raw material and distinguish it from other related or similar substances. Impurities include both process-related substances (e.g. in the case of recombinant proteins: host-cell-derived proteins (HCP), host-cell-derived DNA and vector-derived DNA (residual DNA), other biological or chemical substances) and product-related substances (e.g. aggregates and degradation products).

The content of a raw material may be expressed either in absolute or relative terms. The assay for determination of biological activity may be used to establish the content.

3-3-1 IDENTIFICATION

The identity tests are specific for the particular raw material and address the molecular structure/composition or other relevant physico-chemical, biological or immunochemical properties. Methods used in the determination of biological activity and purity may also serve to identify the raw material. Identification may be carried out by comparison with a defined reference material or a representative batch of the raw material.

3-3-2 TESTS

Tests that may be applicable to raw materials include the following (see also the sections below for specific raw materials):

Appearance
Liquid or reconstituted freeze-dried raw materials comply with the limits defined for the particular raw material with regard to degree of opalescence (*2.2.1*) and degree of coloration (*2.2.2*).

Solubility
Freeze-dried raw materials dissolve completely in the prescribed volume of reconstituting liquid within a specified time, at a specified temperature, as defined for the particular raw material.

Osmolality (*2.2.35*)
Within the limits defined for the particular raw material.

pH (*2.2.3*)
Within the limits defined for the particular raw material.

Elemental impurities
Within the limits defined for the particular raw material.

Total protein (*2.5.33*)
Within the limits defined for the particular raw material.

Related substances
The content of product-related substances is within the limits defined for the particular raw material.

Microbiological control
Depending on the raw material concerned, it complies with the test for sterility (*2.6.1*) or the microbial contamination is determined (*2.6.12*).

Viral contaminants
Depending on the raw material concerned, relevant virus contamination is determined.

Bacterial endotoxins (*2.6.14*)
Less than the limit defined for the particular raw material.

Mycoplasmas (*2.6.7*)
Raw materials are free from mycoplasmas.

Stabiliser
Where applicable, it complies with the limits defined for the particular raw material.

Water (*2.5.12*)
Freeze-dried raw materials comply with the limits defined for the particular raw material.

3-3-3 ASSAY

Content
The content (e.g. protein content)/composition of the raw material is determined by an appropriate qualified method.

Biological activity
Where relevant, the biological activity is determined by a suitable assay. Where relevant (e.g. for enzymes), the biological activity is expressed per milligram of total protein (specific activity).

3-3-4 REFERENCE MATERIAL OR REFERENCE BATCH

An appropriate reference material or a representative batch of the raw material is used to perform the above-mentioned identification, tests and assay. Where available, the use of established reference standards, such as European Pharmacopoeia reference standards or WHO International Standards, is recommended.

3-4 STORAGE

The shelf life and storage conditions are defined.

3-5 LABELLING

The label states the expiry date, conditions for storage and use and any code that may be required for traceability including the biological origin of the raw material.

4 SERA AND SERUM REPLACEMENTS

4-1 DEFINITION

Sera from human or animal sources and serum replacements (including platelet lysates and other undefined growth additives, conditioned media, blood and other cellular components) are used as growth additives for cell culture. Sera and serum replacements used to promote cellular growth are typically complex biological mixtures, whose exact composition is not always possible to define. Due to this complex nature, special attention is given to verifying the consistency and performance of every batch.

Bovine serum
If bovine serum is used, it complies with the monograph *Bovine Serum (2262)*.

Human serum and platelet lysates
Human serum and platelet lysates used as raw materials for the production of cell-based/gene therapy medicinal products are human blood-derived materials, which can originate from the recipient (autologous) or from another individual (allogeneic).

Conditioned media
Conditioned media, isolated and purified from cultured cell supernatant, may also be used to enhance cell proliferation due to various growth factors and cytokines secreted by the cells into the medium.

Other growth additives with undefined composition
Cell and/or tissue lysates may be used as growth additives.

Composite media
Composite media contain growth additives such as bovine serum, growth factors etc. The principles described in this section of the general chapter apply to individual ingredients of biological origin and/or biologically active ingredients of the composite media.

4-2 PRODUCTION

Due to potential differences in quality between batches of serum, cell or tissue lysate, suitable measures are implemented to verify the consistency of each batch before using them as raw materials for the production of cell-based/gene therapy medicinal products.

Because of the inherent risk of transmitting infectious agents from pooled plasma, pooled sera, or other derivatives from pooled allogeneic human blood or plasma, consideration is given to limit the number of donations which are pooled, unless sufficient methods for inactivation/removal of viruses are applied during production, where applicable.

For conditioned media, a cell bank system is preferred. The removal of the cells from the media must be ensured and potential impurities originating from these cells determined if possible.

4-3 IDENTIFICATION

It is recognised that the exact qualitative composition of sera and serum replacements may be difficult to determine. However, the approximate protein composition in both cases may be determined by, for example, protein electrophoresis. Where relevant, tests for total protein content or any chemical additives are performed. For human serum, the electrophoretic pattern corresponds to that of an appropriate serum reference batch. Alternatively, identity may be determined by comparison of albumin content with an appropriate serum reference batch. For serum replacements,

the electrophoretic pattern or the use of markers secreted by cells/platelets may be used. Human origin is determined by a suitable immunochemical method (*2.7.1*), unless otherwise justified.

4-4 TESTS

See section 3-3-2.

Haemoglobin

Where relevant, within the limits defined for the particular raw material.

Cell-derived impurities

Where relevant, within the limits defined for the particular raw material.

Specific tests for viral contaminants

For bovine serum, the tests for viral contaminants specified in the monograph *Bovine serum (2262)* apply. For human serum, the tests for viral safety specified in the monograph *Human plasma for fractionation (0853)* apply.

4-5 ASSAY

The serum or serum replacement must show cell growth promoting properties that are within the limits defined for the particular raw material. More than one type of assay may be necessary to show suitability for the intended use.

5 PROTEINS PRODUCED BY RECOMBINANT DNA TECHNOLOGY

5-1 DEFINITION

Proteins and peptides produced by recombinant DNA technology, which are used as raw materials, include growth factors, cytokines, hormones, enzymes and monoclonal antibodies.

Growth factors, cytokines and hormones They are substances typically used for stimulation or inactivation, growth promotion or differentiation of cells in cell culture systems.

Other proteins Enzymes (e.g. collagenases), as raw materials, may be used for extraction of active substances from tissues and/or fluids. Other proteins (e.g. fibronectin) may be used as culture supports or media components.

Monoclonal antibodies Used as raw materials, they include immunoglobulins and fragments of an immunoglobulin with defined specificity. Antibodies can either be conjugated (chemically modified) or non-conjugated. Typical chemical modifications include fluorescent labelling and conjugation to magnetic beads. Antibodies, as raw materials, may be used for selection, activation/stimulation, isolation or purification of cells in cell culture.

5-2 PRODUCTION

Production of proteins using recombinant DNA technology is based on a well-characterised host-vector system, using a master cell bank and, if applicable, a working cell bank derived from the master cell bank. The expressed protein is extracted and purified using a variety of techniques, such as extraction, precipitation, centrifugation, concentration, filtration and/or chromatography.

During protein production using recombinant DNA technology, process-related impurities including residual host-cell or vector DNA and host-cell proteins must be reduced to acceptable levels. Particular attention must also be given to product-related impurities.

5-3 IDENTIFICATION

Identity is established by appropriate, qualified methods, such as electrophoresis (*2.2.31*), peptide mapping (*2.2.55*), isoelectric focusing (*2.2.54*) or liquid chromatography (*2.2.29*). For antibodies, identification is based on immunoglobulin class, isotype and/or specificity. In addition to the above-mentioned methods, immunochemical methods (*2.7.1*) and determination of activity are also considered suitable for identification.

5-4 TESTS

See section 3-3-2.

Host-cell-derived proteins and residual host-cell or vector DNA

Where relevant for the particular raw material, the content of residual host-cell or vector DNA and/or protein is determined using a suitable method unless the production process has been qualified to demonstrate suitable clearance. The content is within the limits defined for the particular raw material.

Related proteins

Related proteins (e.g. polyclonal antibodies with undefined specificities, glycoforms, degradation and oxidation products, oligomers and aggregates) are determined using liquid chromatography, electrophoretic or immunological methods and are within the limits defined for the particular raw material.

5-5 ASSAY

Content

The protein content is determined by an appropriate qualified method, for example by liquid chromatography (*2.2.29*) or UV spectrophotometry (*2.2.25*).

Biological activity

The biological activity of a recombinant protein is determined using, for example, cell proliferation, cell differentiation or an enzyme assay. Several acceptable bioassays may exist for a particular protein. For antibodies, cell-based immunoassays and assays based on ligand-binding and affinity may be used.

Where relevant, the biological activity is expressed per milligram of total protein (specific activity).

6 PROTEINS EXTRACTED FROM BIOLOGICAL MATERIAL

6-1 DEFINITION

Proteins extracted from biological material and used as raw materials include enzymes (e.g. porcine - derived trypsin and endonucleases), polyclonal antibodies, other proteins of biological origin (e.g. albumin and transferrin) and peptides of biological origin. They may be of human, animal, plant or microbiological origin.

Proteins extracted from biological material are used in a wide range of applications such as growth promotion, differentiation or purification of cultured cells and extraction of active substances from tissues and/or fluids.

6-2 PRODUCTION

Proteins are extracted from the blood or tissue of animals or humans, or from plant or microbiological sources using mechanical and/or chemical techniques. They are then subjected to further purification processes using a variety of techniques such as centrifugation, filtration, chromatography and concentration.

Polyclonal antibodies are produced by immunisation with a specific antigen, followed by purification. Antibody purification involves selective enrichment or specific isolation of antibodies from serum based on physico-chemical fractionation, class-specific affinity and/or antigen-specific affinity.

During production of these proteins, process-related impurities, such as blood components, tissue fragments or contaminating proteins, must be reduced to acceptable levels. Particular attention is given to product-related impurities.

6-3 IDENTIFICATION

Identity is established by appropriate, qualified methods, such as electrophoresis (*2.2.31*), isoelectric focusing (*2.2.54*) peptide mapping (*2.2.55*), liquid chromatography (*2.2.29*) and immunochemical methods (*2.7.1*).

6-4 TESTS

See section 3-3-2.

Process-related impurities

Substances derived from the starting material (e.g. blood components, tissue fragments or contaminating proteins) are determined using suitable methods and are within the limits defined for the particular raw material.

Related proteins

Related proteins (e.g. antibodies with undefined specificity, degradation and oxidation products, oligomers and aggregates) are determined using suitable methods and are within the limits defined for the particular raw material.

6-5 ASSAY

Content

The protein content is determined using an appropriate qualified method, for example by liquid chromatography (*2.2.29*) or UV spectrophotometry (*2.2.25*).

Biological activity

Where relevant, the biological activity of a protein is determined using, for example, enzyme assays, immunoassays or assays based on cell proliferation/differentiation.
For trypsin, the assay may be performed as described in the monograph *Trypsin (0694)*.

Where relevant, the biological activity is expressed per milligram of total protein (specific activity).

7 VECTORS

Vectors that may be used as raw materials in the production of cell-based and gene therapy medicinal products include DNA vectors (e.g. plasmids, transposon vectors) as well as viral vectors and bacteria (e.g. modified *Lactococcus* species). Vectors are usually considered as starting materials, thus not under the scope of this general chapter. In cases where vectors are not considered as starting materials, such as vectors used as helper plasmids or helper viruses, the principles of this general chapter and the principles of production and quality control as outlined in general chapter *5.14.Gene transfer medicinal products for human use* are to be followed.

T. Depyrogenation of Items used in the Production of Parenteral Preparations

(Ph. Eur. general text 5.1.12)

This general chapter covers pyrogen inactivation or removal from items (primary packaging materials and equipment) coming into direct contact with the final sterilised product.

Pyrogens are substances that have the ability to induce fever when infused or injected into the body.

In this general chapter, depyrogenation is defined in terms of a reduction in lipopolysaccharide (endotoxin), the most potent and difficult-to-eliminate of pyrogenic materials.

1. DEPYROGENATION PROCESSES

1-1 DRY HEAT TREATMENT

1-1-1 Treatment conditions

Dry heat treatment is the most common depyrogenation process for glassware and other non-heat-labile materials.

A common dry heat depyrogenation cycle consists of subjecting the items to a temperature of at least 250 °C for at least 30 min; other validated combinations of temperature and time may be used, but the minimum combination required is 180 °C for 3 h.

1-1-2 Equipment

Dry heat treatment is carried out in an oven or tunnel with forced air circulation or using other equipment specifically designed for this purpose.

As a minimum, relevant dry heat depyrogenation parameters (e.g. time, temperature and, where applicable, belt speed) are monitored at the most difficult-to-heat position in the chamber, which is defined during the qualification study on the equipment.

1-1-3 Validation

All processes must be validated by adding suitable endotoxin indicators to the load at the positions identified as being the most difficult to heat (and therefore to depyrogenate) in order to verify the efficacy of depyrogenation.

1-2 OTHER PROCESSES

Other processes may be used only when heat treatment is not possible.

1-2-1 Physical treatment

Depyrogenation may also be achieved by rinsing the items with an appropriate pharmaceutical-grade water such as water for injections (WFI), with or without additional physical means.

Items must be dried following cleaning.

1-2-2 Chemical treatment

Chemical reactions using strong oxidative, alkylating or reducing agents, either liquids or vapour-phase gases, with or without heat, capable of removing pyrogens, may be applied and followed by final rinses with WFI. At the end of the treatment a verification test is carried out to detect any chemical residues.

1-2-3 Validation

All processes must be validated by adding suitable endotoxin indicators to the load at the positions identified as being the most difficult to depyrogenate in order to verify the efficacy of depyrogenation.

2. CHARACTERISTICS OF ENDOTOXIN INDICATORS

Endotoxin indicators consist of purified bacterial endotoxins (lipopolysaccharides). The following are required to be known by the user:

- name of the producer;
- composition of the indicator system if applicable;
- genus and species of the micro-organism (including the culture collection number if available) from which the endotoxin is isolated;
- specific activity of the endotoxin, in International Units per gram, per millilitre or per container;
- storage conditions and expiry date;
- batch number.

Depending on the nature of the item to be depyrogenated (e.g. glass, stainless steel, plastic), a suitable endotoxin indicator (ready-to-use or custom-made) is used during validation of a depyrogenation process.

2-1 READY-TO-USE ENDOTOXIN INDICATORS
These indicators are ampoules, vials or other carriers that have been inoculated with a known amount of endotoxin and are used to measure endotoxin reduction or inactivation in a depyrogenation process.

The type of carrier used must be representative of the item that is being depyrogenated as this may influence the effectiveness of the depyrogenation process.

2-2 CUSTOM-MADE ENDOTOXIN INDICATORS
Custom-made endotoxin indicators are items (e.g. primary packaging materials or equipment) that have been surface-inoculated with a suitable endotoxin solution (e.g. endotoxin reference standard). Once dried, the inoculated item is used to measure endotoxin reduction or inactivation in a depyrogenation process.

3. TEST PROCEDURE

3-1 RECOVERY TEST PROCEDURE OF ENDOTOXIN FROM ENDOTOXIN INDICATORS
The endotoxin recovery rate is determined on items (ready to use or custom made indicators) that are not subjected to the depyrogenation process in order to calculate the percentage recovery and to check any interference from leachables before assessing the effectiveness of a depyrogenation process.

The endotoxin recovery test must be validated.

If the endotoxin recovery rate is insufficient to demonstrate a 3.0 $\log_{10}$ reduction, the amount of endotoxin used must be adjusted accordingly.

3-2 ENDOTOXIN REDUCTION TEST PROCEDURE
Items that have been artificially contaminated with a known amount of endotoxin or ready-to-use endotoxin indicators are placed in the load to be tested. Endotoxin levels are assessed before (initial endotoxin level) and after (residual endotoxin level) the depyrogenation process according to the same validated method. The level of endotoxin per indicator and the number of indicators used for the test must be sufficiently high to allow accurate determination of the reduction in endotoxin due to the depyrogenation process.

4. CRITERIA
Not less than a 3.0 $\log_{10}$ reduction in recoverable endotoxin is used as the criterion for process validation. The $\log_{10}$ reduction is calculated by determining the measured $\log_{10}$ of recoverable endotoxin in the unprocessed item minus the measured $\log_{10}$ of recoverable endotoxin remaining in the processed item.

U. Multivariate Statistical Process Control

(Ph. Eur. general text 5.28)

The following chapter is published for information only. It is an introduction to the use of multivariate statistical process control (MSPC) for monitoring and controlling manufacturing processes. The objective is to provide guidance on good practice. This chapter complements general chapter 5.21. Chemometric methods applied to analytical data.

1 INTRODUCTION
In pharmaceutical manufacturing, statistical process control (SPC) is an established tool to control and improve process performance and to reduce the variation of key parameters. These parameters may be either critical performance characteristics of the process or critical quality attributes (CQA) of the product, which are referred to in the rest of the text as 'process variables' and 'product variables' respectively. SPC is implemented by means of control charts that show the changes in the values of these variables, measured from samples or directly from the process and plotted against sample number or time.

SPC applied to a single or a few independent variables involves a univariate approach (each variable is analysed separately). However, process performance usually depends on many parameters. Moreover, process analytical technology (PAT) now makes it possible to measure directly many variables at high frequency. Hence, the input measurements for SPC are not necessarily simple scalar values (such as temperature or pH), but might also be more complex (such as spectra or analytical data from several sources). Since the set of all measurements at a certain point in time characterises one state of the process, interdependencies among the many measurements are expected and hence the measurement variables may be correlated.

Multivariate statistical process control (MSPC) can be defined as the application of multivariate statistical techniques in order to analyse complex process data with potentially correlated variables. MSPC in combination with automated data collection and analysis may be used to generate control charts based on a multivariate (chemometric) model. These control charts can be used to monitor a process or adjust it. MSPC in combination with a high degree of automation may facilitate continuous manufacturing (CM) as well as real-time release testing (RTRT). It can be combined with PAT (see general chapter *5.25. Process analytical technology*), quality by design (QbD) and design of experiments (DoE), in line with relevant ICH guidelines.

Many different activities in pharmaceutical manufacturing may rely on MSPC, such as:
— process development;
— monitoring of production operations;
— process optimisation, lifecycle management;
— trouble shooting, fault detection, root cause analysis;
— improvement of process understanding.

2 STATISTICAL PROCESS CONTROL (SPC)
Although univariate SPC is not the subject of this chapter, aspects of SPC that are required to understand MSPC are described below.

2-1 DEFINITION
Univariate SPC uses measurements of a variable over time. These measurements may be performed off-line, in-line, at-line, or on-line (see definitions in general chapter *5.25*). The aim is to characterise the time course of the variable when the process is operating normally, which is referred to as the in-control state. With this information, the process can be monitored to ensure that the distribution of the monitored variable does not change as the process runs. If the variable distribution stays constant, the process is said to be in statistical control.

When the process is developed or significantly changed, the variable distribution is not yet known, and process data therefore have to be gathered to develop a model (phase I). In a retrospective analysis of these data, using trial control charts, it is determined if the process was in control when the data were collected. If the process was in control, the data can be used to construct the final control charts. If not, the trial control charts are used to investigate atypical results and outlying data points (root cause analysis) to bring the process under control. Once the final control charts have been

constructed with in-control process data (i.e. a clean set), phase I ends and the monitoring phase starts (phase II). During this phase, control charts are used prospectively to check whether the process is in control or whether action is necessary to keep it in control.

2-2 DEVELOPMENT OF CONTROL CHARTS

Control charts are constructed by collecting a set of samples of n consecutive measurements (rational subgroups). For a control chart of means ($\bar{x}$), here taken as an example, the averages $\bar{x}_i$ ($i = 1, \ldots, m$) of each of the m subgroups are computed next. Averages of sufficiently large subgroups likely follow an approximately normal distribution, which facilitates the setting of the control limits. The averages $\bar{x}_i$ are then plotted against sample number i or time t_i. The $\bar{x}$-control chart consists of a baseline (or centre line) $\bar{\bar{x}}$ equal to the grand mean ($\bar{\bar{x}}$) value of the variable (e.g. the mean content of the product) when the process is in statistical control. There are 2 additional lines, one above and one below the centre line, called the upper control limit (UCL) and the lower control limit (LCL).

The control limits are set so that the average of a rational subgroup $\bar{x}$ has a very low probability of falling outside them if the process is in control. If an average falls outside the limits, this is an indication that the distribution of the variable may have changed, in which case some kind of action is necessary to bring the process back under control.

The control limits (CL) depend on the variable's standard deviation and the size of the subgroups as follows:

$$CL = \bar{\bar{x}} \pm L \times \frac{\hat{\sigma}}{\sqrt{n}}$$

$\bar{\bar{x}}$ = grand mean, the mean of all the subgroup averages $\bar{x}_i$ in the clean data set from phase I;
$\hat{\sigma}$ = pooled standard deviation of the single measurements making up the subgroups;
n = size of the subgroups (constant sample size assumed here);
L = factor derived from the normal distribution that controls the risk of a false alarm.

In this expression, the quotient $\frac{\hat{\sigma}}{\sqrt{n}}$ is the standard deviation of the mean ($\bar{x}$), also referred to as the standard error. The control limits are set at $\pm$ L times this standard deviation above and below the centre line. The choice of L influences the risk of a false alarm when the process is in control (false positive, error of the first kind). With $L = 3$, the risk of false alarms is very low (0.27 per cent) if the averages $\bar{x}_i$ actually follow a normal distribution.

The subgroup size n influences the risk of not detecting a mean shift in the process (false negative). The sampling frequency is also important since the time interval from the occurrence of an event (i.e. an out-of-control signal) to its detection depends on it.

The procedure described above using the $\bar{x}$-control chart as an example is also applicable to other types of control charts of various statistical parameters. Control charts developed according to these principles are often called Shewhart control charts. However, many different extensions and more-complex univariate control charts also exist, for example control charts for dispersion measures such as the standard deviation.

3 MULTIVARIATE STATISTICAL PROCESS CONTROL (MSPC)

3-1 INTRODUCTION

A multivariate approach is necessary if several potentially correlated variables need to be analysed simultaneously. Theoretically, multivariate (high-dimensional) data could be analysed by setting up a univariate control chart for each variable. This approach is in principle correct if all measurement variables are uncorrelated. However, this is rarely the case. If some variables are correlated, the use of many univariate control charts instead of one multivariate chart increases the risk of not detecting an out-of-control state (see Figure 5.28.-1). This is because the use of several univariate control charts can only detect points outside the whole data space, while correlated variables vary only within a certain subspace of the whole data space spanned by the ranges of all individual variables. MSPC makes it possible to find this subspace and identifies values that are outside this subspace, i.e. samples that do not follow the general correlation pattern (see section 4. Theoretical background). MSPC is therefore more sensitive for out-of-control signals and is a better diagnostic tool in such situations.

3-2 DEFINITION

MSPC uses statistical procedures to monitor simultaneously many interrelated variables, combined in a measurement vector (see section 4. Theoretical background). These variables may form a set of variables with a high level of correlation (e.g. a spectrum), but some variables may also be mutually uncorrelated. Importantly, the relationships (correlations) existing between the different variables are exploited in MSPC. Control charts based on multivariate measurements follow the same principles as Shewhart control charts. The main difference is that a summary statistic considering the entire measurement vector is plotted against time or sample number. That way, the multivariate input data are transformed into a control statistic that can be monitored much like the single parameter in a univariate control chart. For instance, mean changes in a process can be monitored in a chart by computing the distance between measurement vectors for newly acquired data and the in-control mean vector. Hotelling's T^2 and Q statistics (squared prediction error or squared residuals) are the most widely used distance measurements. Hotelling's T^2 takes the deviation of all measurement variables from their process mean into account, with each deviation weighted according to the natural variation of that variable and its relationship to all other variables. Q statistics can be used to complement T^2 since it corresponds to the deviations that T^2 does not model. By taking the relationships between variables into account, unusual patterns in the deviations can be detected, which result in large distances from the in-control mean.

The information needed is obtained from the inverse of the sample covariance matrix, where the latter needs to be estimated from in-control process data. If each entry in the multivariate measurement vector is normally distributed, and if each measurement vector is independent of those taken at other time points (or with other samples), the natural variability of T^2 for a process in statistical control follows a known distribution (see section 4. Theoretical background), which can be used to derive the UCL. If T^2 is larger than the UCL, the process is thought to be out-of-control. Since the smallest conceivable value of T^2 is zero (i.e. the measurement vector and the process mean vector coincide), which is considered the ideal case, there is no LCL when using T^2.

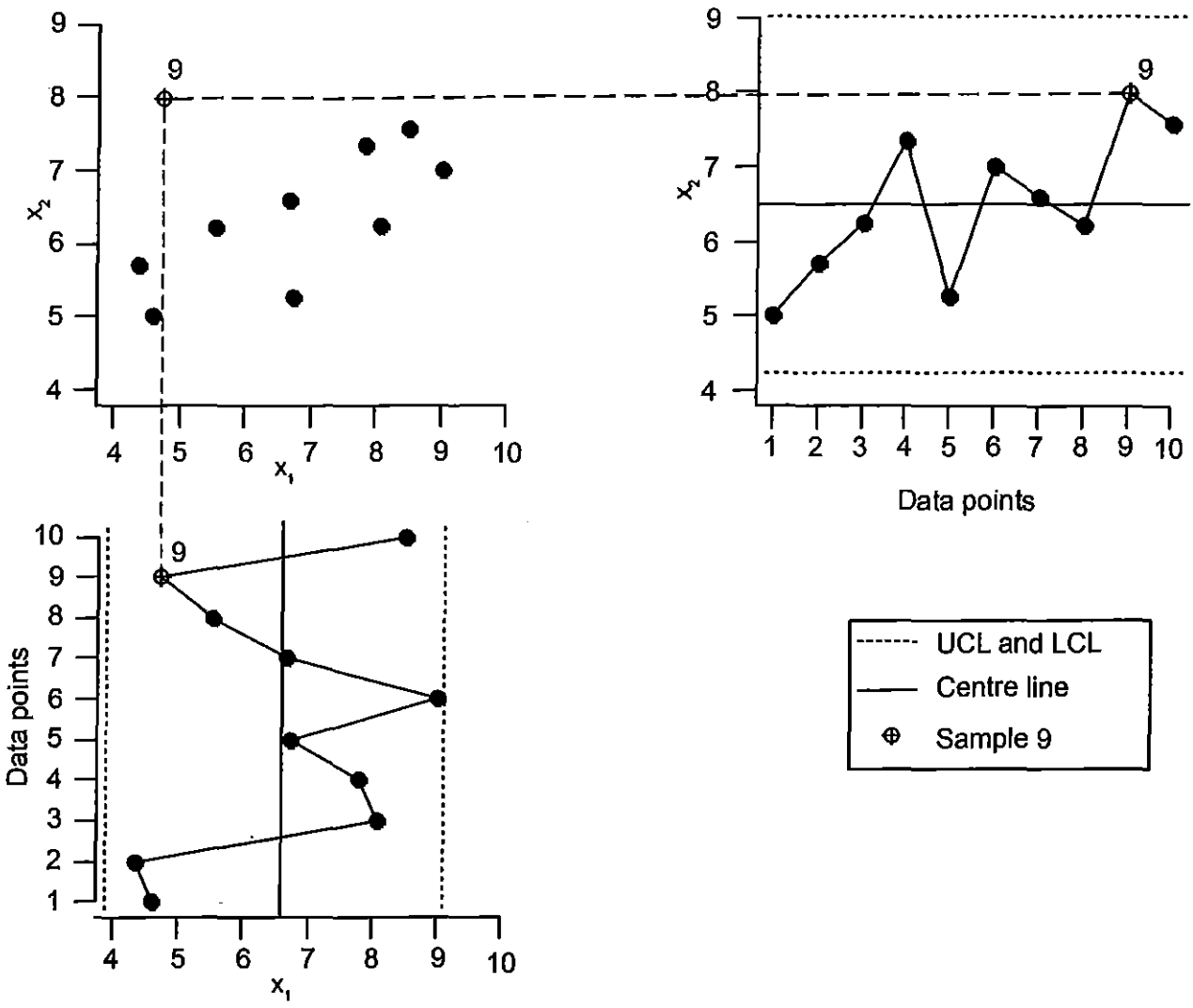


Figure 5.28.-1. – *Univariate and multivariate graphical representations of x_1 and x_2. While sample 9 is inconspicuous (i.e. in-control) in the univariate control charts, sample 9 is noticeable as an anomaly in the multivariate space*

Optimal application of MSPC may require a preliminary evaluation of variables. If all variables cannot be considered as equally important, weighting can be applied to variables. Some variables might be of less importance and at the same time trigger unnecessary signals that might increase the risk of a false alarm. Furthermore, variables can fall into different groups, e.g. raw material quality parameters or parameters associated with different steps of the process. Rather than merely grouping all variables into a single analysis, a separate T^2 plot for each of the distinct groups of variables might be more efficient.

MSPC is applicable to continuous as well as batch processes. In both cases, the production process is monitored over time using T^2 and Q statistics employing the specific control limits for the different stages of production. These limits may be established based on the variability of a set of batches or can be based on the variability of a number of samples within a certain time frame of a continuous process.

The computations for batch data are not described in this chapter.

3-3 DEVELOPMENT OF MULTIVARIATE CONTROL CHARTS

Method development of multivariate control charts is also subdivided into phases I and II. Once again, phase I includes planning, development and estimation of the model. Preliminary data are collected under proper operational conditions and examined for statistical control and potential problems. Due to the high number of variables, preliminary data analysis is far more extensive than in the univariate case. Attention should be paid to data-collection procedures, potential data-transmission errors, theoretical relationships between the variables, necessary pre-processing of the variables, potential for and handling of missing data, detection of statistical outliers, handling of variable correlations, and the potential for autocorrelated errors, i.e. time-dependent, slowly varying error sources, which would cause a violation of the aforementioned independence assumption. Once statistical control for the preliminary data is established, they can be used to estimate the necessary model parameters. In particular, computing T^2 requires estimating and inverting the covariance matrix for this sample of data (to obtain the weighting factors, see above). Estimating the many parameters of a covariance matrix requires a large enough sample of in-control process data

from phase I. Sample size particularly matters if process-dispersion control charts are also to be constructed. These charts display either the determinant (the so-called generalised variance) or the trace of the covariance matrix and require good estimates of the sample covariance matrix to be useful. With highly correlated measurement data, inverting the covariance matrix may not be possible or may be numerically unstable. In such cases, the raw measurement data are first reduced in dimensionality by principal component analysis (PCA) or partial least squares regression (PLS) (see general chapter *5.21*). The raw data are then replaced by PCA or PLS scores after selection of the optimal number of principal components or PLS factors. This reduction step is the rule rather than the exception for high-dimensional measurement data and enables the computation of control statistics such as T^2 even for highly correlated data.

Once control chart development is finished, the chart can be used in phase II for process monitoring. Here, a control statistic such as T^2 or Q is computed for newly acquired measurements to determine if the process is in control. It is plotted against time or sample number and compared with its UCL. It should be noted that the value of the control statistic depends on the entire measurement vector. When an out-of-control situation occurs, the measurement vector is very different from the mean vector. To find out which variable(s) caused the out-of-control signal, the contribution of each variable to T^2 or Q is computed. Variables with large contributions are examined preferentially during root cause analysis (see section 4. Theoretical background).

3-4 MAINTENANCE

Model maintenance ensures ongoing performance such as maintaining a low risk of false alarms and false negatives. Changes are required if the correlation structure or the mean of some variables slowly change during long-term periods without the control statistic falling outside the control limits. Maintenance encompasses updating model parameters and control limits. In the case of T^2 charts, this amounts to re-estimating the sample covariance matrix and adapting the control limits to the sample size of the new phase I. Maintaining the model can be done by periodically rebuilding the model off-line or by automatically updating it. Setting the parameters for automatic methods is critical and requires a deep understanding of the process, since automatic updates may mask real trends in the process. Maintenance may also be necessary if process conditions or raw materials have changed or if instruments have been exchanged or recalibrated.

4 THEORETICAL BACKGROUND

4-1 BASIC PRINCIPLES OF MULTIVARIATE CONTROL CHARTS

MSPC uses multivariate statistical procedures, the basic principles of which are given in general chapter *5.21. Chemometric methods applied to analytical data*. Key techniques for MSPC are the latent variable methods PCA (general chapter *5.21*, section 2-1) and PLS (general chapter *5.21*, section 2-8), which reduce the dimensionality of the raw input data by projecting them onto a lower dimensional subspace. This dimension reduction is possible whenever the raw input data are correlated, which is almost always the case. The signals monitored in the low dimensional space are referred to as latent variables and can be thought of as source signals of the process. Since the number of latent variables is rarely known *a priori*, another key technique for MSPC is the data-driven choice of the number of latent variables, which is commonly accomplished by resampling techniques such as cross-validation (general chapter *5.21*, section 1-3-2-1).

4-1-1 Hotelling's T^2

Let $\mathbf{X}$ be an $n \times m$ matrix that holds, for example, m process variables measured at n different time points. Define the vector $\bar{\mathbf{x}}$ as the column means of $\mathbf{X}$:

$$\bar{\mathbf{x}} = (\mathbf{X}^T \mathbf{1}_n) / n$$

and let $\mathbf{X}_c$ be the column mean-centred measurement matrix:

$$\mathbf{X}_c = \mathbf{X} - \mathbf{1}_n \bar{\mathbf{x}}^T$$

The estimator of the sample covariance $\mathbf{C}$ is then given by:

$$\mathbf{C} = \frac{1}{n-1}(\mathbf{X}_c^T \mathbf{X}_c)$$

An important multivariate control chart is based on T^2:

$$T^2 = (\mathbf{x}_o - \bar{\mathbf{x}})^T \mathbf{C}^{-1} (\mathbf{x}_o - \bar{\mathbf{x}}) = \mathbf{x}_{c,o}^T \mathbf{C}^{-1} \mathbf{x}_{c,o}$$

where $\mathbf{x}_o$ is an $m \times 1$ vector of raw measurements measured at a new time point o and $\mathbf{x}_{c,o}$ is the corresponding mean-centred measurement vector. T^2 represents the squared Mahalanobis distance of the new measurement vector $\mathbf{x}_o$ from the process mean vector $\bar{\mathbf{x}}$. T^2 can only be used directly on the raw measurements for a few uncorrelated variables. If the dimension m becomes large and the measurement variables are correlated, matrix $\mathbf{C}$ becomes ill-conditioned or singular and can no longer be inverted. If this happens, the raw variables are replaced by latent variables. With PCA, the raw data are replaced by the scores $\mathbf{T}$ or $\mathbf{t}_o$:

$$\mathbf{T} = \mathbf{X}_c \mathbf{P}$$

$$\mathbf{t}_o = (\mathbf{x}_{c,o}^T \mathbf{P})^T = \mathbf{P}^T \mathbf{x}_{c,o}$$

Here, the score matrix $\mathbf{T}$ and the score vector $\mathbf{t}_o$ are of dimension $n \times q$ and $q \times 1$ respectively ($q < m$), and the principal component loadings matrix $\mathbf{P}$ is of dimension $m \times q$. The number of latent variables (q) is a hyperparameter of the model and needs to be estimated, e.g. by cross-validation. Postmultiplication of the raw process data $\mathbf{X}_c$ (or $\mathbf{x}_{c,o}^T$) by the orthonormal matrix $\mathbf{P}$ projects them into a lower dimensional subspace where the main process characteristics can be monitored. $\mathbf{P}$ holds the first q eigenvectors of the sample covariance matrix $\mathbf{C}$. It is chosen to capture all relevant information of the process (i.e. common cause variation) while omitting noise. With the substitution of the raw process variables by the respective principal component scores, T^2 in the process-relevant subspace becomes:

$$T^2 = \mathbf{t}_o^T \Lambda^{-1} \mathbf{t}_o = \sum_{i-1}^{q} \frac{t_{o,i}^2}{\lambda_i}$$

where Λ is the $q \times q$ diagonal matrix of eigenvalues of the sample covariance matrix $\mathbf{C}$ and λ_i is the i^{th} diagonal element of Λ. In principal component space, T^2 is defined in terms of the eigenvalues and eigenvectors of the sample covariance matrix $\mathbf{C}$. Here, λ_i reflects the variance of the i^{th} score vector $\mathbf{T}_i$:

$$\lambda_i = \frac{1}{n-1}(\mathbf{T}_i^T \mathbf{T}_i)$$

where $\mathbf{T}_i$ is the i^{th} column of matrix $\mathbf{T}$. In general, for T^2 to be properly scaled in latent variable space, λ_i has to reflect the variance of the i^{th} score vector. This scaling is important for the transition from PCA to PLS in order to keep the

distributional properties of T^2 compliant with the definition of the UCL.

Under the assumption that the measurement vectors X follow a multivariate normal distribution and the covariance matrix has been estimated from phase I data, the critical value $T^2_{UCL,1-\alpha}$ for phase II (monitoring) follows an F-distribution:

$$T^2_{UCL,1-\alpha} = \frac{(n^2-1)\times q}{n\times(n-q)} \times F(q, n-q, 1-\alpha)$$

where $F(q, n-q, 1-\alpha)$ is the $1-\alpha$ percentile of the F cumulative distribution function with q and $n-q$ degrees of freedom and α is the probability of an error of the first kind (i.e. the risk of a false alarm). This critical value for the UCL is valid under the assumption that the sample covariance matrix has been estimated in phase I and is thus independent of the monitored measurement vector $\mathbf{x}_o$. Hence, the latter critical value is valid for phase II. For trial control charts in phase I, where this assumption is not fulfilled, the critical value follows a beta distribution.

PCA projects the raw process variables into a lower dimensional subspace. However, the out-of-control signal does not necessarily lie in that subspace. If it is known that such a signal manifests itself in certain latent variables, then these latent variables may be monitored individually in addition to T^2.

4-1-2 Q statistics (squared prediction error or squared residuals)

In general, solely monitoring the process by T^2 is not sufficient since an out-of-control process may move away from the subspace being monitored. New events of this kind can be detected by assessing how well the q-dimensional score vector $\mathbf{t}_o$ approximates the mean-centred measurement vector $\mathbf{x}_{c,o}$. This is done by projecting $\mathbf{t}_o$ back into the original data space and comparing this approximation of the measurement vector $\hat{\mathbf{x}}_{c,o}$ to the actual $\mathbf{x}_{c,o}$:

$$Q = \| \mathbf{x}_{c,o} - \mathbf{P}\,\mathbf{t}_o \|^2 = \| \mathbf{x}_{c,o} - \hat{\mathbf{x}}_{c,o} \|^2$$

Here, $\|a\|$ refers to the Euclidean norm of vector a. Q is the squared prediction error of $\hat{\mathbf{x}}_{c,o}$ which is routinely monitored in addition to T^2. The control limit for Q is difficult to obtain, but different approximations can be found in the literature.

Let $\mathbf{Y}$ be an $n \times r$ matrix of r product variables measured at the same n time points as the process variables in the aforementioned matrix $\mathbf{X}$. Using the $\mathbf{X}$ and $\mathbf{Y}$ data, PLS makes it possible to extract latent variable scores $\mathbf{T}$ that explain the process variables (matrix $\mathbf{X}$) and are predictive for the product variables (matrix $\mathbf{Y}$).

Apart from the construction of the latent variable scores, which is different in PLS compared with PCA, the variability of novel data $\mathbf{x}_o$ is monitored in the same manner as when the product variables are not considered (see previous section). What changes is the way the data $\mathbf{x}_o$ are projected into the q-dimensional subspace to yield $\mathbf{t}_o$, since PLS uses different criteria for doing this. In addition to the analogous Q statistics for the process variables (X), in PLS there are also Q statistics for the prediction error of the product variables (Y):

$$Q = \| \mathbf{y}_{o,c} - \hat{\mathbf{y}}_{o,c} \|^2$$

where $\mathbf{y}_{o,c}$ is a mean-centred new vector of product variables and $\hat{\mathbf{y}}_{o,c}$ is the prediction of the q-dimensional PLS model. Again, q is a hyperparameter of the model, which needs to be estimated, e.g. by cross-validation. Since the construction of the Q statistics in PLS differs from PCA, the approximation of the critical values also changes.

4-1-3 Linear and non-linear methods

In most cases, linear methods (such as those described here) are sufficient for monitoring a process trajectory since small process changes can be approximated reasonably well by linear techniques. If certain measurement variables are non-linearly related to the process, it may be necessary to re-express them by a transformation to obtain a better linear relationship. Non-linear PCA is needed for applications where the variables display non-linear interrelationships in in-control state. In this case kernel PCA is often used in non-linear MSPC since determining the UCL for kernel PCA is particularly straightforward.

4-1-4 Critical aspects

The latent variable methods described in this chapter are sensitive to the scale of the variables. Process variables, or even product variables, may have to be weighted differently.

Latent variables associated with a small variance of the scores may easily cause out-of-control signals even though the observed variation is practically negligible. This is because small values for λ_i in the denominator of T^2 amplify small (practically negligible) deviations in $t_{o,i}$. This fact emphasises the importance of a careful selection of the number of latent variables q.

T^2 is intended to monitor changes in the process mean characteristics. However, it measures shifts in mean and variance of the recorded measurements simultaneously and is very sensitive towards changes in variance (see above). While it is important to know about shifts in variance, it would be preferable to have separate control charts for location (i.e. the process mean) and dispersion (i.e. the process covariance matrix).

In the case of MSPC applied to batch production, it is important to synchronise the monitoring of the various batches, in terms of either the process time or the process stage, to ensure that the batch trajectories are compared at the same stages defined in physical chemical terms rather than temporally.

4-2 BASIC PRINCIPLES OF FAULT DIAGNOSIS

Latent variable methods provide process operators with tools to diagnose which variables caused the out-of-control signal, which in turn may help to investigate the root cause (fault diagnosis). For T^2 charts, the scores where the out-of-control signal manifests itself in $\mathbf{t}_o$ are first identified. This is done by plotting the i^{th} normalised score $t^2_{o,i}/\lambda_i$ against i ($i = 1, \ldots, q$), and those scores with high values are further inspected by computing variable contributions. Let k be the dimension where the score vector shows an unusually high value. It is possible to re-express the k^{th} contribution to T^2 so that the impact of each raw variable can be seen:

$$T^2_k = \frac{t^2_{o,k}}{\lambda_k} = \frac{\sum_{j=1}^{m} \left(P_{kj} \times (x_{oj} - \bar{x}_j)\right)^2}{\lambda_k}$$

where $p_{k,j}$ is the loading of the k^{th} latent variable for the j^{th} original variable and $x_{o,j}$ and $\bar{x}_j$ are the corresponding components of the measurement vector $\mathbf{x}_o$ and the column mean vector $\bar{\mathbf{x}}$. By plotting each individual normalised summand $\left(P_{kj} \times (x_{oj} - \bar{x}_j)\right)^2/\lambda_k$ against the corresponding variable index j, the contribution of each measurement $x_{o,j}$ to T^2_k can be displayed in order to identify highly influential variables. The variables causing the out-of-control signal will be far from the corresponding variable mean in either

direction. The procedure for PLS is very similar. The loading $p_{k,j}$ needs to be replaced by the corresponding weight $w_{k,j}$, where $\mathbf{w}_k$ is the weight vector that generates the k^{th} PLS score vector $\mathbf{t}_k = \mathbf{X}_c \mathbf{w}_k$.

For Q charts the contribution of the j^{th} variable is given by:

$$\left(x_{c,o,j} - \hat{x}_{c,o,j}\right)^2,\ j = 1, \ldots,\ m$$

where $x_{c,o,j}$ and $\hat{x}_{c,o,j}$ are the j^{th} elements of the vectors $\mathbf{x}_{c,o}$ and $\hat{\mathbf{x}}_{c,o}$, respectively. Again, the individual contributions can be plotted against j to identify influential variables visually.

4-2-1 Critical aspects

Contribution plots help to identify variables or groups of variables that contribute numerically to the out-of-control signal. Hence, they highlight variables that are related to the fault instead of revealing the actual cause of it. The root cause still needs to be elucidated by finding the event that caused the state of the highlighted variables.

Supplementary Chapter V

Unlicensed Medicines

This section gives guidance to prescribers, manufacturers and suppliers of unlicensed medicines on the legal and ethical considerations of such medicines. It also provides guidance on the standards for the preparation and manufacture of unlicensed medicines.

Introduction

The term "unlicensed medicine" means either a medicine prepared under a Manufacturer's 'Specials' Licence or a medicine that is prepared extemporaneously under the supervision of a pharmacist.

Unless exempt, medicines for human use must have a Marketing Authorisation (also known as a Product Licence) before being placed on the market in the UK. There is an exemption provided by the medicines legislation to allow the supply of unlicensed medicines which are also known as 'Specials'[1]. A 'Special' may only be supplied to meet the special needs of an individual patient and may only be manufactured by the holder of a Manufacturer's 'Specials' Licence. The decision on whether an individual patient has special needs is made by the authorised healthcare professional responsible for the patient's care.

The UK medicines legislation also allows a pharmacist to prepare an unlicensed medicine for supply in accordance with an authorised healthcare professional's prescription[2]. Such circumstances can arise where no licensed product is available or where the formulation of the licensed product is not suitable. This activity is known as extemporaneous preparation.

The use of unlicensed medicines in the UK is widespread, mainly in the hospital sector and also in primary care. There are a number of reasons for this including:

1. requirements for liquid formulations for paediatric and geriatric populations;
2. discontinued supply of licensed medicines;
3. specialist products used in hospitals;
4. novel therapies in hospitals and clinics;
5. low demand critical care products.

An unlicensed medicine by its very nature will not have been licensed by the UK Licensing Authority or the European Commission and, therefore, will not have been assessed by them for its safety, quality or efficacy. Since 'Specials' are manufactured in accordance with Good Manufacturing Practice (GMP), in facilities licensed by the Medicines and Healthcare products Regulatory Agency (MHRA), there is some assurance of the manufacturing quality and hence the safety related to quality. However, the intrinsic safety and efficacy of 'Specials' are not assessed and are the responsibility of the prescriber.

Legal Requirements

'Specials'

The legislative provision for the supply of unlicensed medicines known as 'Specials' is contained within Regulation 167 of the Human Medicines Regulations 2012 ("Exceptions to Requirement for Marketing Authorisation etc"). This states *inter alia* that, in order to fulfil special patient needs, medicinal products supplied in response to a *bona fide* unsolicited order, and formulated in accordance with the specifications of an authorised healthcare professional, and for use by an individual patient under their personal direction, may be excluded from the requirements to have a Marketing Authorisation. Regulation 167 is subject to a number of conditions which are:

(a) the product is supplied to a doctor, dentist, nurse independent prescriber, pharmacist independent prescriber or supplementary prescriber or for use in a registered pharmacy, a hospital or a health centre under the supervision of a pharmacist;

(b) no advertisement or representation relating to the product is issued;

(c) the manufacture or assembly of the product is carried out under the supervision of such staff and such precautions are taken to ensure that the product meets the specifications of the doctor, dentist, or supplementary prescriber who requires it;

(d) written records as to the manufacture or assembly are made and maintained and are available to the licensing authority;

(e) the relevant medicinal product is manufactured or assembled by the holder of a Manufacturer's 'Specials' Licence;

(f) the product is distributed by way of wholesale dealing by the holder of a Wholesale Dealer's Licence.

It should also be noted that 'Specials' may be imported to fulfil the special needs of an individual patient. Importation should be in accordance with the 2012 regulations relating to Manufacturing and Wholesale Dealing[3] and importers of such products must hold the requisite licences. Imported 'Specials' are outside the scope of the General Monograph on Unlicensed Medicines. However, where an individual monograph exists for an imported 'Special', then the product must comply.

Extemporaneous Preparations

The 1968 Medicines Act ("the Act") allows a pharmacist to prepare or dispense a product which does not have a Marketing Authorisation in response to a prescription from an authorised healthcare professional. The exemptions which apply to pharmacists are contained in Section 10 of the Act. Under the exemptions, the licensing requirements of the 2012 Regulations for a Marketing Authorisation or relevant Manufacturer's Licence do not apply when preparing or dispensing a medicinal product in a registered pharmacy, a hospital, a care home service or a health centre, by or under the supervision of a pharmacist in accordance with a prescription given by an authorised healthcare professional. Under Section 10 of the Act a pharmacist may also prepare a stock of medicinal products for dispensing under the same conditions and procure the preparation or dispensing of an unlicensed product in accordance with the prescription of an authorised healthcare professional.

In addition, Regulation 4 of the 2012 Regulations sets out further provisions in relation to activities conducted under Section 10 of the Act which pharmacists have to follow. These provisions relate to information and labelling provisions that must appear on the packaging of a pharmacy prepared and dispensed medicine.

Ethical Considerations and Guidance

The underlying principle of medicines legislation is that, subject to specific exemptions, no medicinal product may be placed on the market without an appropriate Marketing Authorisation. The exemptions from the formal licensing requirement allow a practitioner to prescribe and a

pharmacist to supply an unlicensed medicine subject to certain conditions. However, in these circumstances both the prescriber and the pharmacist have a duty of care to the patient receiving the unlicensed medicine.

In developing an unlicensed medicine an appropriate risk assessment should be undertaken, which includes consideration of the suitability and fitness of purpose of the drug product. The risk assessment should also consider the contribution of the excipients to the safety profile of the unlicensed medicine.

Following recommendations from the Commission on Human Medicines restricting the amount of *chloroform* in oral medicines, the use of *chloroform* as an ingredient in unlicensed preparations should be avoided.

The MHRA have issued guidance on the supply of Unlicensed Medicines known as 'Specials' in the UK which advises on the manufacture, importation, distribution and supply of such products[4]. It also advises that where a suitable licensed equivalent product is available, an unlicensed product must not be used.

The Royal Pharmaceutical Society (RPSGB) has issued guidance for registered pharmacies on preparing unlicensed medicines, the procurement and supply of 'Specials' and on the prescribing of 'Specials'[5,6,7]. This guidance aims to support pharmacists and their teams to work with prescribers, patients and carers to ensure the safe and appropriate procurement and supply of 'Specials'.

Since there is a greater demand for unlicensed medicines for the treatment of children, due to the lack of suitable licensed formulations, a joint committee of the Royal College of Paediatrics and Child Health (RCPCH) and the Neonatal and Paediatric Pharmacists Group (NPPG) has published a statement providing guidance to health professionals and parents who prescribe, dispense or administer medicines for children[8]. It recognises the need to use some unlicensed medicines in paediatric practice since for many medicines authorised for children there is no appropriate dosage form, whilst for other medicines there is no paediatric authorisation but their use is established. The RCPCH and NPPG Joint Committee on Medicines have also produced information leaflets for parents and older children on the use of unlicensed medicines[9].

Communications to those patients or carers of patients who receive unlicensed medicines can be of paramount importance. Patients need to be reassured that unlicensed medicines are not "second-class" healthcare products and that the treatment has been carefully thought out by the prescriber and is in the best interests of the patient. Where a medicine has been manufactured in a licensed 'Specials' facility, the manufacturing activities will have been approved by the MHRA, thus providing some assurance of product quality. Patients and carers may not always know that the medicines they have received are unlicensed but both prescriber and pharmacists should be prepared to discuss these issues if they are raised.

Labelling of Unlicensed Medicinal Products

The labelling of medicines is a critical contributor to patient safety. Licensed medicines must comply with the requirements set out in Part 13 of the Human Medicines Regulations in respect of their labelling and Patient Information Leaflets[10]. However, this regulatory requirement does not extend to unlicensed medicines. There is a need for a standard, consistent approach to the labelling of unlicensed medicines so that purchasers and suppliers are aware of the composition and status of the product and can make a more informed judgement with regard to their liabilities. This is particularly relevant to those products produced on a batch scale by licensed 'Specials' manufacturers.

Best practice guidance on the labelling and packaging of licensed medicines advises that certain items of information are deemed critical for the safe use of the medicine[11]. These critical items of information, which should be located together on the pack and appear in the same field of view, are: name, strength, route of administration, dosage and warnings. This guidance is equally applicable to unlicensed medicines. Advice on warning statements is also available and may be applicable to unlicensed medicines[12].

Standards for Preparation and Manufacture of Unlicensed Medicines

In the UK unlicensed medicines will be prepared either by the holder of a Manufacturer's 'Specials' Licence, within the exemption provided by Regulation 167 of the 2012 Regulations, or under the supervision of a pharmacist, within the exemption provided by Section 10 of the Act.

As a general rule, it is expected and advised that batch manufacture of medicines for human use should be undertaken in licensed manufacturing units and that preparation under the Section 10 exemption will be of individual products to fulfil the requirement of an authorised healthcare professional's prescription. It is recognised, however, that there will be overlap between these two activities. Manufacturer's 'Specials' Licence holders can manufacture single individual preparations and limited batch preparation is possible under the exemption provided in Section 10 of the Act. Manufacturer's 'Specials' Licence holders are inspected regularly by MHRA GMP inspectors. The continuation of their licence is conditional upon compliance with GMP requirements. It is for this reason that it is advised that batch manufacture of unlicensed medicines is carried out in such licensed units. Unlicensed preparation activities under the supervision of a pharmacist are not inspected by the competent authority and are not independently audited. The following guidance applies equally well to products manufactured under a Manufacturer's 'Specials' Licence and those prepared under the exemption provided by Section 10 of the Act , but is more relevant to preparation under the Section 10 exemption.

The manufacture or preparation of unlicensed medicines should be in accordance with appropriate current standards of GMP and, where applicable, good dispensing practice.

The manufacture or preparation of unlicensed medicines should only be undertaken by competent staff within suitable facilities and using equipment appropriate for the scale of manufacture and specific dosage form.

Where such a monograph is available, the medicinal substance and any excipients must comply with the specific monograph requirements of the Pharmacopoeia.

The medicinal substance and any excipients must also comply with the General Monograph for Substances for Pharmaceutical Use and, where appropriate, the provisions of Supplementary Chapter IV J on the Control of Impurities in Substances for Pharmaceutical Use and the General Monograph for Products with Risk of Transmitting Agents of Animal Spongiform Encephalopathies.

Unlicensed medicinal products must comply with the requirements of the General Monograph for Pharmaceutical Preparations, with the requirements of the General Monograph for Unlicensed Medicines and with the requirements of the relevant General Monograph for the

specific dosage form. Where a BP monograph for a formulated preparation is available, the product must comply.

Further guidance on tests to be applied to the finished dosage form is also contained in Supplementary Chapters III C (Monograph Development: Guidance to Manufacturers), III D (Monograph Development: Methods of Analysis) and III F (Validation of Analytical Procedures).

Bibliography

[1] Regulation 167 of The Human Medicines Regulations 2012 (Supply to fulfil special patient needs).

[2] Section 10 of the Medicines Act 1968 (Exemptions for pharmacists).

[3] Part 3 of The Human Medicines Regulations 2012 (Manufacturing and Wholesale Dealing).

[4] MHRA Guidance Note No. 14: The Supply of Unlicensed Medicinal Products ('Specials').

[5] Medicines Ethics and Practice – The professional guide for pharmacists, Royal Pharmaceutical Society, July 2016.

[6] Professional Guidance for the Procurement and Supply of Specials, Royal Pharmaceutical Society, December 2015.

[7] Prescribing Specials – Guidance for the prescribers of Specials, Royal Pharmaceutical Society, April 2016.

[8] The use of unlicensed medicines or licensed medicines for unlicensed applications in paediatric practice, February 2000, Royal College of Paediatrics and Child Health.

[9] Medicines for Children: Information for parents and carers, 2000, Royal College of Paediatrics and Child Health.

[10] Part 13 of the Human Medicines Regulations 2012 (Requirements for packaging and package leaflets relating to medicinal products).

[11] Best Practice Guidance on the Labelling and Packaging of Medicines; MHRA, 2012.

[12] British National Formulary, Appendix 3: Cautionary and advisory labels for dispensed medicines.

A. Monograph Selection: Unlicensed Medicines

This section provides an outline of the criteria by which monographs for unlicensed products that are produced to meet particular patient needs are selected for inclusion in the British Pharmacopoeia. Such products are manufactured under a Manufacturer's 'Specials' Licence or prepared extemporaneously under the supervision of a pharmacist.

The British Pharmacopoeia Commission will consider a monograph for inclusion in the British Pharmacopoeia in the following circumstances:

1. The formulation is widely used (for example: products in the top 100 list of items used in hospitals; products identified by members of the Expert Advisory Group on Unlicensed Medicines).

2. There is a particular need based on the therapeutic category and/or the importance of the material concerned; the latter being particularly relevant to small patient populations.

3. A request is received from the Competent Authority [Medicines and Healthcare products Regulatory Agency (MHRA)].

4. A request is received from a Specials manufacturer for one of their own products.

5. A request is received from official bodies [such as the World Health Organization (WHO)].

6. Other circumstances considered on a case-by-case basis.

It should be noted that compliance with any of the above criteria will not necessarily mean that a monograph will be included in the British Pharmacopoeia. The British Pharmacopoeia Commission may decide not to elaborate a monograph for a number of reasons, including resource limitations or other circumstances, decided on a case-by-case basis.

B. Preservative-free Unlicensed Medicines

Pharmaceutical products that are intended for multiple-use after opening are required to contain suitable preservatives that will minimise the risk of microbiological contamination during manufacture and storage, after opening and during use. There is limited information on the acceptability and safety of preservatives for neonates and young children. In the first few months of life, immature physiology and metabolism may mean that preservatives considered safe in adults or older children are not appropriate for the very young. One of the reasons that an unlicensed form of a licensed formulation is developed is the need for a preservative-free preparation. Oral medicines for neonates and children, eye drops and nebuliser solutions are examples of these.

There are a number of situations where the use of preservatives in certain patient populations has been shown to cause adverse reactions and should be avoided. A risk assessment should be undertaken on the use of the preservative in the intended population. There should be evidence of acceptability and safety; absence of evidence of harm should not be taken to mean that a preservative is acceptable.

In addition, for aqueous formulations the risks and benefits of presenting the medicine in either single- or multi-dose containers, with or without an antimicrobial preservative, must be assessed.

Preservative-free Eye Drops

Eye drops are required to be sterile until opened and so preservative-free eye drops must be manufactured as sterile solutions. BP monographs for eye drops are open monographs that do not specify a formulation or a concentration and so will apply equally to preserved and preservative-free preparations. In the absence of a preservative, however, there is a risk of microbiological contamination immediately after the container is opened. Preservative-free eye drops should be for single patient use within one clinical session. The product should be applied immediately after opening and, subject to the above, any remaining solution discarded unless an alternative procedure has been justified and validated.

Preservative-free Oral Liquids

It is widely recognised and accepted that oral liquids for children and neonates should not contain antimicrobial preservatives, if possible. Toxic reactions have been reported to all of the commonly used preservatives. Formulation of oral liquids for children is further complicated by the preference for sugar-free and alcohol-free preparations.

Minimal risk is considered achieved for preservative-free oral liquids if these are presented in single-dose containers, which

have been subjected to a process that renders the contents sterile, and the product is used immediately after first opening. Where this is not possible, appropriate means of preparation, suitable packaging and storage conditions should be defined that achieve and maintain the expected quality of the product throughout its period of use.

In addition, suitable measures should be in place to ensure that users of the product are aware of, and have knowledge of, the optimum procedures for handling and storage of the product in use to minimise the risk of microbial contamination and the subsequent risks to patients.

The following options need to be considered to reduce the risk of microbial contamination during manufacture and the risk of microbial growth and contamination during storage and in use.

To minimise microbial contamination within the product at the time of preparation:

1. Use constituents with low viable counts. Pay particular attention to the microbial quality of water, excipients, containers and closures. The total viable counts of all production batches should be controlled by tight limits that have been justified.

2. Assess the potential for native pathogens to survive and grow in the formulation.

3. Investigate the possibility of sterilisation of the final product. This can lead to problems with stability of the preparations and process validation, especially for more complex formulations, but is often a viable option for less complex formulations.

To minimise microbial growth within the product on storage before first opening:

4. Develop formulations with inherent bactericidal or bacteriostatic properties, for example high osmotic pressure solutions. However, such formulations may irritate the gut and cause nausea/vomiting and have been associated with the development of necrotising enterocolitis in neonates. Consideration may be given to including directions to dilute the preparation with liquid or food immediately before administration and, if acceptable, this should be evaluated.

5. Store in a refrigerator (unless sterile).

To minimise microbial contamination and growth within the product after first opening:

6. Provide the preparation in single-dose containers.

7. Store in a refrigerator.

8. Introduce a suitable expiry time after first opening, supported by appropriate in use testing.

C. Bioequivalence of Oral Liquids

Patients receiving medicines with a low therapeutic index may require their blood levels to be monitored to ensure they remain within the required therapeutic range. This therapeutic drug monitoring is essential for substances such as those used for anticoagulant or neuroleptic therapy, because of the potential for non-bioequivalence between different products, and is particularly important in the case of unlicensed oral suspensions.

Where the medicinal products are licensed, appropriate information will be provided in the Summary of Product Characteristics (SmPC) and/or in the Patient Information Leaflet (PIL). No such information will be provided, however, for unlicensed medicines.

It is possible that different formulations of oral suspensions may not be bioequivalent, particularly if the particle size of the active ingredient in the formulations is significantly different. Differences may occur between batches of the same product from a single source or between formulations from different sources.

For unlicensed oral solutions and suspensions of pharmaceutical substances where bioequivalence is a concern and therapeutic drug monitoring is a routine practice, the following statement has been included in relevant British Pharmacopoeia monographs:

"Patients should be monitored in accordance with appropriate clinical guidelines."

D. Storage and Stability of Unlicensed Medicines

For many unlicensed medicines there will be little or no data on the physico-chemical stability of the formulation that is prepared. By contrast, products with a marketing authorisation are required to have robust and validated stability data to justify their shelf life and storage conditions. Consequently, the assigned shelf life for unlicensed medicines will, in many cases, be based on the professional judgement of the person responsible for its preparation or manufacture, using either available scientific information and published data or a limited amount of stability testing data.

Assigning storage conditions for unlicensed medicines is a complex issue for a number of reasons.

1. Degradation pathways for chemical actives are often complex and are not always predictable.

2. Many unlicensed preparations require the reformulation of solid oral dosage forms as oral liquids or suspensions to facilitate their administration to paediatric and geriatric populations and via naso-gastric tubes. Thus, environmental and physicochemical factors such as heat, light, moisture, and pH will become more critical since, in many cases, very little will be known about the stability profile of the active substance in liquid formulations.

3. Whilst extemporaneous preparations will normally be freshly prepared, the storage and shelf life during use needs to be considered if it becomes impractical to prepare the product each time, immediately prior to dispensing.

4. Excipients such as chemical and microbiological preservatives that prolong the in-use shelf life of products are sometimes deliberately omitted from unlicensed formulations due to the possibility of adverse reactions. This may limit the period over which the product can be stored and used.

5. One of the reasons for the absence of a licensed medicinal product can be that large scale batch manufacture is impractical due to poor stability profiles and hence short shelf life. The product, therefore, becomes uneconomical to manufacture commercially, resulting in the need to prepare an unlicensed version.

6. Whilst it might be possible to produce a stable formulation under suitable controlled conditions prior to use, once the container is opened the risk of degradation can increase significantly, particularly where the in-use storage conditions differ from those applied before opening.

7. The stability of an unlicensed medicine depends not only on the intrinsic stability characteristics of the active substance but also on the influence of formulation excipients and

potential interactions with the packaging components. These factors may be especially complex in cases where a liquid formulation is prepared from a previously formulated solid dosage form.

8. The type and size of the container closure system, and the number of doses it contains, should be consistent with the known stability and dosage regimen of the product.

In the monographs for unlicensed medicines, a storage statement has been included where data on the stability of the formulation are available. Storage statements of the British Pharmacopoeia are non-mandatory but give a recommended shelf life under defined storage conditions. Similarly, where information on the shelf life during use is known, this is also included in the storage statement. As a general rule the information in the storage statement relates only to the physico-chemical stability of the formulation and not to its microbiological quality. Such information applies only to the product formulation described in the monograph. Where a different formulation is used, it cannot be assumed that the storage statement in the monograph will still apply.

Where no storage statement is given in the monograph no assumptions should be made about the stability or shelf life of the product. Pharmacopoeial monographs apply throughout the shelf life of the product and so any assigned storage conditions and shelf life should be validated by the persons responsible for the preparation or manufacture of the product, who should satisfy themselves that the product complies with the monograph during storage and use, and that any assigned shelf life is appropriate.

E. Extemporaneous Preparations

This section provides information and guidance on well-established formulae for widely used unlicensed medicines.

This section includes examples of well-established formulae and/or methods that may be used to prepare a medicinal product extemporaneously. Formulae other than those specified in this section may also be available and the medicinal product may be prepared by other means.

An article intended for medicinal use that is described by means of an official title must comply with the requirements of the relevant monograph, regardless of how it is prepared.

The examples provided have been taken from former fixed strength monographs of the British Pharmacopoeia. These monographs have been amended to an open strength format in order to allow a single monograph to apply to the range of strengths available.

Dithranol Paste

Extemporaneous preparation

Dithranol	1 g, or a sufficient quantity
Zinc and Salicylic Acid Paste	Sufficient to produce 1000 g

Mix the Dithranol with a portion of the Zinc and Salicylic Acid Paste until a smooth, even dispersion is obtained and gradually incorporate the remainder of the Zinc and Salicylic Acid Paste.

Levomenthol Cream

Extemporaneous preparation

Grind the Levomenthol to a *fine powder*. If necessary, a small amount of Glycerol may be added to minimise the creation of airborne menthol dust. Add the Aqueous Cream gradually and mix until smooth.

Salicylic Acid Ointment

Extemporaneous preparation

Salicylic Acid, finely sifted	20 g
Wool Alcohols Ointment	980 g

Melt the Wool Alcohols Ointment, gradually add the Salicylic Acid and stir until cold.

F. Aseptic Preparation of Unlicensed Medicines

This section provides general guidance on the Aseptic Preparation of Unlicensed Medicines. It includes guiding principles that should be followed for all aseptically prepared unlicensed medicines. It also includes specific information relating to Parenteral Nutrition Solutions, Ready-to-Administer Injections and Intraocular Injections.

1. GENERAL GUIDANCE

Introduction

It is now an accepted principle that the aseptic preparation of medicines that are intended for parenteral administration should be undertaken in specialised aseptic preparation units, under the supervision of a pharmacist, and not in clinical areas. The Breckenridge report, published in 1976, recommended centralisation of IV additive services in hospital pharmacy units and the British National Formulary (BNF) supports this advice. There is now a wide range of diverse product types prepared in aseptic units. These can be parenteral nutrition solutions (PN), cytotoxic preparations, radiopharmaceutical injections and additives for parenteral administration. As aseptic preparation services have developed, the benefits of preparing these products in specialised, dedicated facilities has been quickly recognised and the demand for these products has increased. This has led to some standardisation of treatments, enabling batch preparation processes, and as a consequence commercial units operating under Manufacturer's ('Specials') Licences have been developed to meet this demand. Home parenteral nutrition services that supply PN therapy direct to the patients' homes, together with other homecare aseptic therapy services, are a feature of this service development.

The benefits that result from the preparation of these products in specialised facilities are well recognised: dose calculations can be automated or checked and confirmed; products are labelled correctly; documentation allows a treatment audit trail; sterility assurance is increased. There are, however, risks associated with centralisation of aseptic preparation services. Process failures, particularly those that result in product contamination can impact on a wider population compared with individually prepared medicines when a single individual patient is at risk. Furthermore, centralisation of aseptic activities into a small number of specialist facilities can significantly affect capacity and supply. All aseptic units have a finite capacity and increasing supply beyond this capacity, in response to increasing demand, may impact upon product quality and compromise patient safety. It is accepted, however, that the benefits of aseptic preparation in specialised units outweigh the risks and so it is now routine practice.

Aseptic preparation of unlicensed medicines is exempt from the licensing requirements of the Human Medicines Regulations 2012. This includes preparation for individual

patients and the preparation of stocks from which to dispense to patients in accordance with a prescription. Nonetheless, it is expected that aseptic preparation activities for stocks are undertaken in units that possess a Manufacturer's ('Specials') Licence that covers their activities. Aseptic preparation units that are dispensing preparations for individual patients are exempt from these requirements but should be operating in accordance with good practice and appropriate guidance (for example, the NHS guidelines on Quality Assurance of Aseptic Preparation Services[1]).The guidance provided in this Chapter applies to all products prepared aseptically for administration to patients. PN, cytotoxic injections, radiopharmaceuticals and additives for parenteral administration are the most common examples but other dosage forms e.g. irrigations, eye drops, are also applicable.

Patient safety is of paramount importance for aseptic preparation units. Incorrect dosage, inappropriate administration, product degradation, product contamination or lack of sterility can and has caused serious adverse reactions in patients and in some cases has been fatal. Published standards are needed for these high risk products. This should include advice on their safe preparation and the application of current guidelines including, where appropriate, compliance with any general monographs that define analytical requirements.

Standards for Aseptic Preparation Services

The following are the principles that should be followed in order to ensure a comprehensive quality management system (QMS) is implemented for aseptic preparation services that is fit for purpose. There is an abundance of guidance documents that should be referred to for the detail that the QMS should include. These general principles will be the basis for the QMS.

1. FACILITIES

All aseptic manipulations should be performed in a workstation with a controlled workspace environment conforming to EC grade A. This should be sited in facilities that protect and maintain the grade A environment during use and when transferring materials into and out of the workstation. Ideally, clean air devices should run continuously. All rooms and equipment used for aseptic preparation should be cleaned and disinfected regularly. Equipment should have written operating instructions and be part of a planned preventative maintenance programme. The facilities should be commissioned when installed and monitored at regular intervals.

2. FORMULATION AND STABILITY

The range of formulations encountered in aseptic preparation units is very wide and can range from simple two component systems to complex mixtures with in excess of 50 constituents. For a substantial number of these, product stability data is limited or not available. However, an assessment of stability is needed to ensure the quality of the product is suitable for the patient at the time of administration. This assessment should include the chemical and physical stability of the product's components, together with potential incompatibilities with containers and materials used during preparation. Where no assessment of stability can be made, the decision to prepare the product should be made in the context of the clinical needs of the patient, and the shortest possible shelf life that is compatible with the administration procedure and supply logistics should be assigned.

Expiry periods given to products must be evaluated in accordance with local conditions. Data obtained from the literature or from the manufacturer of the products used for the preparation should be carefully assessed to ensure its applicability and validity. As a general principle, the shortest expiry date consistent with the usage pattern of the product should be used.

Microbiological integrity must also form a part of the shelf life assessment.

3. DOCUMENTATION

A comprehensive documentation system should be prepared and approved. These documents should be clear and detailed. They should include standard operating procedures (SOPs) for all the critical activities and functions that will affect the quality of the product. A list of SOPs that should be available can be found in the various guidance documents.

All products that are prepared in the unit should have their own worksheet and labels that ensure product traceability from preparation to administration and identify the person(s) responsible for its preparation and release. Within any one unit, worksheets and labels should have a standardised style and presentation. Batch worksheets should be reproduced from an approved master document. The batch documentation should be sufficiently detailed to allow traceability of starting materials and components to establish an audit trail for the product. Completed worksheets should be retained for a sufficient period to satisfy regulatory requirements. For example worksheets for paediatric medicines should be retained for 30 years.

Operation, cleaning, maintenance and fault logs should be maintained. All Planned Preventative Maintenance (PPM) and breakdown maintenance should be recorded. A permit to work system should be used.

4. PERSONNEL AND TRAINING

An aseptic preparation service must be managed by a person who has up-to-date practical and theoretical experience in aseptic preparation. In unlicensed units this must be a pharmacist. In NHS units the responsible pharmacist is designated as the Accountable Pharmacist. He/she should have practical experience in Good Manufacturing Practice (GMP), formulation of aseptic preparations, validation of aseptic processes and QMS.

All staff working in an aseptic preparation unit should have the knowledge and skills required to perform their duties. A documented training programme should record and confirm the competencies of all staff working in the unit. Staff should not work in the unit until training (or retraining) has been satisfactorily completed. There should be a system of accreditation linked to specific activities for all staff involved in aseptic preparation services.

Media fill trials should be used to validate and confirm the competence of the operators in different aseptic activities.

5. MONITORING

Regular monitoring of the environment, processes and finished products is an essential and fundamental part of the QMS. Standards and guidelines are available for many of the physical and microbiological aspects. Where definitive standards do not exist it is important to set in-house standards and action limits based on meaningful results and monitoring trends.

Senior personnel within the aseptic preparation unit must have an understanding of clean room technology, together with a thorough knowledge of the design and operation of their department e.g. ventilation systems, position and grade of HEPA filters, work stations, etc. A documented programme of monitoring should be in place and should be

adhered to. This will include test limits for environmental monitoring both at rest and in use.

When the unit is in use, the critical zone of the controlled workspace should be monitored continuously.

There should be a planned programme of physical, chemical and microbiological monitoring of finished products to further validate the aseptic processes. Samples may be obtained from unused products, additional samples that are specially prepared, or in process samples taken at the end of the compounding procedure. Sampling of the final container after completion of preparation and prior to issue may be a threat to product integrity and is therefore not recommended.

Media fill trials should be used to validate the process initially and subsequently on a regular basis. These should mirror the batch sizes in the unit and cover the number and types of manipulations performed on the actual batch.

6. CLEANING AND DISINFECTION

All areas of an aseptic preparation unit should be regularly cleaned and, where necessary disinfected, according to written approved procedures and a log kept. Workstations should be cleaned and disinfected before and after each work session and appropriate measures put in place to prevent chemical cross-contamination. The effectiveness of cleaning and disinfection should be routinely demonstrated by microbiological monitoring. The surfaces of all items that are to be used in the grade A workstation should be disinfected by means of swabbing, spraying or immersion prior to their introduction. A sporicidal agent should be used for the transfer process. Gaseous sterilisation of enclosed isolator workstations is also possible.

7. STARTING MATERIALS, COMPONENTS AND CONSUMABLES

Where available, starting materials should be licensed products. Where unlicensed products are used, it is the responsibility of the pharmacist to ensure that the product is of an appropriate quality by means of Certificates of Analysis, analytical testing, or a combination of both, in accordance with appropriate Royal Pharmaceutical and GPhC Specials guidance. Unlicensed materials should be obtained from a supplier with a Manufacturer's ('Specials') Licence. Imported products that are licensed in the country of origin may also be used, subject to appropriate import controls.

Components, including reconstitution devices, syringes and needles, and the product contact parts of filling systems, transfer tubing and final containers, should be purchased sterile from the manufacturer or sterilised prior to use. All such products should be UKCA or CE marked. Components should be packaged in such a way that they can be transferred into the grade A workstation environment without risk of contamination to the product or the environment

8. STORAGE AND DISTRIBUTION

A close examination should be made of all stages between product approval and product use to ensure that the quality of the product is not compromised before its expiry. Distribution should be controlled and validated as rigorously as storage. Where necessary, the security of the cold chain should be assessed and assured. Staff involved in storage and distribution should be aware of their responsibilities and trained appropriately. Distribution records of all products should be kept to ensure that effective recall can take place if necessary.

Management Principles for Aseptic Preparation Units

Aseptic preparation departments must ensure that the products they supply are fit for their intended use and do not place patients at risk. Achieving this objective is the responsibility of senior managers and pharmacy managers and requires the commitment, understanding, and participation of all staff who are involved in the ordering, preparation, storage and supply of aseptic products. Some units may not have the facilities, equipment or competencies to do certain tasks and some products or processes may present too high a risk for the resources available. Appropriate controls are needed to manage this. There must be a comprehensive and correctly implemented QMS. It should be fully documented and its effectiveness monitored. It is the responsibility of the management of the aseptic unit to ensure that standards within the unit are maintained and that any faults or deficiencies, once identified, are rectified.

Senior managers should be aware of current Legislative and Regulatory requirements for aseptic preparation services and ensure that the unit(s) comply with these requirements.

Management of aseptic preparation services should be the responsibility of a named Accountable Pharmacist. Management of day-to-day functions can be delegated but all line-management responsibilities should be defined in the QMS. Senior pharmacy managers and senior managers in the organisation with management accountability for the aseptic preparation service should be familiar with the QMS and support the delivery of safe and effective aseptic products.

The prescribing physician must also understand his/her responsibility for the clinical decision to prescribe an unlicensed medicine and the liability for the efficacy of the product.

1. QUALITY MANAGEMENT SYSTEM

The QMS should fully document the procedures whereby the standards for aseptic preparation outlined above will be applied and achieved. All staff involved in the preparation and supply of aseptically prepared products should have a knowledge and understanding of the QMS and be able to apply it to their areas of work. Evidence of staff competencies in their fields of work should be documented and continuously reviewed. The QMS should be continuously reviewed and the outcome of the review documented and implemented.

All departments undertaking aseptic preparation activities should have a documented organisational structure which indicates clearly the responsibilities and accountability of each member of staff. All staff should be clear about their level of responsibility.

2. PRODUCT APPROVAL

A formal recorded decision of approval should be made by a person authorised to do so before a preparation can be released for use. The person who authorises the approval for use, or his/her deputy, should be suitably trained and have the appropriate knowledge to fulfil this role.

There should be a written procedure for dealing with preparations that fail to comply with the required standard. The investigation should be documented and senior managers informed.

3. CAPACITY PLANNING

Managers must know and document the maximum capacity at which their aseptic preparation units can operate. This will vary depending upon staff resources, the size and design of the unit and storage facilities, but managers need to understand these variables and plan accordingly. No unit should operate at more than 80% capacity for a sustained period. There needs to be sufficient flexibility to accommodate unplanned critical demand. Increasing output

beyond capacity represents a critical risk to the integrity of the product. These decisions to operate at over-capacity are normally taken to minimise costs or increase profits but neither of these reasons are justified at the risk of patient safety. The QMS should define the capacity of the unit and should be constantly updated to reflect changes in staff numbers, staff competencies and facilities.

There should be a contingency plan in place to manage situations when the risk assessment of an over capacity concludes that the risk to product quality, product integrity and patient safety is unacceptable. This should include measures to maintain the supply chain until the capacity of the facilities is restored. The plan should be documented and approved through appropriate management structures and developed with the agreement of pharmacy, medical and nursing staff.

Diversity of supply is also an important aspect of capacity management. Aseptic preparation covers a wide range of product types as evidenced by Section 2 of this chapter. These different product types have different requirements regarding facilities design, equipment, preparation procedures, storage and operator competencies. Activities are not necessarily transferable between units. The capacity for each type of activity should be individually defined in the quality system.

4. INSPECTION AND AUDIT

Internal and external audits should be carried out routinely. Audits should cover all areas where aseptic products are prepared, stored or distributed. They should include examination of documentation, preparation and quality control methods, validation, training and complaints and recall procedures. The Accountable Pharmacist for the unit should take responsibility for all remedial actions.

Internal audits are part of the QMS and should be carried out at regular intervals. An external audit, performed by a suitably trained and qualified person should be undertaken at least every two years.

In the UK, NHS hospitals collaborate in an independent audit programme for aseptic preparation units, which was initiated in 1997. This programme continues and the NHS guidelines for aseptic preparation services provide the standards against which the audits are performed.

2. TYPES OF FORMULATION

2.1 Parenteral Nutrition Solutions

Parenteral Nutrition Solutions are one of the most widely prescribed, and one of the most frequently prepared, unlicensed medicines. Due to the complexity of the formulations, there is only limited routine testing performed on these products. However, the demand for end-product testing is increasing notwithstanding the challenges that sampling of Parenteral Nutrition (PN) solutions presents. The provision of a BP monograph provides a suitable standard for such testing.

Parenteral Nutrition Solutions provide essential nutrition requirements in the form of protein (amino acids), carbohydrate (glucose), and fat (lipids), together with essential elements, trace elements and vitamins.
The composition of PN solutions varies according to the needs of the individual patient with regard to calorific requirements, metabolic status, fluid and electrolyte balance, acid-base status, as well as other specific PN requirements. As a consequence, many are formulated uniquely to the patient's requirements. PN solutions will typically contain the following components:

Amino acids
Glucose
Lipids
Sodium
Potassium
Calcium
Magnesium
Acetate
Chloride
Phosphate
Trace elements (Zn, Cu, Mn, Cr, Se, Fl, I)
Water-soluble vitamins
Fat-soluble vitamins

Antioxidants are not usually added to PN solutions. However, ascorbic acid may be added in doses larger than those required physiologically to act as an antioxidant and to help stabilise the preparation.

The composition of PN solutions will vary significantly depending upon individual patient needs. Requirements for adult PN differ significantly from those for neo-natal and paediatric populations. Therapeutic ranges and established dosage regimens will limit the ranges for the various components, but defining limits is not always possible or practical. The upper range limits of some constituents may be controlled by other factors, such as stability. For example, interaction between phosphate and calcium will cause precipitation above a certain concentration.

STANDARDS FOR PREPARATION

Starting Materials

All starting materials used in the preparation of parenteral nutrition solutions should comply with relevant pharmacopoeial standards (including the monographs for Substances for Pharmaceutical Use and Pharmaceutical Preparations). Licensed preparations should be used, where available. Any unlicensed preparations should have been manufactured in a licensed Specials manufacturing unit in compliance with the conditions of the licence.

Manual Preparation

Due to the complexity of the formulation, and the preparation process, PN should be prepared or manufactured in aseptic preparation facilities. No preparation should be performed in uncontrolled environments and all processes should be automated or semi-automated. Manual preparation carries the greatest risk of error and should only be undertaken in emergency situations.

Automated Systems

Automated compounding devices are routinely used for the preparation of PN solutions. A number of containers can be attached to such devices using specific, single-use tubing. The system's information technology can control the identity of products and components (bar codes), pump flow rates and addition sequence. The systems also incorporate an audit record to facilitate quality checks.

Automation of the preparation process for PN solutions reduces the risk of microbiological contamination since the numbers of manual manipulations are reduced, but increases the risks of reconstitution or mixing errors because when these occur they can affect a wider population. Such errors can arise from:

— Incorrect solutions being used
— Incorrect volumes being set
— Incorrect strength of solutions being used

— Incorrect sequence of addition of solutions
— Inappropriate mixing in the tubing set
— Incompatibilities

Fatalities due to the use of incorrect concentrations of glucose and electrolytes have been reported.

These risks apply equally to manual preparation but are multiplied in automated systems.

Validation of automated filling apparatus should be part of the QMS and should be repeated at appropriate intervals. Process checks should confirm the products and containers are connected correctly.

Incompatibilities

Monovalent cations do not cause physical incompatibility of PN solutions unless they are in high concentrations. Positively charged divalent and trivalent ions neutralise the zeta potential of lipid droplets leading to aggregation, coalescence and phase separation.

Admixture of calcium and phosphate ions in PN solutions may result in the precipitation of calcium phosphate, similarly with magnesium and phosphate. The precipitate will not normally be detected by visual examination in lipid-containing solutions and needs to be prevented by the sequence and rate the ions are added to the solution, the phosphate salt used, the phosphate concentration and the volume of the PN solution. Organic complexes of calcium and phosphate can reduce the risk of precipitation and may be preferred.

It is not appropriate to make further additions to PN solutions. The complexity of the formulation makes assessment of interactions and incompatibilities very difficult. There are reports confirming the use of some additives; these include heparin, insulin, ranitidine and iron, but, in general, all additions should be avoided.

PN solutions are packaged in plastic containers and so container compatibility for PN solutions needs to be considered. Interactions of PN solutions with the surfaces of containers and administration devices can occur. PN solution components can be adsorbed or absorbed to plastics, for example vitamin A is absorbed by PVC bags and tubing. Components of the plastic material can leach into the solution. There are a variety of specialised container-closure systems for use with PN. The most appropriate should be selected for the application concerned. Ethyl-vinyl-acetate (EVA) bags are a better option than PVC, but multilayer bags are now the preferred choice. These provide a barrier to oxygen and therefore have the potential to improve stability.

PN solutions may be supplied in:

— flexible plastic containers with all components admixed in a single container and administered together through an intravenous line;
— a combination of two containers with the lipid emulsion supplied in a separate container. The contents of the two containers are mixed just prior to administration;
— a combination of three containers with the carbohydrate solution, amino acid solution and lipid emulsion provided in separate containers. The contents of the three containers are mixed prior to administration by breaking the separation seals between the three containers.

Stability

The large number of components (up to more than 50) and the metastable lipid emulsion present significant challenges to the stability of PN solutions. Physicochemical reactions that will impact on the stability include:

— Aggregation of lipid droplets; lipid coalescence; phase separation of the emulsion
— Precipitation of calcium phosphate
— Complexation of trace elements
— Oxidation of oxygen-sensitive components
— Chemical degradation of amino acids (glutamine, cysteine)
— Interaction between amino acids and glucose
— Oxidation of lipids, catalysed by trace elements
— Light-catalysed degradation of vitamins

SAFETY CONSIDERATIONS

Aluminium in Parenteral Nutrition Solutions

Aluminium is a polyvalent cation and is found in its ionic form in almost every animal and plant tissue. The general population is exposed to aluminium from a variety of sources including food, water, beverages, canned products, containers and cooking utensils. Exposure to aluminium can also occur through medication such as antacids, buffered analgesics and through parenteral nutrition.

The body has a natural protective barrier to prevent systemic absorption of aluminium. Both skin and lungs are effective in limiting aluminium exposure as is the GI tract. However, these protective mechanisms are bypassed when aluminium is administered parenterally. In adults, 40% of aluminium that is infused intravenously is retained; in neonates, this figure increases to 75%[2]. Aluminium becomes bound to transferrin and albumin and is eliminated via the kidneys. It follows, therefore, that patients with reduced or impaired renal function are at the greatest risk of aluminium toxicity. Premature and neonatal babies who have immature renal function are especially prone to aluminium toxicity.
No current UK guidelines are available on the daily limit for aluminium exposure or aluminium content in parenterals. However, in the US, to limit patients' exposure to aluminium, the Food and Drug Administration (FDA) has restricted the aluminium content of large-volume parenterals for PN to 25 µg/L (but no limit currently exists for small-volume parenterals, although labelling of the aluminium content is required)[3]. Other publications refer to aluminium toxicity following parenteral nutrition, particularly in neonates[4,5]. Aluminium toxicity is more likely to occur in patients with impaired kidney function, including premature infants, who receive parenteral levels of aluminium at greater than 4 to 5 µg/kg/day[5].

The risk of aluminium exposure can increase substantially due to abnormally high levels of aluminium in some parenteral preparations. Calcium Gluconate Injection in glass ampoules is one such product. Calcium Gluconate Injection is routinely used as a source of calcium ions for PN preparations. In September 2010, the MHRA published a Public Assessment Report entitled *"Calcium gluconate injection 10% in 10 mL glass containers: risk of aluminium exposure"*. It reported that, when the aluminium content of Calcium Gluconate Injection 10%, packed in 10 mL (type 1) glass ampoules, was compared to those packed in 10 mL plastic ampoules, there was around 200 times more aluminium in Calcium Gluconate Injection packed in glass ampoules than in that packed in plastic ampoules. Type 1 glass ampoules contain a significant amount of aluminium which may be leached into Calcium Gluconate Injection during autoclaving and storage. As a result of these data, the report made the following recommendations:

1. Do not use Calcium Gluconate Injection packed in small-volume (10 mL) glass containers for repeated or prolonged treatment, including as an intravenous

infusion, in children aged younger than 18 years or in patients with renal impairment;

2. Do not use Calcium Gluconate Injection in small-volume glass containers in the preparation of PN solutions;

3. Use Calcium Gluconate Injection packed in plastic containers to reduce the aluminium burden in vulnerable patients.

High aluminium levels may also be found in other parenteral products used as additives in PN solutions including preparations containing magnesium or containing inorganic phosphates.

2.2 Ready-to-Administer Injections

It is now routine practice to prepare injection solutions in their administration device for direct administration to patients. Examples include intravenous fluid (IV fluid) bags, pre-filled capped syringes and ambulatory infusion pumps. Initially, these were the most commonly reconstituted powders for injection, or cytotoxic doses, but preparation services have now extended to include a wider range of ready-to-use injection solutions. Examples of these include injections supplied as part of a home therapy programme and injections provided in continuous ambulatory infusion devices. A major advantage of preparing these medicines within a properly managed aseptic preparation service is the benefit to patient safety, product quality and operator protection that can be achieved. There are also cost/efficiency benefits in batch preparation of high value medicines such as monoclonal antibodies. These benefits have been detailed in the introductory section of this chapter and so will not be restated. This section will focus on specific aspects of quality and safety that relate to aseptic preparation of injections in a ready-to-administer form. It is recognised that the preparation of injections for immediate administration to patients is also performed by healthcare professionals outside the aseptic preparation unit as a part of their routine duties and in accordance with the Summary of Product Characteristics (SmPC). These will normally be single-dose injections that are administered immediately after preparation and are not intended to be stored for any length of time prior to administration. This chapter is intended for aseptic preparation units where products are prepared in anticipation of demand and are stored in a ready-to-administer form until administered to the patient(s).

Standards for Preparation

Starting Materials

All materials used for the preparation of the injections must be sterile licensed medicinal products. This applies to all injection products, diluents and diluent aids that may be used. In the UK aseptic manipulation of unlicensed materials or any non-sterile solutions is considered as part of manufacturing operations and, as such, must be undertaken in a facility holding an appropriate manufacturing licence.

All administration devices used for the preparation of the injection must be sterile and carry the appropriate UKCA or CE mark. Since the injections are in a ready-to-administer form, the injection device is also the container for the product in its final presentation. Therefore, it must be able to maintain the chemical and microbiological integrity of the product throughout the assigned shelf life. Container integrity must be validated for the intended shelf life of the product in accordance with NHS guidance[6].

All devices and equipment that are used for the manipulation of the product during the aseptic preparation process, and that come into contact with the product, must be sterile and carry the appropriate UKCA or CE mark. This includes any devices used for preparing and transferring the injection from its original formulation into the final container for administration.

Preparation

Generally, preparation of ready-to-administer injections will be a manual operation. Economies of scale are possible where multiple injections can be prepared from the same container, for example in paediatric medicine or where bulk products that allow the preparation of multiple injections from one container are available, but these situations are relatively rare. For the majority of these injections, manual manipulation has been the norm.

This is changing with the introduction of automated filling systems and robotic systems for the aseptic preparation of injections. Robotics allow batch preparation of a single product on a large scale but can also be used to prepare individual single patient doses. Robotics reduce the need for manual manipulations and so should improve sterility assurance of the process. Batch processing does, however, increase the size of the population at risk in the event of process failure and so suitable controls should be in place to mitigate this. These will include validation of the precision and accuracy of the robotics operation.

Preparation can be a simple process of transferring a sterile solution from one container to another or a more complex process involving reconstitution, dilution, mixing and transfer. Protocols for the training and validation of staff and for process validation should cover the full range of products and processes used in the aseptic preparation unit. Current published guidance on standards for aseptic preparation services should be followed.

Batch Preparation

Ready-to-administer injections will be unit dose presentations. However, there are circumstances where the preparation process produces multiple doses from the same injection container, or more than one injection of the same product is prepared as a single batch process from a number of individual containers. Batch sizes can vary from 5-1000.

Although multiple preparations of injections from single containers are considered as batch processes in the context of aseptic preparation, within each batch the value of the quality attributes for individual injections may vary. Within any batch of licensed dry powder injection, the amount of active ingredient will vary, within approved limits, and so the concentration of the active ingredient in any batch of reconstituted prepared injections will be different. This is the reason why setting meaningful limits for the concentration of active ingredient in unlicensed medicines prepared from dry powder licensed products is difficult and must allow for the uniformity of dose variation in the licensed product. Furthermore, where the transfer of the injection into its final container is a manual procedure, the sterility assurance for each container within the batch will differ. Microbiological data from a single container cannot be extrapolated to the rest of the batch. It is for this reason that sterility tests should be performed as part of an on-going quality assurance programme rather than a product quality control test.

Dose banding of cytotoxic injections has been specifically introduced to allow batch preparation of these injections rather than individual preparation of specific patient doses. For safety reasons cytotoxic injections should, as a minimum, be prepared in a Class 2 safety cabinet or a negative pressure isolator. Positive pressure isolators with demonstrable low leakage rates are available, but need to be safely validated.

Preparing individual patient doses for busy clinics creates a heavy workload which can affect product quality and patient safety. Dose banding regimes allow preparation and storage in advance of its use. However, it introduces issues with stability and compatibility and integrity associated with the storage of the injection. National NHS specifications for dose-banded cytotoxic injections are available[7].

Incompatibilities

Solutions of injections will usually be salts of weak acids or bases. Others, like high molecular weight proteins, will contain weak acid or base moieties. They are presented in solution at a pH which maintains the physicochemical stability of the injection and any change to the solution pH will affect the solubility and stability of the injection components. This applies both to the active ingredient and to any excipients.

Injections should be prepared as single component solutions. Admixtures to form multiple product solutions in their final container will affect the pH equilibrium and should be avoided, due to the risk of both physical and chemical instability. Nonetheless, admixtures are a convenient means of administering multiple drugs and their use is increasing in cytotoxic therapy (eg. vincristine and doxorubicin) and in other commonly used drug combinations (e.g. fentanyl and bupivacaine). In circumstances where multiple component solutions are prepared, a full evaluation of the stability and compatibility of the admixture must be performed. This should include particle size analysis, including sub-visible particles.

Injection solutions can also be incompatible with the administration device and tubing used to administer them and it is advised to refer to information in the SmPC of the licensed product. Licensed injections are normally supplied in glass containers whereas the administration device will be plastic or rubber. Absorption or adsorption of the active ingredient onto the administration system is possible and can result in the dose being reduced. Absorption is most prevalent with PVC bags and tubing and, where absorption does occur, the high surface area to volume ratio of the narrow bore connective tubing will increase the degree of absorption.

Leaching of extractable plastic components of containers and administration devices may also be a potential source of product contamination and be a risk to patient safety. This will be particularly pertinent when storage times in administration devices are extended beyond licenced recommendations. Assessments of administration devices should take account of assigned shelf lives and storage conditions and consider the potential leaching of contaminants into the injection solution.

Stability

Shelf lives for injections in ready-to-use preparations need to be based on both the physicochemical stability of the active ingredient(s) and critical excipients, and the microbiological stability of the final product. Dry powder injections are usually formulated as such because of their instability in solution. Furthermore, the product SmPC contains recommendations on the stability and shelf life of injections after preparation and advises that, following preparation, the product should be used immediately. Extending the shelf life beyond these recommendations transfers liability for the quality and safety of the product to the user. If aseptic preparation services are to operate economically, they must be able to assign shelf lives that are relevant to the required storage and use of the product. Suitable shelf lives may be assigned to enable effective provision of the service. This may be longer than the SmPC recommendations, but should not exceed 7 days unless justified. All shelf lives for prepared injections should be based upon robust validated data. Peer reviewed published data can be used but, in the absence of this, in-house studies should be performed. NHS standards for the assessment and conduct of stability studies are now available[8,9].

The physicochemical stability of injection solutions is dependent upon a number of individual and interacting factors, which should be taken into account during stability studies and assignment of shelf life. These factors include, but are not limited to:

- *Chemical stability in solution* Injections that are presented as aqueous solutions in their licensed formulation are stable to the end of their assigned shelf life, when stored in their original container, and are demonstrated as such through the licensing process. On the other hand, stability profiles for reconstituted dry powder injections will vary significantly. Once removed from its licensed packaging, and manipulated to prepare a ready-to-use injection, the product composition will be altered and the licensed shelf life will not be applicable.
- *Concentration* Dilution of the active ingredients can affect their stability profile and increase degradation rate. Excipients such as solubilising agents and antimicrobial preservatives can become less effective.
- *Oxygen* Many injections are presented in oxygen-free containers. Preparation introduces oxygen into the solution which may significantly affect the stability.
- *Light* Products that are sensitive to light will be protected from light in their licensed packaging. This may not apply to the packaging for injections in their ready-to-use form.
- *Temperature, Storage, Administration* Following preparation, ready-to-use injections should be stored at 2-8°. There will be circumstances where this is either not possible or is inappropriate. In these cases, suitable storage should be defined and supported by evidence. During administration, the temperature will increase. This will rise to room temperature for some devices and for continuous infusion pumps worn next to the body this will be nearer to body temperature. Stability testing protocols should take account of this variation and the time outside refrigerated storage. Current regulatory advice is to conduct stability studies for body-worn devices at 32°.
- *pH* Aqueous solutions of injections are generally unbuffered. Altering the pH of the solution can affect both the chemical and the physical stability of the product; solubility can be reduced and degradation rates can change. Such changes can occur when using multi-component injection admixtures or can be due to the dilution vehicle used for its preparation. This should be considered during formulation development.

Microbiological stability refers to the ability to maintain the microbiological integrity of the product from its preparation to the point of its administration. Where the product has been prepared in a controlled and validated aseptic preparation unit, quality assurance programmes should provide guarantees of product sterility at the point of release. No aseptic process will be 100% effective but, for aseptic preparation, sterility assurance levels of at least 10^{-3} would be expected. During storage, the sterility of the preparation will be determined by the integrity of the container-closure

system and the storage conditions. These should have been validated.

For cytotoxic preparations, information on the stability of preparations is published in The Handbook of Cytotoxic Medicines[10]. Part 1 of the Handbook is a series of chapters on best practice for cytotoxic preparation services and Part 2 contains monographs on injectable cytotoxic medicines used in cancer chemotherapy. The objective of the monographs was to provide the user with information on the preparation, administration and stability of cytotoxic injections and so enable a valued judgement on the use of these products. In addition to sections on the chemistry, clinical use and preparation of injections there was a section on the stability profile of the products. This was a summary of the known stability profile including degradation pathways, parameters that influence stability after reconstitution and packaging, incompatibilities, and container compatibilities. Although some of the information in the Handbook is no longer current, it remains a reference point for those products covered by the monographs.

2.3 Intraocular Injections

Intraocular injections include intracameral injections that are administered into the anterior chamber of the eye and intravitreal injections that are administered into the vitreous chamber of the eye. They also encompass sub-retinal injections, corneal injections and intravitreal implants, but these are outside the scope of the current chapter. Due to the unique physiological composition of the eye, intraocular injections have very specific requirements that would not necessarily apply to other parenteral preparations. It is important to recognise these differences and to apply quality standards that are appropriate and achievable.

Intraocular injections must be free from preservatives and from excipients that may cause toxicity to the corneal epithelium, endothelium, lens or the retina.

Endotoxin levels, osmolality, isotonicity and pH are particularly critical in these preparations.

PREPARATION

Preparation may involve manufacture from active pharmaceutical ingredients or preparation from licensed medicinal products, often followed by several dilution stages. Preservative-free diluents should be used at all stages of preparation. The diluent used will influence the osmolality and pH of the final preparation, which should comply with the requirements for Intraocular Injections stated under the General Monograph for Unlicensed Medicines.

When withdrawing solutions from glass ampoules, a suitable filter needle should be used to prevent spicules of glass entering the preparation.

DOSE

Intracameral Injections

Intracameral injections are administered into the aqueous humour, which has a volume of 200 to 250 microlitres in adults. Volumes of 0.1 mL are usually administered. A volume of 0.1 mL is routine for administration that is left *in situ* but larger volumes may be administered during surgical procedures.

Intravitreal Injections

Intravitreal injections are administered into the vitreous humour, which has a volume of 4 mL in adults. Volumes of 0.05 to 0.1 mL are administered, although 0.2 mL may be administered in vitrectomised eyes.

It may be necessary to adjust the dose in paediatric patients due to the smaller volume of the vitreous humour in such patients.

CONTAINER

Intraocular injections must be supplied in single-dose containers. Silicone-free syringes are recommended. A pre-filled Luer lock syringe with a blind hub should be used, preferably double wrapped. A sterile double wrap is preferred, particularly for use during surgery.

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Supplementary Chapter VI

Pharmacopoeial Quantitative Analysis

A. Pharmacopoeial Calculations

Introduction

This Chapter provides information on methods of calculating numerical limits specified in monographs of the British Pharmacopoeia. To facilitate calculations, information on Weights and Measures is included in Appendix XXIII.

The presentation of this Chapter follows the order in which the tests appear in monographs for medicinal substances and formulated preparations. The calculations will apply where techniques are common for tests and assays. Examples provided are those specified in the British Pharmacopoeia, with varying degrees of complexity.

The majority of pharmacopoeial calculations use the ratio of the measured sample response (that is, peak area, titration volume, UV absorbance, etc) to the standard response (measured, stoichiometric or a fixed value).

LIMIT TESTS FOR ANIONS

Limit tests for anions are usually specified in parts per million(ppm) in British Pharmacopoeia monographs unless the limit exceeds 500 ppm in which case the limits are expressed in per cent weight in weight (% w/w).

The key to determining the nominal limit set in the test is to calculate the weight of the sample in the same volume as the standard solution and convert this to µg per g. It is also helpful to associate parts per million in terms of the number of µg of the impurity being limited perg of substance being examined.

The standard solutions used as reference standards in limit tests for anions are published in Appendix I C and their concentrations are expressed in ppm. These concentrations may more conveniently be considered in terms of µg per mL so that the calculations are made in the same numerical value or units and are based on the assumption that 1 g is approximately equivalent to 1 mL in dilute aqueous solutions since the density of water is about 1.0 g per mL.

For example, in the Limit Test for Chlorides (Appendix VII), the standard solution contains 50 µg of Cl (10 mL of 5 ppm Cl). Thus, if the sample solution contains 1g of the substance being examined in the same volume as the standard solution, the limit being set is 50 µg per g, that is, 50 ppm.

As an illustration of the above, in the monograph for Aluminium Glycinate, the test for Chlorides is as follows.

Dissolve 1.0 g in 10 mL of 2M *nitric acid* and dilute to 100 mL with *water*. 15 mL of the resulting solution complies with the limit test for chlorides, Appendix VII (330ppm).

To calculate the limit, determine the actual weight of the substance being examined. In the above example, it is:

(15 ÷ 100) × 1.0 (the original weight taken for the test) = 0.15 g.

This gives a sample weight of 0.15 g.

In order to compare like with like, 15 mL of the standard solution in the limit test for Chlorides contains 10 mL of a 5 ppm chloride solution equivalent to 50 µg of Cl. Therefore 1 g of the substance being examined contains:

(1 ÷ 0.15) × 50 = 333.33 µg (nominally 330 ppm).

An illustration of how to calculate the limit in tests where no value is specified is given below using the test for Inorganic phosphates in the monograph for Hydrocortisone Sodium Phosphate.

Inorganic phosphate Dissolve 25 mg in 10 mL of *water*, add 4 mL of 1M *sulfuric acid*, 1 mL of a 10% w/v solution of *ammonium molybdate* and 2 mL of *methylaminophenol-sulfite reagent* and allow to stand for 15 minutes. Add sufficient *water* to produce 25 mL and allow to stand for a further 15 minutes. The absorbance of a 4-cmlayer of the resulting solution at 730 nm, Appendix II B, is not more than that of a 4-cm layer of a solution prepared by treating 10 mL of a 0.0036% w/v solution of *potassium dihydrogen orthophosphate* in the same manner, beginning at the words 'add 4mL ...'.

As a first step, determine the percentage content of phosphate(PO_4) in potassium dihydrogen orthophosphate (KH_2PO_4) using the atomic weight of phosphate (PO_4) (P + O_4; i.e. 30.97 + 64.00 = 94.97) and the molecular weight of KH_2PO_4(136.10). Thus:

$$(94.97 \div 136.1) \times 0.0036 = 0.0025$$

0.0036% w/v of potassium dihydrogen orthophosphate is equivalent to 0.0025% of phosphate. Therefore 10 mL of a 0.0036% w/v solution contains 0.00025 g of phosphate.

Ensure that the weights for comparison purposes are in the same units. Therefore, convert the sample weight (25 mg) to grams. Thus:

$$25 \div 1000 = 0.025$$

The sample solution contains 0.025 g in 10 mL.

To calculate the percentage limit, use the values obtained above, compare the weight of the standard solution with the weight of the sample and multiply by 100. Thus:

$$(0.00025 \div 0.025) \times 100 = 1\%$$

LIMIT TESTS FOR CATIONS

The principles for determining the limit for anions apply when determining the limit for cations. The following example is taken from the European Pharmacopoeia monograph for Bismuth Subcarbonate.

Silver To 2.0 g add 1 mL of *water R* and 4 mL of *nitric acidR*. Heat gently until dissolved and dilute to 11 mL with *waterR*. Cool and add 2 mL of 1M *hydrochloric acid*. Allow to stand for 5 min, protected from light. Any opalescence in the solution is not more intense than that in a standard prepared at the same time in the same manner using a mixture of 10 mL of *silver standard solution (5 ppm Ag) R*, 1mL of *nitric acidR* and 2 mL of 1M *hydrochloric acid* (25 ppm).

In the reference solution, 10 mL of 5 ppm Ag is specified. This is equivalent to 10 mL of a 5 ppm solution:

$$5 \times 10 = 50\ \mu g$$

50 µg in 2 g is 25 µg per gram which is 25 ppm.

The limit is calculated by dividing the maximum amount of Ag permitted inmg by the weight of the sample taken in mg and multiplying this by 1000000. Thus:

$$(0.05 \div 2000) \times 1\,000\,000 = 25\ ppm$$

Note that, as a check, the calculated 'weight equivalent' of the limit in ppm should be the same as the calculated weight of impurity present in the reference solution.

RELATED SUBSTANCES

The key to determining the nominal limit set in the test is to compare the absolute concentrations (e.g. in mg/mL or % w/v) of the test solution and the limiting solution. The relative concentration may then be calculated as a percentage and this gives the limit.

Examples below are based on the various chromatographic techniques used in British Pharmacopoeia monographs.

1. The following test is included in the monograph for Choline Theophyllinate.

Carry out the method for *thin-layer chromatography*, Appendix III A, using *silica gel* HF_{254} as the coating substance and a mixture of 95 volumes of *chloroform* and 5 volumes of *ethanol (96%)* as the mobile phase. Apply separately to the plate 5 µL of each of two solutions of the substance being examined in *ethanol(96%)* containing (1) 1.0% w/v and (2) 0.010% w/v. After removal of the plate, allow it to dry in air and examine under *ultraviolet light (254 nm)*. Any secondary spot in the chromatogram obtained with solution (1) is not more intense than the spot in the chromatogram obtained with solution (2) (1%).

To calculate the nominal limit, compare the concentration of the limiting solution with the test solution prescribed and multiply by 100 to obtain the percentage content of related substances permitted. Thus, for the above example, the calculation is as follows:

[Concentration of solution (2) ÷ Concentration of solution (1)] × 100

$$(0.010 \div 1) \times 100 = 1\% = \text{nominal limit}$$

2. The example below is an extract from the British Pharmacopoeia monograph for Ethanolamine.
The chromatographic conditions included in the monograph are not reproduced below.

Prepare a 0.1% w/v solution of *3-aminopropan-1-ol* (internal standard) in *dichloromethane* (solution A). Carry out the method for *gas chromatography*, Appendix III B, using the following solutions prepared in suitable sealed reaction vials. For solution (1) prepare a solution containing 0.05% w/v of ethanolamine and 0.1% w/v each of *diethanolamine* and *triethanolamine* in solution A. To 0.5 mL of this solution add 0.5 mL of *trifluoroacetic anhydride*, mix and allow to stand for 10 minutes. For solution (2) prepare a 10% w/v solution of the substance being examined in solution A. To 0.5 mL of this solution add 0.5 mL of *trifluoroacetic anhydride*, mix and allow to stand for 10 minutes.

In the chromatogram obtained with solution (1) the peaks eluting after the solvent peak in order of emergence are due to (a) ethanolamine, (b) 3-aminopropan-1-ol, (c) diethanolamine and (d)triethanolamine. In the chromatogram obtained with solution (2) calculate the content of diethanolamine and triethanolamine by reference to the corresponding peaks in the chromatogram obtained with solution (1). Calculate the content of any other impurity by reference to the peak due to ethanolamine in the chromatogram obtained with solution (1). The content of diethanolamine and triethanolamine is not more than 1.0% w/w of each, the content of any other impurity is not more than 0.5% w/w and the sum of the contents of all the impurities is not more than 2.0% w/w.

The limits are expressed in exactly the same way as previously, by comparing the absolute concentrations of the impurities in solution (1) to the concentration of solution (2). Thus:

For ethanolamine:

$$(0.05 \div 10) \times 100 = 0.5\%$$

For diethanolamine and triethanolamine:

$$(0.1 \div 10) \times 100 = 1\%$$

However, as this method includes an internal standard, the ratios of the peak areas of the impurity and the internal standard are determined before the results are calculated.

3. The following example is taken from the British Pharmacopoeia monograph for Amantadine Capsules.
The chromatographic conditions included in the monograph are not reproduced below.

Carry out the method for *gas chromatography*, Appendix III B, using 1 µL or other suitable volume of the following solution. Dissolve a quantity of the contents of the capsules containing 0.1 g of Amantadine Hydrochloride in 2 mL of *water*, add 2 mL of a 20% w/v solution of *sodium hydroxide* and 2 mL of *chloroform* and shake for 10 minutes. Separate the chloroform layer, dry over *anhydrous sodium sulfate* and filter.

The area of any secondary peak is not greater than 0.3% and the sum of the areas of any secondary peaks is not greater than 1% by normalisation.

The impurity content in the above example is determined by normalisation. The percentage content of any secondary peak is calculated by determining the area of the peak as a percentage of the total area of all the peaks, excluding those due to solvents or any added reagents.

$$\%\ \text{Impurity} = [(\text{Impurity response}) \div (\text{total responses})] \times 100$$

4. The following example is taken from the British Pharmacopoeia monograph for Lormetazepam.
The chromatographic conditions included in the monograph are not reproduced below.

Carry out the method for liquid chromatography, Appendix III D, using five solutions in *methanol (70%)* containing (1) 0.25% w/v of the substance being examined, (2) 0.0005% w/v of the substance being examined, (3) 0.00025% w/v of the substance being examined, (4) 0.0005% w/v of *lormetazepam BPCRS* and (5) 0.00025% w/v each of *lormetazepam BPCRS* and *lorazepam BPCRS*.

In the chromatogram obtained with solution (1) the area of any *secondary peak* is not greater than that of the principal peak in the chromatogram obtained with solution (2) (0.2%) and not more than two such peaks have an area greater than the area of the principal peak in the chromatogram obtained with solution (3) (0.1%). The sum of the areas of all such peaks is not greater than 2.5 times the area of the principal peak obtained with solution (2) (0.5%).

To calculate the first limit, compare the concentrations of the two solutions specified, that is, solutions (1) and (2) and multiply by 100 to obtain the percentage limit. Thus:

[Concentration of solution (2) ÷ Concentration of solution (1)] × 100%

$$(0.0005 \div 0.25) \times 100 = 0.2\%$$

For the second limit, compare the concentrations of the two solutions specified, that is, solution (1) and (3) and multiply by 100 to obtain the percentage limit. Thus:

[Concentration of solution (3) ÷ Concentration of solution (1)] × 100%

$$(0.00025 \div 0.25) \times 100 = 0.1\%$$

For the third limit, the value obtained for the first limit multiplied by 2.5 will give the sum. Thus:

$$0.2 \times 2.5 = 0.5\%$$

Note that certain solutions specified within the test are not used to calculate the limits but are used for system suitability purposes.

COMMON SOLVENTS AND OTHER RESIDUES

The following example is taken from the British Pharmacopoeia monograph for Azapropazone.

Acetic acid Not more than 0.2%, determined by the following method. Dissolve 10 g in 25 mL of *methanol*, add 75 mL of *water* and carry out a *potentiometric titration*, Appendix VIII B, using 0.1M *sodium hydroxide VS* as titrant to a pH of 5.9. Each mL of 0.1M *sodium hydroxide VS* is equivalent to 6.005 mg of acetic acid, $C_2H_4O_2$.

The formula to calculate the result is as follows:

$$[V \times (6.005 \times C / 0.1) / w] \times 100$$

V = the volume used (mL)

C = the exact molarity of *sodium hydroxide VS*

w = weight in mg of the substance being examined

RESIDUE ON IGNITION

The following example is taken from the British Pharmacopoeia monograph for Calamine.

68.0 to 74.0%, when ignited at a temperature not lower than 900° until, after further ignition, two successive weighings do not differ by more than 0.2% of the weight of the residue. Use 1 g.

To calculate the percentage Residue on ignition in the example above, the formula to use is as follows.

$$[(w_1 - w_2) / (w_3 - w_2)] \times 100$$

w_1 = weight in g of the ignited crucible and residue

w_2 = weight in g of the crucible

w_3 = weight in g of the crucible and sample

B. Titrimetric Analysis

This Supplementary Chapter provides general guidance regarding the practice of titration in quantitative pharmaceutical analysis. It is not intended as an exhaustive guide to the underlying theory.

Basic concepts

Titration is the process of dissolving an analyte and reacting with another species in a solution of known concentration (titrant). Once the reaction between the analyte and the titrant is well understood, the stoichiometry is characterised and the point at which the titrant amount is equivalent to the analyte amount (the equivalence point) is known, the exact quantity of the analyte can be determined by simple calculation.

Acid-base titrations The determination is based on the neutralisation reactions that occurs between an acid and a base. A measured volume of an acid or base of known concentration is reacted with a sample to the equivalence point. The relative acidity (basicity) of an aqueous solution can be determined using the relative acid (base) equivalents. An acid equivalent is equal to one mole of H^+ ions. Similarly, a base equivalent is equal to one mole of OH^- ions. Some acids and bases are polyprotic, which means that each mole of the acid or base is capable of releasing more than one acid or base equivalent. Examples of monoprotic acids are hydrochloric acid (HCl) and nitric acid (HNO_3). Examples of polyprotic acids are sulfuric acid (H_2SO_4) and phosphoric acid (H_3PO_4).

When the solution of known concentration and the solution of unknown concentration are reacted to the point where the number of acid equivalents equals the number of base equivalents (or vice versa), the equivalence point is reached. The equivalence point of a strong acid or a strong base will occur at pH 7. For weak acids and bases, the equivalence point need not occur at pH 7. There will be several equivalence points for polyprotic acids and bases.

Redox titrations The determination is based on the transfer of electrons between a donor (reducing agent) and an acceptor (oxidising agent). During the reaction, the oxidising ion, whether it is the analyte or the titrant, is reduced by gaining one or more electrons and the reducing ion is oxidised, losing one or more electrons.

These reactions are less common than acid/base reactions but involve a wider range of titrants including oxidising agents (such as iodine, potassium dichromate, potassium permanganate solutions, cerium(IV) salts, hydrogen peroxide, oxidised chlorine (for example ClO^-, ClO), and reducing agents (such as sodium thiosulfate, oxalic acid, ammonium iron(II) sulfate ("Mohr's salt"), hydrogen peroxide, phenylarsine oxide).

Complexometric titrations The determination is based on the formation of a complex usually between a chelating agent and a metal cation. The most frequent use of complex reactions is to determine the concentration of divalent cations such as calcium, magnesium, copper, lead, zinc and cadmium as well as other cations such as aluminium.

The most commonly used complexing reagents are ethylenediaminetetraacetic acid (EDTA) and ethylenebis (oxyethylenenitrilo)tetracetic acid (EGTA).

Although these reactions are easy to perform, it is necessary to work within a well-defined pH interval.

Precipitation titrations The determination is based on the formation of an insoluble salt under certain conditions. During a titration, the end of the precipitation reaction means that excess titrant is present and a coloured complex appears immediately. They are performed in slightly acidic conditions (pH about 4.5) and solvents such as ethanol or acetone may be added to reduce the solubility of the precipitate.

The most frequent use of precipitation reactions in analytical chemistry is the titration of halides (in particular chloride ions) with silver ions.

Direct titrations

In a direct or forward titration the analyte reacts directly with the titrant. For example, a known volume of a solution of unknown acidity may be titrated with a base of known concentration until complete neutralization has occurred. This point is called the equivalence point and is generally determined by observing a colour change in an added

indicator such as phenolphthalein. From the volume and concentration of added base and the volume of acid solution, the unknown concentration of the solution before titration can be determined.

Titrations can also be used to determine the number of acidic or basic groups in an unknown compound. A specific weight of the compound is titrated with a known concentration of acid or base until the equivalence point has been reached. From the volume and concentration of added acid or base and the initial weight of the compound, the equivalent weight, and thus the number of acidic or basic groups, can be determined.

Examples of direct titrations include the Assay for Fusidic Acid and the Assay for Ketoconazole.

Back titrations

The term back titration is used when a titration is done "backwards"; instead of titrating the original analyte, a volumetric solution of a reagent is added to the solution to react with the analyte, then the excess reagent is titrated.

Back titrations are useful if the end point of the reverse titration is easier to identify than the end point of the normal titration. They are also useful if the reaction between the analyte and the titrant is very slow. They may also be used where direct titrations are unsuitable for technical reasons, including: when the sample is not soluble in water, or when the sample contains impurities that interfere with forward titration.

Examples of back titrations include the Assay for Aspirin Tablets and the Assay for Cloxacillin Benzathine.

Measuring the equivalence point

The equivalence point may be determined in a number of ways. The most common is the use of an indicator that shows a colour change at the equivalence point.

Alternatively, the pH or potential difference of the solution can be plotted against the amount of added acid or base on a graph; such a plot is called a titration curve and is usually sigmoid (S-shaped), with the inflection point where the curve changes direction corresponding to the equivalence point. From the pH at the equivalence point, the dissociation constant of the acidic or basic group can be determined. If a compound contains several different acidic or basic groups, the titration curve will show several sigmoid-shaped curves and the dissociation constant of each group can be obtained from the pH at its corresponding equivalence point.

Indicators

Indicators are used to provide a visual determination of the end point of a reaction. This may be by a change in colour or the formation of a precipitate.

Because they are used in low concentrations, indicators do not appreciably alter the equivalence point. Sometimes the volume difference (error) is ignored; in other cases a correction factor may be applied. Often a sensitivity test may be used as a suitability check before an indicator is used.

Examples of indicators where sensitivity tests are specified include Methyl Orange Solution, Phenolphthalein Solution, Starch Solution and Thymol Blue Solution.

Acid/base Indicators Acid-base indicators are weak acids or weak bases. The undissociated form of the indicator has a different colour to the dissociated form. Indicators do not change colour from pure acid to pure alkali at a specific hydrogen ion concentration; rather the colour change occurs over a range of hydrogen ion concentrations. This range is often termed the *colour change interval* and is expressed as a pH range.

Acid-base indicators include Bromocresol Green, Cresol Red, Crystal Violet, Methyl Orange, Phenolphthalein and Thymol Blue.

An example of this type of titration is the Assay for Aspirin Tablets.

Redox indicators Redox indicators undergo a definite colour change at a specific electrode potential. As they have different colours in their oxidised and reduced states, their colour changes according to the redox potential of the solution. As for those used in pH measurement, these indicators have specific *colour change intervals* expressed in mV. They may be dependant or independent of pH.

Redox indicators include Nitrophenanthroline, Diphenylamine, Indigo Carmine, Methylene Blue and Ferroin.

An example of this type of titration is the Degree of unsaturation test in the monograph for Undecenoic Acid.

Complexometric indicators Complexometric indicators are ionochromic dyes that undergo a definite colour change in the presence of specific metal ions. They form a weak complex with the ions present in the solution, which has significantly different colour than the uncomplexed form. They are also called metallochromic indicators.

Complexometric indicators are water-soluble organic molecules, including Eriochrome Black T, Xylenol Orange, Murexide, Eriochrome Blue SE, Methylthymol Blue and Naphthol Green B.

An example of this type of titration is the Assay in the monograph for Hydrotalcite Tablets.

Precipitation indicators Precipitation indicators precipitate from solutions in a readily visible form at or near the equivalence point of a titration.

Precipitation indicators include Fluorescein and Eosin.

The *colour change intervals* for several common indicators are given in Supplementary Chapter VI C.

C. Indicator Colour Changes

This Supplementary Chapter provides a list of colour change intervals for some common indicators, with approximate pKa values and colours. It is provided for the convenience of the analyst in selecting appropriate indicators for their applications. Values given are approximate and are the modal or median averages of several reported figures. Where "not reported" is stated the value has not been reported in the sources consulted in the compilation of the list.

ALIZARIN S

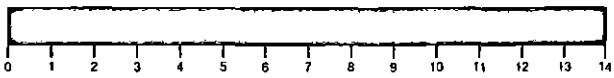
Approximate pKa: not reported

Approximate pH range for colour change: 3.7-5.2

Colour of acid form: yellow

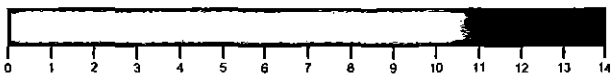
Colour of base form: red

ALIZARIN YELLOW GG



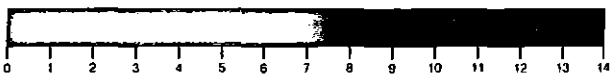
Approximate pKa: 11.0
Approximate pH range for colour change: 10.0-12.0
Colour of acid form: clear
Colour of base form: yellow

ALIZARIN YELLOW R



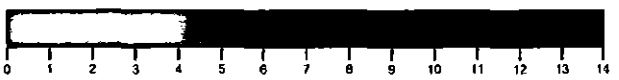
Approximate pKa: 11.0
Approximate pH range for colour change: 10.1-12.0
Colour of acid form: yellow
Colour of base form: red

BRILLIANT YELLOW



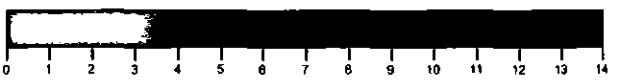
Approximate pKa: not reported
Approximate pH range for colour change: 6.6-7.8
Colour of acid form: yellow
Colour of base form: orange

BROMOCRESOL GREEN



Approximate pKa: 4.7
Approximate pH range for colour change: 3.8-5.4
Colour of acid form: yellow
Colour of base form: blue

BROMOPHENOL BLUE

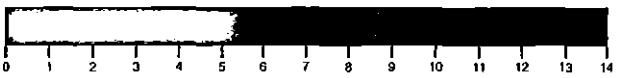


Approximate pKa: 4.0
Approximate pH range for colour change: 3.0-4.6
Colour of acid form: yellow
Colour of base form: blue

BROMOTHYMOL BLUE

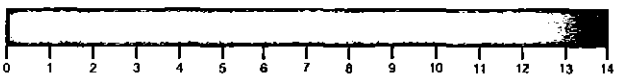
Approximate pKa: 7.1
Approximate pH range for colour change: 6.0-7.6
Colour of acid form: yellow
Colour of base form: blue

CHLOROPHENOL RED



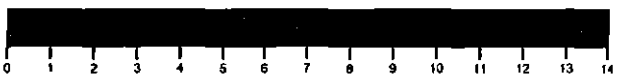
Approximate pKa: 6.0
Approximate pH range for colour change: 4.8-6.4
Colour of acid form: yellow
Colour of base form: red

CLAYTON YELLOW



Approximate pKa: not reported
Approximate pH range for colour change: 12.2-13.2
Colour of acid form: yellow
Colour of base form: amber

CONGO RED

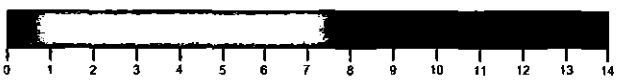


Approximate pKa: not reported
Approximate pH range for colour change: 3.0-5.0
Colour of acid form: blue
Colour of base form: red

CRESOL RED (IN ACID SOLUTION)

Approximate pKa: not reported
Approximate pH range for colour change: 0.0-1.0
Colour of acid form: red
Colour of base form: yellow

CRESOL RED (IN BASIC SOLUTION)

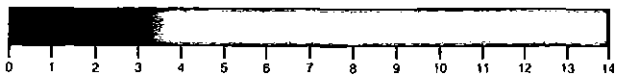


Approximate pKa: not reported
Approximate pH range for colour change: 7.2-8.8
Colour of acid form: yellow
Colour of base form: red

CRYSTAL VIOLET

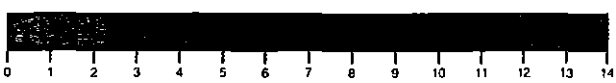
Approximate pKa: 0.8
Approximate pH range for colour change: 0.0-2.0
Colour of acid form: yellow-green
Colour of base form: blue

DIMETHYL YELLOW



Approximate pKa: 3.3
Approximate pH range for colour change: 2.9-4.0
Colour of acid form: red
Colour of base form: yellow

ERYTHROSINE (DISODIUM SALT)

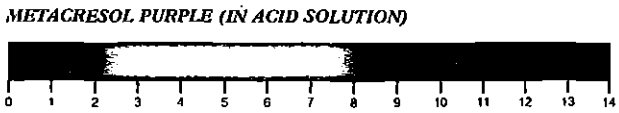


Approximate pKa: not reported
Approximate pH range for colour change: 2.2-3.6
Colour of acid form: orange
Colour of base form: red

MALACHITE GREEN

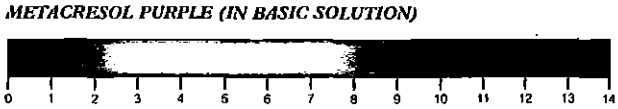
Approximate pKa: 1.0
Approximate pH range for colour change: 0.2-1.8
Colour of acid form: yellow
Colour of base form: blue-green

METACRESOL PURPLE (IN ACID SOLUTION)



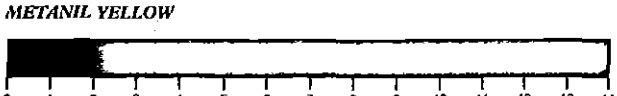
Approximate pKa: 2.0
Approximate pH range for colour change: 1.2-2.8
Colour of acid form: red
Colour of base form: yellow

METACRESOL PURPLE (IN BASIC SOLUTION)



Approximate pKa: 8.3
Approximate pH range for colour change: 7.4-9.0
Colour of acid form: yellow
Colour of base form: purple

METANIL YELLOW

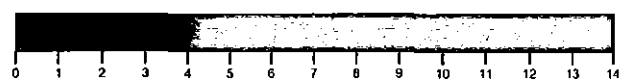


Approximate pKa: not reported
Approximate pH range for colour change: 1.2-2.4
Colour of acid form: red
Colour of base form: yellow

METHYL GREEN

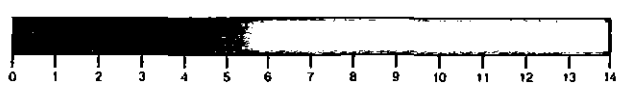
Approximate pKa: not reported
Approximate pH range for colour change: 0.2-1.8
Colour of acid form: yellow
Colour of base form: blue

METHYL ORANGE



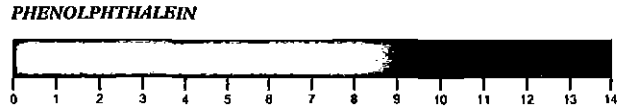
Approximate pKa: 3.8
Approximate pH range for colour change: 3.0-4.4
Colour of acid form: red
Colour of base form: yellow

METHYL RED



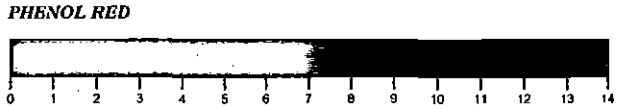
Approximate pKa: 5.0
Approximate pH range for colour change: 4.4-6.0
Colour of acid form: red
Colour of base form: yellow

PHENOLPHTHALEIN



Approximate pKa: 9.7
Approximate pH range for colour change: 8.3-10.0
Colour of acid form: clear
Colour of base form: red-violet

PHENOL RED



Approximate pKa: 7.4
Approximate pH range for colour change: 6.8-8.4
Colour of acid form: yellow
Colour of base form: red

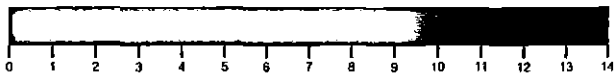
QUINALDINE RED

Approximate pKa: not reported
Approximate pH range for colour change: 1.0-2.2
Colour of acid form: clear
Colour of base form: red

RESAZURIN

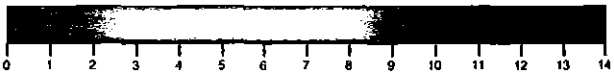
Approximate pKa: not reported
Approximate pH range for colour change: 3.8-6.4
Colour of acid form: orange
Colour of base form: violet

THYMOLPHTHALEIN



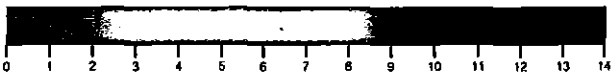
Approximate pKa: 9.9
Approximate pH range for colour change: 9.3-10.5
Colour of acid form: colourless
Colour of base form: blue

THYMOL BLUE (IN ACID SOLUTION)



Approximate pKa: 1.7
Approximate pH range for colour change: 1.2-2.8
Colour of acid form: red
Colour of base form: yellow

THYMOL BLUE (IN BASIC SOLUTION)



Approximate pKa: 8.9
Approximate pH range for colour change: 8.0-9.6
Colour of acid form: yellow
Colour of base form: blue

Supplementary Chapter VII

Traditional Herbal Medicines

This Supplementary Chapter provides information on Traditional Herbal Medicines.

A. Traditional Herbal Medicines

Introduction

This Supplementary Chapter provides information to help the users of the British Pharmacopoeia gain a greater understanding of the process behind the elaboration of monographs for traditional herbal medicines.

Traditional Herbal Medicinal Products (THMP) are classed as medicines under Part 7 of the Human Medicines Regulations 2012, as amended. The publication of monographs is intended to provide objective and publicly available standards of quality. It is emphasised that, although the monographs provide standards for quality, the British Pharmacopoeia Commission has not assessed the safety or efficacy of the herbal medicines.

British Pharmacopoeia monographs for traditional herbal medicines are identified for development in accordance with criteria referred to in Supplementary Chapter III B, in particular the degree of use of a traditional herbal medicinal product.

Some commercially available samples of herbal materials are processed herbal drugs. Where the herbal material is used in the processed form, only the processed form is readily available as an item of commerce in Europe. Where the processed herbal drug is used and where it is possible to obtain authenticated reference herbal samples for both the unprocessed and the processed herbal materials, two separate monographs have been elaborated. Processing methods may have the ability to alter the physical characteristics and/or the chemical constituents of a herbal drug. The two types of monographs are distinguished by means of the monograph titles. Monographs for the processed herbal drug include a 'Production statement' providing information on the method of processing.

The ultimate aim is to ensure that the quality of the monographs for the unprocessed and processed herbal drugs is of an equivalent standard to those of established monographs for herbal drugs.

The herbal samples used for the development of the monographs are authenticated independently. Specific limit tests may be prescribed to detect potential contaminants in processed herbal drugs. Other than the monograph titles the quality of the monographs for traditional herbal medicines are the same as established pharmacopoeial monographs for herbal drugs.

Monograph Titles

For traditional herbal medicinal ingredients, the monograph title is usually a combination of the binomial name together with a description of use (see the paragraph below).

A monograph carries the title of the herbal ingredient and any appropriate synonyms. Because of regional variations in names, spelling variations and the likelihood of existence of other established terms in the countries of origin, Anglicised versions of the traditional names, for example Pinyin names, Ayurvedic names and also Chinese and Ayurvedic symbols will not be included as monograph titles.

To distinguish between monographs for unprocessed and the processed herbal drug, the word 'Processed' is included in the relevant monograph title.

Control Methods

Where applicable, the control methods outlined below will be applied:

(1) macroscopical and microscopical descriptions and chemical/chromatographic tests for identification;

(2) tests for absence of any related species;

(3) microbial test to assure microbial quality;

(4) where appropriate, tests for inorganic impurities, pesticides, aflatoxins and heavy metals;

(5) where appropriate, non-specific purity tests, including extractive tests and sulfated ash;

(6) wherever possible, a method for assaying the active constituent(s) or suitable marker substance(s);

(7) when more than one species/subspecies of the same plant are included in the Definition, individual differences between the two are indicated where possible.

Macroscopical Description The macroscopical description includes those features that can be seen by the unaided eye or by the use of a hand lens.

Microscopical Description The microscopical description of the powdered herbal drug gives information on the diagnostic characters. Example micrographs are being introduced on the website of the British Pharmacopoeia (http://www.pharmacopoeia.com).

Chromatographic tests Diagrammatic representation of the TLC plate will be included in the monograph as an aid to identification of the various constituents.

Other tests In the tests for Acid-insoluble ash, Ash, Extractive soluble in ethanol, Loss on drying, Sulfated ash, Water, Water-soluble ash and Water-soluble extractive of herbal drugs, the calculations will be made with reference to the herbal drug that has not been specifically dried unless otherwise prescribed in the monograph. Samples used for the development of the monographs are free from moulds, insects, decay, animal matter and animal excreta. Unless otherwise prescribed the amount of foreign matter will not be more than 2% w/w. Microbial contamination is such that a traditional herbal medicinal product complies with the acceptance criteria for the stated route of administration as recommended in Appendix VII D and G.

Assay When determining the content of the active constituents or the suitable marker substances measurements are made with reference to the dried or anhydrous herbal drug.

B. Names of Herbal Drugs Used in Traditional Chinese Medicine

(Ph. Eur. general text 5.22)

This general chapter is published for information.

For transparency, this general chapter provides cross-references to the Chinese names in pinyin and in sinograms for herbal drugs used in traditional Chinese medicine for which a monograph is published in the Ph. Eur.

However, the titles in English, French and Latin are the only official names; the samples of herbal drugs must be labelled with at least one official title.

Monograph number	Latin title	English title	Pinyin	Sinogram
2827	Abelmoschi corolla	Abelmoschi corolla	*huangshukuihua*	黄蜀葵花
2432	Acanthopanacis gracilistyli cortex	Acanthopanax bark	*wujiapi*	五加皮
2999	Achyranthis bidentatae radix	Achyranthes bidentata root	*niuxi*	牛膝
2472	Akebiae caulis	Akebia stem	*mutong*	木通
2554	Amomi fructus	Amomum fruit	*sharen*	砂仁
2555	Amomi fructus rotundus	Round amomum fruit	*doukou*	豆蔻
2712	Andrographidis herba	Andrographis herb	*chuanxinlian*	穿心莲
2661	Anemarrhenae asphodeloides rhizoma	Anemarrhena asphodeloides rhizome	*zhimu*	知母
2556	Angelicae dahuricae radix	Angelica dahurica root	*baizhi*	白芷
2557	Angelicae pubescentis radix	Angelica pubescens root	*duhuo*	独活
2558	Angelicae sinensis radix	Angelica sinensis root	*danggui*	当归
2435	Astragali mongholici radix	Astragalus mongholicus root	*huangqi*	黄芪
2559	Atractylodis lanceae rhizoma	Atractylodes lancea rhizome	*cangzhu*	苍术
2560	Atractylodis macrocephalae rhizoma	Atractylodes rhizome, largehead	*baizhu*	白术
1797	Aucklandiae radix	Aucklandia root	*muxiang*	木香
2561	Belamcandae chinensis rhizoma	Belamcanda chinensis rhizome	*shegan*	射干
2384	Bistortae rhizoma	Bistort rhizome	*quanshen*	拳参
2562	Bupleuri radix	Bupleurum root	*chaihu*	柴胡
2386	Carthami flos	Safflower flower	*honghua*	红花
2713	Chaenomeles fructus	Chaenomeles fruit	*Mu Gua*	木瓜
2430	Citri reticulatae epicarpium et mesocarpium	Mandarin epicarp and mesocarp	*chenpi*	陈皮
2463	Clematidis armandii caulis	Clematis armandii stem	*chuanmutong*	川木通
2714	Codonopsidis radix	Codonopsis root	*dangshen*	党参
2454	Coicis semen	Coix seed	*yiyiren*	薏苡仁
2715	Coptidis rhizoma	Chinese goldthread rhizome	*huang lian*	黄连
2976	Corydalis rhizoma	Corydalis rhizome	*yan husuo*	延胡索
2998	Cyathulae radix	Cyathula root	*chuanniuxi*	川牛膝
2890	Dioscoreae nipponicae rhizoma	Dioscorea nipponica rhizome	*chuanshanlong*	穿山龙
2473	Dioscoreae oppositifoliae rhizoma	Dioscorea oppositifolia rhizome	*shanyao*	山药
2563	Drynariae rhizoma	Drynaria rhizome	*gusuibu*	骨碎补
2564	Ecliptae herba	Eclipta herb	*mohanlian*	墨旱莲
2451	Ephedrae herba	Ephedra herb	*ma huang*	麻黄
2412	Eucommiae cortex	Eucommia bark	*duzhong*	杜仲
2718	Evodiae fructus	Evodia fruit	*wuzhuyu*	吴茱萸
2720	Forsythiae fructus	Forsythia fruit	*lianqiao*	连翘
2452	Fraxini chinensis cortex	Fraxinus chinensis bark	*qinpi*	秦皮
3001	Ganoderma lucidum	Ganoderma	*lingzhi* (*reishi*)	灵芝 (赤芝)
2565	Gardeniae fructus	Cape jasmine fruit	*zhizi*	栀子
2721	Gastrodiae rhizoma	Gastrodia rhizome	*tianma*	天麻
2722	Houttuyniae herba	Houttuynia herb	*yuxingcao*	鱼腥草
2566	Isatidis radix	Isatis root	*banlangen*	板蓝根
2634	Ligustici chuanxiong rhizoma	Szechwan lovage rhizome	*chuanxiong*	川芎
2431	Ligustici radix et rhizoma	Ligusticum root and rhizome	*gaoben*	藁本
2612	Lycii fructus	Barbary wolfberry fruit	*gouqizi*	枸杞子
2723	Lycopi herba	Lycopus lucidus herb	*zelan*	泽兰
2742	Magnoliae biondii flos immaturus	Magnolia biondii flower bud	*xinyi*	辛夷
2567	Magnoliae officinalis cortex	Magnolia officinalis bark	*houpo*	厚朴
2568	Magnoliae officinalis flos	Magnolia officinalis flower	*houpohua*	厚朴花
2977	Morindae officinalis radix	Morinda root	*bajitian*	巴戟天
2474	Moutan cortex	Moutan bark	*mudanpi*	牡丹皮
2383	Notoginseng radix	Notoginseng root	*sanqi*	三七
3000	Ophiopogonis radix	Dwarf lilyturf tuber	*maidong*	麦冬
2424	Paeoniae radix alba	Peony root, white	*baishao*	白芍
2425	Paeoniae radix rubra	Peony root, red	*chishao*	赤芍
2727	Persicariae tinctoriae folium	Indigo plant leaf	*liaodaqingye*	蓼大青叶
2477	Piperis fructus	Pepper	*hujiao*	胡椒
2453	Piperis longi fructus	Long pepper	*bibo*	荜茇
2660	Platycodonis radix	Platycodon root	*jiegeng*	桔梗
2724	Polygoni cuspidati rhizoma et radix	Polygonum cuspidatum rhizome and root	*huzhang*	虎杖
2433	Polygoni multiflori radix	Fleeceflower root	*heshouwu*	何首乌
2726	Polygoni orientalis fructus	Polygonum orientale fruit	*shuihonghuazhi*	水红花子
2475	Poria	Poria	*fuling*	茯苓
2439	Prunellae spica	Common selfheal fruit-spike	*xiakucao*	夏枯草
2434	Puerariae lobatae radix	Kudzuvine root	*gegen* (*yege*)	葛根 (野葛)

Monograph number	Latin title	English title	Pinyin	Sinogram
2483	Puerariae thomsonii radix	Thomson kudzuvine root	*fenge*	粉葛
2569	Rehmanniae radix	Rehmannia root	*dihuang*	地黄
2663	Salviae miltiorrhizae radix et rhizoma	Salvia miltiorrhiza root and rhizome	*danshen*	丹参
2385	Sanguisorbae radix	Sanguisorba root	*diyu*	地榆
2428	Schisandrae chinensis fructus	Schisandra fruit	*wuweizi* (*bei wuweizi*)	五味子 (北五味子)
2438	Scutellariae baicalensis radix	Baical skullcap root	*huangqin*	黄芩
2450	Sinomenii caulis	Orientvine stem	*qingfengteng*	青风藤
2440	Sophorae flavescentis radix	Lightyellow sophora root	*kushen*	苦参
2639	Sophorae japonicae flos	Sophora flower	*huaihua*	槐花
2427	Sophorae japonicae flos immaturus	Sophora flower-bud	*huaimi*	槐米
2478	Stephaniae tetrandrae radix	Fourstamen stephania root	*fenfangji* (*hanfangji*)	粉防己 (汉防己)
2937	Typhae pollis	Typhae pollen	*puhuang*	蒲黄
2729	Uncariae rhynchophyllae ramulus cum uncis	Uncaria stem with hooks	*gou teng*	钩藤
2656	Zanthoxyli bungeani pericarpium	Zanthoxylum bungeanum pericarp	*huajiao*	花椒

C. Monographs on Herbal Drug Extracts (Information Chapter)

(Ph. Eur. general text 5.23)

Basis for elaboration of monographs on herbal drug extracts

European Pharmacopoeia monographs on herbal drug extracts are elaborated on the basis of extracts present in medicinal products that have been authorised and/or registered by the competent authorities of Parties to the Convention on the Elaboration of a European Pharmacopoeia. However, these monographs do not necessarily cover all extracts which may be available on the market.

European Pharmacopoeia monographs on herbal drug extracts are elaborated by groups of experts and working parties in collaboration with national pharmacopoeia authorities, the competent authorities for marketing authorisation, Official Medicines Control Laboratories (OMCLs) and the EDQM laboratory; they are also assisted by the producers of herbal drug extracts and/or the pharmaceutical manufacturers that use these extracts.

During the elaboration of an extract monograph, the group of experts works on the basis of a number of extracts from the specified herbal drug that are incorporated into authorised and/or registered medicinal products originating from different sources. These extracts may have been produced using different extraction solvents and/or extraction processes and may include different types and contents of excipients (added for technological purposes during production of the extract).

Where the results from the analysis of the extracts indicate that all of the extracts are in compliance with all of the quality parameters, the monograph is intended to apply to all types of production of that specific herbal extract (for example, *Devil's claw dry extract (1871)*, where differences in the extraction solvent used, as stated in the Production section of the monograph, and any differences between the extracts in terms of their production process have no significant effect on the quality parameters).

Where there is a definable difference in the quality parameters between the extracts due to one or more aspects of the production process, the monograph is presented as a family monograph in which there will be a qualitative or quantitative difference applicable to one or more analytical parameters (for example, *Boldo leaf dry extract (1816)*, where differences in the extraction solvents used, as stated in the Production section of the monograph, necessitates defining a lower minimum content of assayed constituents in the aqueous extract in comparison with the minimum content in the hydroalcoholic extract; all other quality parameters are identical).

Where there is a significant difference in the quality parameters between the extracts due to one or more aspects of the production process, more than one extract monograph is elaborated (for example, *Valerian dry aqueous extract (2400)* and *Valerian dry hydroalcoholic extract (1898)*, where differences in the extraction solvents used and the processing method, as stated in the Production section of the monographs, necessitates defining different minimum values for the assayed constituents and different chromatographic profiles for the aqueous and the hydroalcoholic extracts).

Types of extract

The general monograph *Herbal drug extracts (0765)* distinguishes different types of extracts. This classification is based on the principles applied by European competent authorities during the assessment of extracts in applications for marketing authorisation/registration of medicinal products. These principles are summarised in Table 5.23.-1.

This classification of extracts implies that, for each of these types of extract, distinct principles of production and of defining or adjusting the content of assayed constituents are required by the general monograph *Herbal drug extracts (0765)*.

Genuine (native) extract The concept of the genuine (native) drug extract ratio ($DER_{genuine}$) (see the Glossary in the monograph *Herbal drug extracts (0765)*) was originally devised for application to dry extracts where, after extraction of the herbal drug, all solvent is removed leaving only the extracted dry matter from the herbal drug, that is, the genuine (native) extract. However, this extracted dry matter often requires additives (inert excipients as processing aids) to produce a technologically suitable extract. Such extracts made by different manufacturers from a given herbal drug may have been produced using different solvents and processing methods and contain different amounts of excipients relative to the quantity of extracted dry matter. In order to permit comparison of such extracts, the declaration of the drug extract ratio (*DER*) on the basis of the genuine (native) extract was introduced. Thus, for those dry extracts which can be produced without excipients, the DER_{total} and the $DER_{genuine}$ are identical, but where excipients are required, the DER_{total} and the $DER_{genuine}$ are different.

Table 5.23.-1. – *Classification and principles of production of extracts*

Information available during assessment as regards pharmacological/therapeutic relevance of constituents	Extract type	Extract concept		Extract adjustment
		Quantitative parameters		
		Constituent to be analysed	Quantity of genuine (native) extract that is included in finished products	
Constituents with known therapeutic activity	Standardised	Constituents with known therapeutic activity *Constant*	*Variable*	1) By addition of inert excipients (dry extracts) or solvents (liquid extraction preparations or soft extracts) 2) By blending batches
Constituents which are generally accepted to contribute to the therapeutic activity	Quantified	Active marker *Range*	*Constant*	By blending batches
Constituents chosen solely for analytical purposes irrespective of any pharmacological or therapeutic activity which they may be reported to possess	Other	Analytical marker *Variable*	*Constant*	None

It was then thought necessary to apply this concept to soft extracts and liquid extraction preparations where solvent and, in some cases, other substances are present as an integral part of the extract. For these extracts, the concept of basing the $DER_{genuine}$ on extracted dry matter was abandoned and the extract in its entirety (including solvents, processing aids, etc.) is considered to be the genuine (native) extract. Therefore, for these extracts, the DER_{total} and the $DER_{genuine}$ are identical. For the declaration of the active substance in the finished product, only the $DER_{genuine}$ is to be used.

Constituents for assay

For the purposes of quality control, monographs of the European Pharmacopoeia generally include an assay. The choice of the constituent(s) to be assayed is linked, wherever possible, to the regulatory process and is based upon the following criteria:

— where constituents with known therapeutic activity are present, these are selected for assay.

Constituents with known therapeutic activity are chemically defined substances or groups of substances which are generally accepted to contribute substantially to the therapeutic activity of a herbal drug, a herbal drug preparation or a herbal medicinal product.

— where constituents with known therapeutic activity are absent, marker constituents are selected for assay.

Active markers are constituents or groups of constituents which are generally accepted to contribute to the therapeutic activity of an extract.

Analytical markers are constituents or groups of constituents that serve solely for analytical purposes, irrespective of any pharmacological or therapeutic activity which they may be reported to possess.

Use of analytical markers in 'other' extracts

The assay method described in an individual monograph:

— is usually derived from the method used to assay the corresponding herbal drug;
— is considered appropriate to determine the content of the chosen analytical marker;
— serves as a general analytical platform for determining the content of the marker in the individual extracts covered by the monograph for the purpose of quality control. The method may also be suitable for stability studies;
— may also be suitable as a basis for establishing assay methods to quantify the content of an extract in a finished medicinal product.

The minimum content of the analytical marker when using the assay method:

— is established by assaying extracts used in authorised and registered medicinal products;
— is given based on the final extract (genuine (native) extract with, where present, excipients);
— and thus:
  — represents the minimum value found in the extracts used for establishing the monograph after confirmation or modification following comments received from users, producers or national authorities during the public enquiry;
  — is related to the minimum value as stated in the corresponding herbal drug monograph;
  — reflects the range of yields of that particular marker from the extraction process as well as the stability of that marker during manufacturing;
  — is dependent on the amount of excipients added for technological reasons.

Since the monograph may encompass a broad range of extracts, the minimum value given for the analytical marker is not regarded as a stand-alone quality criterion (as compared to other minimum values given in the Pharmacopoeia). However, it gives an indication of the minimum values that can be expected when producing an extract from a herbal drug compliant with its monograph.

Based on the information given above, manufacturers may be requested by competent authorities to complement this information with a minimum value for their own extract depending on their individual manufacturing process and excipients added.

NOTE: information on the characteristics of the extracts (for example, strength of extraction solvent, percentage of genuine extract, etc.) analysed during the elaboration of an individual extract monograph will be made available to users of the European Pharmacopoeia in the Knowledge database on the EDQM website.

D. DNA Barcoding as a tool for Botanical Identification of Herbal Drugs

This Supplementary Chapter concerns the use of DNA barcoding as a tool for botanical identification of herbal drugs. For the purposes of identification, DNA barcoding has been widely accepted as sufficiently robust and is considered an appropriate method for molecular identification of herbal materials.

INTRODUCTION

In generating the reference barcode sequences published for a herbal drug, several barcode regions are examined including the nuclear Internal Transcribed Spacer (*nrITS*) and several plastid regions; *trnH-psbA, rbcL, trnL-F* and *matK*. The most informative region is identified for each herbal drug, based on the specificity of the DNA sequence to the named species. The species specific sequence for that region is published, highlighting the bases that are essential for identification. Further barcode regions will be examined when no species specific sequences occur in the regions named above.

When a DNA-based identification technique is specified in a monograph for a herbal drug, the identified barcode region and its species specific sequence (reference sequence) will be published as part of the monograph. Where the barcode analysis for a herbal drug has been completed by the BP, but the DNA-based identification technique is not specified as an identification method in the monograph, the barcode region and reference sequence will form part of this Supplementary Chapter.

The usual method of obtaining a barcode sequence from plant material is by DNA extraction, Polymerase Chain Reaction (PCR) and Sanger sequencing. Appendix XI V 'Deoxyribonucleic Acid (DNA) Based Identification Techniques for Herbal Drugs' provides further details. This Appendix encompasses the theory of DNA-based techniques, the infrastructure required, controls and general methods. When a reference sequence is published in a herbal drug monograph or in this Supplementary Chapter the chosen barcode region and any deviations from the published Appendix XI V method for that barcode region will be given either in the herbal drug monograph or in this Supplementary Chapter, as applicable.

Sanger Sequencing: Application and Limitations

Sanger sequencing is the most widely used and easily accessible form of DNA sequencing. However, there are limitations to this technique when applied to herbal drugs, including the inability to detect adulterants, lack of information on purity, potential problems of application to degraded DNA samples, and higher cost, time and technical skill requirements when compared with other DNA-based techniques. To address these limitations, the feasibility of the use of alternative techniques is being investigated and will be applied when appropriate. For example, where adulteration is known to be an issue, consideration will be given to designing a technique that identifies and quantifies the target species and the adulterant species simultaneously. This Supplementary Chapter will be updated to reflect other DNA-based methods as and when they are applied within the BP.

REFERENCE MATERIAL – BRITISH PHARMACOPOEIA NUCLEIC ACID REFERENCE MATERIAL (BPNARM)

To confirm the suitability of the DNA extraction, the PCR chemistry and working practices, appropriate British Pharmacopoeia Nucleic Acid Reference Materials (BPNARM) will be available to end users. Reference electrophoresis results (Figure 1) will be produced in the leaflets for BPNARMs showing the pattern of banding that satisfies the criteria for acceptance.

TABLE 1
REFERENCE ELECTROPHORESIS RESULTS

Lane 1	Amplification product from herbal drug
Lane 2	Amplification product showing both the herbal drug and the BPNARM band. This is the DNA extraction control
Lane 3	Amplification product of the BPNARM alone, the PCR positive control
Lane 4	Negative control with no amplification product visible

TABLE 2
CRITERIA FOR ACCEPTANCE

Lane 1	Visible band from the plant DNA
Lane 2	245 bp band from the BPNARM. The band from the plant DNA may or may not be present
Lane 3	245 bp band from the BPNARM
Lane 4	No band

The suitability of the DNA extraction process used is shown by the results in Lanes 1 to 3 by the banding pattern present. More specifically the BPNARM band is shown in Lanes 2 and 3. As this material is provided as a known DNA sample that amplifies with the method given, an absence of the 245 bp band in both Lanes 2 and 3 shows that the process has not been completed satisfactorily. It is possible for compounds present in the herbal drug to co-purify with the DNA sample, inhibiting the PCR so that no band is formed. Should this occur the subsequent banding pattern would show no bands in Lane 2, due to inhibition, but a 245 bp band in Lane 3, because no plant material is present and therefore no inhibitory compounds. Without the use of the BPNARM it is difficult to discern whether the DNA extraction or the presence of inhibitory compounds has caused the failure of the PCR. Another possibility is that the DNA in the herbal drug may be too degraded to amplify; this would result in the BPNARM bands being formed, but no bands from the plant DNA (Lanes 1 and 2 in Figure 1). The suitability of the PCR chemistry and instrumentation used is verified by the production of the 245 bp BPNARM band in Lane 3. The reaction that produces the band in Lane 3 is the PCR positive control. If a band is not produced from this reaction then there is a problem with either: the components used to make up the reaction, the thermal cycler or the program entered into it, or a user error in the preparation of the reaction mixture.

The suitability of the working practices used to produce the PCRs and the agarose gel electrophoresis system by which to view the amplification products is verified by the presence of a band from the positive control reaction and also by the absence of any other unexplained banding. Lane 4 on Figure 1 is the PCR 'negative control' for which the acceptance criterion is the absence of a band. Should a band be formed in Lane 4, this is likely to be due to contamination of the PCR with extraneous DNA. Similarly, if an unexplained band is found in any of the lanes, this may show contamination of the PCR and/or the DNA extraction, or the adulteration of the herbal drug from which the DNA was extracted.

For the *Ocimum tenuiflorum* barcoding method specified in Appendix XI V a reference material *trnH-psbA* BPNARM has been developed and is listed in the BP Reference Standards

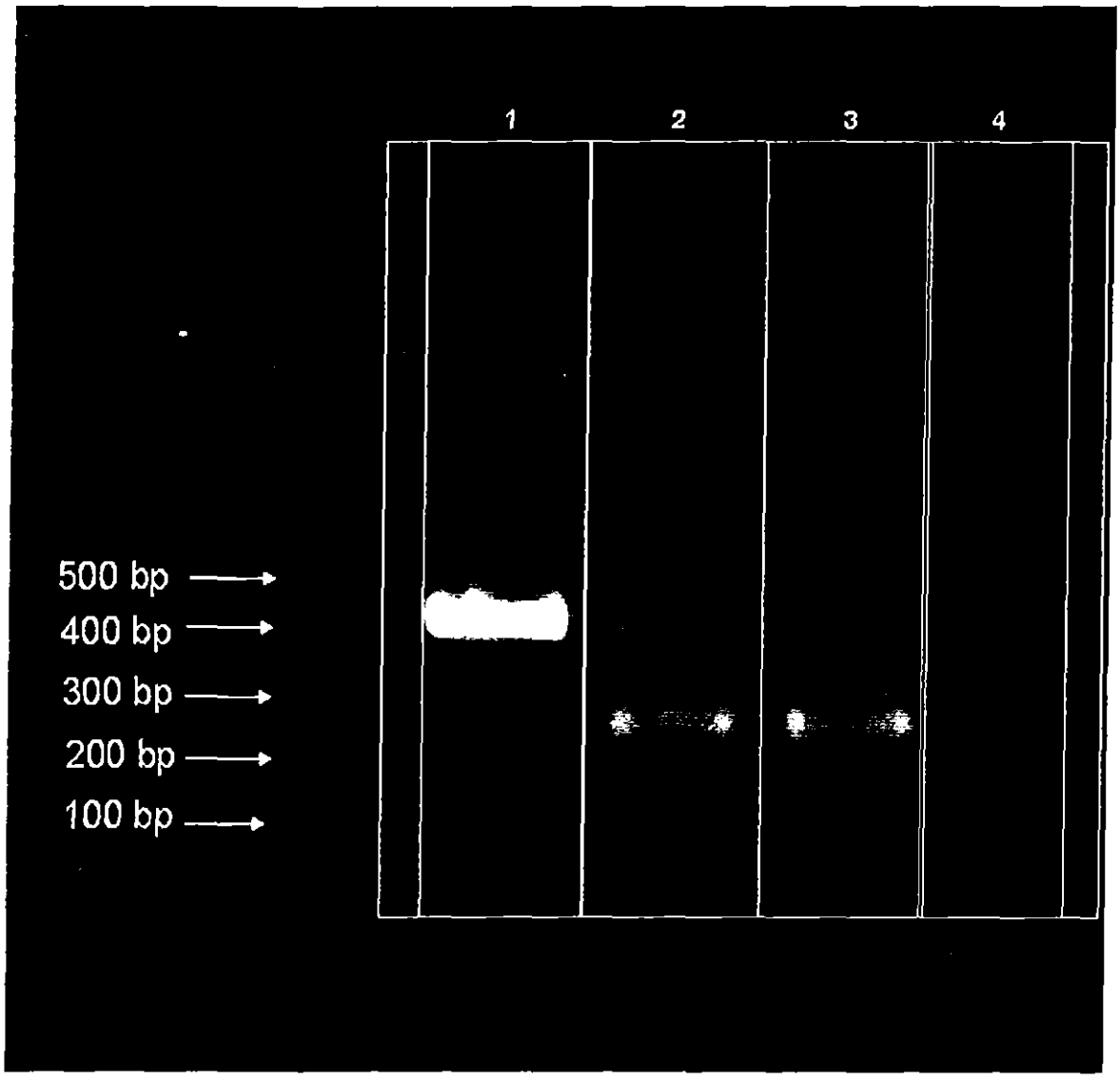


Figure 1 - *Reference electrophoresis results*

Catalogue. This is available through the BP Website (http://www.pharmacopoeia.com). A BPNARM is not tied to one species, but to the chosen DNA barcode region. Therefore, the *trnH-psbA* BPNARM will be applicable to all DNA-based identification techniques based on sequencing the *trnH-psbA* plastid region.

REFERENCE SEQUENCES

When reference DNA sequences are provided, either as one of the identification methods in a monograph or in this Supplementary Chapter, they are published with the key bases for identification indicated by lower case text. In some reference sequences the degenerate DNA code is used, this gives information about the permitted bases at any one position when variation is known to occur. For example, in sequences where variation is seen between a Cytosine (C) and a Thymine (T) at a base position, the degenerate code Y is shown. This means that either a Cytosine (C) or a Thymine (T) would be permitted at the base position, but rules out a Guanine (G) or an Adenine (A) base. The full degenerate code is shown in Table 3.

TABLE 3
DEGENERATE DNA CODE

Code	Meaning
K	G or T
M	A or C
R	A or G
Y	C or T
S	C or G
W	A or T
B	C or G or T
V	A or C or G
H	A or C or T
D	A or G or T

The matching of DNA sequences produced from samples against a published BP Reference Sequence can be achieved by the use of DNA sequence alignment software. Many applications are available for this including internet based, free access portals. Query sequences should be aligned with the relevant BP Reference Sequence and the results are given in both percentage similarity of sequences and full images of the entire alignment. It is possible to upload query sequences from multiple samples to be aligned with a BP Reference Sequence simultaneously. Careful attention must be paid to

the key bases for identification as these must match exactly and be checked manually.

The BP Reference Sequences are provided along with any additional information considered helpful to the analyst in the analysis of samples using an Appendix XI V method. This will include adaptations to optimise DNA extraction and purification methods, and details of sequencing primers. Sequencing is often carried out using the PCR amplification primers, but it can be useful to use different primers at this stage depending on the DNA sequence.

ITS BARCODE REGION REFERENCE SEQUENCES
TRIBULUS TERRESTRIS FRUIT
***TRIBULUS TERRESTRIS* L.**
SEQUENCING PRIMERS
Forward 5′ TCCGTAGGTGAACCTGCGG 3′
Reverse 5′ TCCTCCGCTTATTGATATGC 3′
REFERENCE SEQUENCE:

```
>Tribulus_terrestris_ITS_reference_sequence
CACtcgygcgATGCGTTCCACGctctccaCGGGGACTTGgccaccgcgcg
tTGCTTTATCGGATCATAACAAACCCCGGCGCGGAATGCGTCAAGGAATC
TtwaaATGCGTCGGCACGGCCTTGTGACCCTATCGCAGGGCGTCAGTGCC
AGTGCACTATTactACACGAACGACTCTCGGCAACGGATATCTCGGCTCT
CGCATCGATGAAGAACGTAGCGAAATGCGATACTT
```

ITS2 BARCODE REGION REFERENCE SEQUENCES
ANETHUM GRAVEOLENS SOWA FRUIT
***ANETHUM GRAVEOLENS* SOWA**
SEQUENCING PRIMERS
Forward 5′ ATGCGATACTTGGTGTGAAT 3′
Reverse 5′ GACGCTTCTCCAGACTACAAT 3′
REFERENCE SEQUENCE:

```
>Anethum_graveolens_Sowa_ITS2_reference_sequence
TTTGCTTGCCCCAACCACTCACTCCTTGATGAGATGTGCTGGTTTTTGGG
CGGAAATTGGCCTCCCGTGCCTTGTTGTGCGGTTGGTGCAAAAGCGAGTC
TCCGGCGTTGGACGTCGTGACATCGGTGGTTGTAAAAGACCCTCTTGACT
TGTCGCACGAATCCTCGTCATCTAAGTGAGCTCTAGGACCCTTgggcacc
acACAATCTGTTTGCCCTAACTGTGACCCCAGGTCAGGCG
```

GLEHNIA LITTORALIS ROOT
***GLEHNIA LITTORALIS* F. SCHMIDT EX MIQ.**
SEQUENCING PRIMERS
Forward 5′ ATGCGATACTTGGTGTGAAT 3′
Reverse 5′ GACGCTTCTCCAGACTACAAT 3′
REFERENCE SEQUENCE:

```
>Glehnia_litoralis_ITS2_reference_sequence
CGCATcgtcttgcccacaaACCACTCACACCTGAGAAGTTGTGCCGGTTT
GGGGCGgaaactgGCCTCCCGTaccttgtcttgcGGTTGGCGGAAAAACG
AGTCTCCGGCgacggatgTCGCGGCATCGGTGGTTGTAAAAGACCCTCTT
GTCTTGTCGCGCGAATCCTCGTCATCYTAGMGAGCTCCAGGACCCttagg
cagcacacactctGTGCGCTTCGACTG
```

***TRNH-PSBA* BARCODE REGION REFERENCE SEQUENCES**
CLIVERS
***GALIUM APARINE* L.**
SEQUENCING PRIMERS
trnH 5′ CGCGCATGGTGGATTCACAATCC 3′
psbA 5′ GTTATGCATGAACGTAATGCTC 3′
REFERENCE SEQUENCE:

```
>Galium_aparine_trnH-psbA_reference_sequence
CTGCTATAGATGCTCCATCTATAAATGGATAAGACTtcaGTCTTATtttT
TATTGTATAATAAGCATAGgggTTTTtgaAAAagaAAATAAAGTAGCAAT
AAGAACCTCTTATTGCTACTTTATTTtctTTTTCTTATTTTACacttTAtt
atTTacgaTAAATActgTTTTAAaatTTGTAAayctTAAAGAAAGCAAAA
TTTCCGATATTTTTTtgttgcATTTATTTTATTTTgcaaTAGTAAAACAT
TCAAAattGAAGTAGGGGCGGA
```

HOLY BASIL LEAF
***OCIMUM TENUIFLORUM* LINN.**
SEQUENCING PRIMERS
trnH 5′ CGCGCATGGTGGATTCACAATCC 3′
psbA 5′ GTTATGCATGAACGTAATGCTC 3′
REFERENCE SEQUENCE:

```
>Ocimum_trnH-psbA_reference_sequence
GTTATGCATGAACGTAATGCTCATAACTTCCCTCTAGACCTAGCTGCTAT
TGAAGCTCCAACAAATGGCTAAGacttgttCTTGGTGTGTAGAAGTTTTT
GAAATATAGATAAATATAAGGAGCAATAAACCCTTTCTTGTTCTATCAAA
AGAGGGTTTATTGCTCCTTTCTTTTCTTTTCAATTATGAGTcctttTCTA
TTTTTCTagtAGTATTGGACTTACCTAGACTTTTCTTTCCATAAAAGAAA
GAAGATAAAAAAAATGATTGAAATTTTATCTTTTGTTTTACAATTTTGCA
AAAAAAATTCAAATTGAAAAAGTGAATTCGTaaataAATAGAAAATTTTC
TTTTTAGATTTTTAGAGTAGAGGGGCGGATGTAGCCAAGTGGATCAAGGC
AGTGGattgtGAATCCACCCATGCGCG
```

NUTMEG
***MYRISTICA FRAGRANS* HOUTT.**
SEQUENCING PRIMERS
trnH 5′ CGCGCATGGTGGATTCACAATCC 3′
psbA 5′ GTTATGCATGAACGTAATGCTC 3′
REFERENCE SEQUENCE:

```
>Myristica_fragrans_trnH-psbA_reference_sequence
TACTTCGGTATTAGTGTATACGAGTCATTGAAGGATCAGATCAATACCCa
gaagtGGGTATTGATCTGGTTCACTAGTGGTTTACTCACATAAGATTTTT
TTTTTTCAATTTMARAAAatattaattatttggtTCATGATCGAACATCA
TACCTTTTTTTTTTATTTTAACTGATCTTCAGTTTATGTACATTCCTAAA
ATGCATTTTTTCCTATTGTCAATTTTATAAAGGGTATAAATTAGTATGAA
AAAAAAAAAAAA
```

PHELLODENDRON AMURENSE BARK
***PHELLODENDRON AMURENSE* RUPRECHT**
Due to the presence of polysaccharides, DNA extraction is optimal using less starting material, for example 2 mg rather than 20 mg.
SEQUENCING PRIMERS
Forward 5′ CCATGAAGATCGAAGGGCAC 3′
Reverse 5′ GGGGGTCGGTATTAATCCGTT 3′
REFERENCE SEQUENCE:

```
>Phellodendron_amurense_trnH-psbA_reference_sequence
TATTWAATTAACTGTAAAAATTCAAAAATTAGAATCTAATTAATAAATTA
AKAAAATTAAAATTTTCTTAGTAGTATTAGCGCATACCAAATATATGATA
CTAAATCAAAGAAAAGAACGAAAAGCATAAAAATACTTCAAATTAAAATA
AAAAAAAAAAAAAGAWGTAAATAAAAAACTACTAAAGAAACCGACTCAATA
ACGGATTAATACSGACCCCCCttgcGGGGGGTCGGTATTRATCCKTTWTT
TTTAAAAACTCGTCTACACAAAGATCAAAATCTTATCCATTTGTAGATGG
A
```

Phellodendron chinense Schneid, a closely related species, is known to occur as a substitute for or as an adulterant in *Phellodendron amurense*. The barcode reference sequence for *Phellodendron chinense* is presented below.

REFERENCE SEQUENCE:

```
>Phellodendron_chinense_trnH-psbA_reference_sequence
CTGTAAAAATTCAAAAATTAGAATCTAATTAATAAATTAAKAAAATTAAA
ATTTTYTTAGTAGTATTAGCGCATACCAAATATATGATACTAAATCAAAG
AAAAGAAMGAAAAGCATAAAAATACTTCAAATTAAAATAAAAAAAAAAAA
GAKGTAAATAAAAAACTACTAAAGAAMCCGACTCAATAACGGATTAATAC
CGACCCCCCgcaaGGGGGGTCGGTATTRATCCGTTATTTTTAAAAACTCG
TCTACACAAAGATCAAAATCTTATCCATTTGTAGAT
```

PHELLODENDRON CHINENSE BARK
PHELLODENDRON CHINENSE SCHNEID

Due to the presence of polysaccharides, DNA extraction is optimal using less starting material, for example 2 mg rather than 20 mg.

SEQUENCING PRIMERS

Forward 5′ CCATGAAGATCGAAGGGCAC 3′

Reverse. 5′ GGGGGTCGGTATTAATCCGTT 3′

REFERENCE SEQUENCE:

```
>Phellodendron_chinense_trnH-psbA_reference_sequence
CTGTAAAAATTCAAAAATTAGAATCTAATTAATAAATTAAKAAAATTAAA
ATTTTYTTAGTAGTATTAGCGCATACCAAATATATGATACTAAATCAAAG
AAAAGAAMGAAAAGCATAAAAATACTTCAAATTAAAATAAAAAAAAAAAA
GAKGTAAATAAAAAACTACTAAAGAAMCCGACTCAATAACGGATTAATAC
CGACCCCCCgcaaGGGGGGTCGGTATTRATCCGTTATTTTTAAAAACTCG
TCTACACAAAGATCAAAATCTTATCCATTTGTAGAT
```

Phellodendron amurense Ruprecht, a closely related species, is known to occur as a substitute for or as an adulterant in *Phellodendron chinense*. The barcode reference sequence for *Phellodendron amurense* is presented below.

REFERENCE SEQUENCE:

```
>Phellodendron_amurense_trnH-psbA_reference_sequence
TATTWAATTAACTGTAAAAATTCAAAAATTAGAATCTAATTAATAAATTA
AKAAAATTAAAATTTTCTTAGTAGTATTAGCGCATACCAAATATATGATA
CTAAATCAAAGAAAAGAACGAAAAGCATAAAAATACTTCAAATTAAAATA
AAAAAAAAAAAAAGAWGTAAATAAAAAACTACTAAAGAAACCGACTCAATA
ACGGATTAATACSGACCCCCCttgcGGGGGGTCGGTATTRATCCKTTWTT
TTTAAAAACTCGTCTACACAAAGATCAAAATCTTATCCATTTGTAGATGG
A
```

GLOSSARY
TABLE 4

Term	Definition
Amplicon	The DNA product of a PCR.
Amplification	The copying of DNA during a PCR.
Base call	The identification of a DNA base by sequencing software.
Base pair (bp)	The complementary pairing of two nucleotides, A&T or G&C, which forms the unit of measurement for the length of a DNA molecule.
BPNARM	British Pharmacopoeia Nucleic Acid Reference Material.
Consensus sequence	The product of the combining of several individual DNA sequencing reads, providing a consensus of the correct sequence.
Contig	A set of overlapping DNA sequencing reads from one sample which can be used to produce a consensus sequence.
Deoxynucleotide (dNTP)	The monomer or individual unit of DNA; Adenine (A), Cytosine (C), Guanine (G) and Thymine (T).
di-deoxynucleotide (ddNTP)	A modified form of the DNA monomer without an -OH group present on the 3′ carbon of the deoxyribose sugar which is required to bind a subsequent nucleotide.
DNA	Deoxyribonucleic Acid, a double stranded, helical molecule.
DNA ladder	Mixture of DNA molecules of known base pair length. These provide a measure of how far a DNA molecule travels during gel electrophoresis.
Master mix	A mixture containing the common components for several PCRs, this is made in a large batch or master mix which is then divided between individual reactions. Master mixes contain enough reagents for the required number of tests, typically plus one to allow for pipetting errors.
Mix by pipetting	Drawing up and expelling a substance up to ten times using an automatic pipette, with the aim of mixing the solutions.
Water MB	Deionised, filtered and autoclaved water.
Negative control	A reaction which comprises all but one essential component, thereby proving the necessity of the absent substance.
PCR	Polymerase Chain Reaction - an enzyme driven reaction where DNA molecules are replicated.
Phred score	The likelihood that a base call in a DNA sequence is incorrect, a score of 20 has a 1 in 100 probability of being an incorrect call, 30 is 1 in 1000 etc.
Positive control	A reaction comprising all common PCR components and a known DNA sample, thereby proving the suitability of all reagents.
Primer (oligonucleotide)	A short single stranded DNA molecule which binds to the DNA to be amplified in a PCR. This enables the enzyme to commence replication, and therefore the binding positions define the start and finish point of the PCR.
Probe hybridisation	The complementary binding of an oligonucleotide to a target DNA molecule causing a measurable response.
Sanger sequencing	The method by which a DNA sequence is resolved, developed by Frederick Sanger and colleagues.
Sequencing	Identifying the order of the nucleotide sequence of DNA.
Thermal cycler	The machine that performs the cycling of temperatures required for a PCR.

E. Methods of Pretreatment for Preparing Traditional Chinese Drugs: General Information

(Ph. Eur. general text 5.18)

The following chapter on methods of pretreatment (炮制 paozhi) for preparing traditional Chinese drugs is published for information only.

On the European market, traditional Chinese drugs (herbal, mineral or animal drugs) exist in both pretreated and crude forms. The different forms may be covered by a single monograph or, preferably, by separate monographs.

Regarding herbals, depending on the pretreatment methods used, the products of pretreatment can be herbal drugs or herbal drug preparations. The structure of the method descriptions below is not related to any classification of the resulting products as herbal drugs or herbal drugs preparations. The competent authorities may require a detailed description of the method of pretreatment, including information on the quality of the excipients used.

Terminology: the original Chinese technical terms describing the methods of pretreatment are used, with their English translation. Sinograms and pinyin names (the latter expressing the phonetic transcription) are kept in the text as a reference to guarantee the correct translation of the original terms in case of translation to languages other than French and English. The technical term 'processing', which is widely used in international literature on traditional Chinese medicine (TCM), cannot be used in this context, as it is misleading with respect to the definitions given in the European laws.

1 INTRODUCTION

In contrast to other herbal drugs, drugs used by practitioners of traditional Chinese medicine are rarely in the genuine crude state but in a pretreated form instead.

The pretreatment procedures are different from the common methods already described in the general monograph *Herbal drug preparations (1434)*.

This chapter provides a brief description of the traditionally used pretreatment procedures, as defined in the Chinese Pharmacopoeia.

The major formulation in traditional Chinese therapy is the decoction.

Due to the often very voluminous drug material but also due to other reasons stated below, crude drugs are in many cases not suitable as a raw material for decoctions, and are pretreated before use.

Pretreatments may be used to:

- achieve drugs of consistent quality and size for greater, reproducible effect and better storability (cutting or fragmenting mainly under wet conditions followed by drying procedures);
- reduce toxic effects or undesirable effects of toxic crude drugs;
- modify the effect of the drug;
- increase the effect of the drug;
- modify the extractability of the drug material;
- increase stability and remove unpleasant taste;
- prepare new and modified drug material by fermentation.

Only cleaned drugs are to be used as raw material for pretreatment.

2 CLEANING AND REFINING PROCEDURES: (净制 *JINGZHI*)

Cleaning and refining procedures include sorting (挑选 *tiaoxuan*), sifting (筛选 *shaixuan*), sorting by wind (风选 *fengxuan*), sorting by rinsing with water (水选 *shuixuan*), cutting (剪 *jian*), scraping (刮 *gua*), paring (削 *xue*), rejecting foreign matter (剔除 *tichu*), fermenting (酶法 *meifa*), peeling (剥离 *boli*), pressing (挤压 *jiya*), blanching and rubbing (燀 *chan*), brushing (刷 *shua*), scrubbing (擦 *ca*), singeing with fire (火燎 *huoliao*), throwing in boiling water (烫 *tang*), crushing (撞 *zhuang*), and deburring (碾串 *zhanchuan*).

3 CUTTING (切制 *QIEZHI*)

Unless cut in fresh or dry form, crude traditional Chinese drugs are moistened to soften them prior to cutting. Moistening is preferable to soaking in water which should be avoided in order to prevent the loss or reduction of active principles. The crude drugs are treated according to their size, diameter and hardness, noting the temperature, quantity of water and duration of treatment. The drugs are dried directly after cutting. Dried crude traditional Chinese drugs may be cut into slices, sections, pieces, slivers and other fragments. Their size and thickness are generally as follows.

Slices Less than 0.5 mm in thickness for very thin slices; 1-2 mm in thickness for thin slices; 2-4 mm in thickness for thick slices.

Sections 10-15 mm in length.

Pieces Cubes of 8-12 mm.

Slivers 2-3 mm in width for narrow slivers; 5-10 mm in width for broad slivers.

Crude traditional Chinese drugs other than those treated by cutting are usually pounded or crushed.

4. TREATMENT BY HEATING

4-1 Dry-frying

(炒 *chao*). This method can be divided into simple dry-frying (清炒 *qingchao*, 单炒 *danchao*) and dry-frying with excipients. When dry-frying, stir constantly under homogeneous heating, controlling the temperature, the duration and the condition of individual drugs.

4-1-1 Simple dry-frying

(清炒 *qingchao*, 单炒 *danchao*). Clean crude drugs are placed in a suitable container and dry-fried under gentle heat, taken out and allowed to cool. Crude drugs that should be dry-fried to charring are treated at a relatively high temperature until the surface of the drug turns brown and the fractures become dark. Crude drugs that are inflammable on dry-frying to char may be sprayed with a small amount of water, then dry-fried to dryness or dried in the sun.

4-1-2 Dry-frying with bran

(麸炒 *fuchao*). Bran is put in a suitable, hot container and heated until smoke appears; the clean crude drugs are added and stirred quickly until their surface turns yellow or dark, then taken out. The bran is sifted out and the drugs allowed to cool.

10-15 parts of bran are usually used for 100 parts of clean crude drugs.

4-1-3 Dry-frying with sand

(砂炒 *shachao*). Clean sand is put in a suitable container and heated at high temperature until the sand can move smoothly. The clean crude drugs are added and stirred until the drugs become crisp or reach a specified condition, then taken out. The sand is sifted out and the drugs allowed to cool. The quantity of sand usually used is just enough to cover the crude drugs. Drugs that require tempering with

vinegar are dipped into vinegar while hot until they become crisp.

4-1-4 Dry-frying with powdered clam-shell
Meretricis/Cyclinae concha
(蛤粉炒 *gefenchao*). Powdered and sifted clam-shell is put in a suitable container and heated at medium temperature until the powder can move smoothly. The clean crude drugs are added and dry-fried until the drugs become crisp, their surface becomes yellow or they reach a specified condition, then taken out immediately. The powdered clam-shell is sifted out and the drugs allowed to cool.

30-50 parts of powdered clam-shell are usually used for 100 parts of clean crude drugs.

4-1-5 Dry-frying with talc
(滑石粉炒 *huashifenchao*). Talc is put in a suitable container and heated at medium temperature until the powder can move smoothly. The clean crude drugs are added and dry-fried until the drugs become crisp, their surface becomes yellow or they reach a specified condition, then taken out immediately. The talc is sifted out and the drugs allowed to cool.

40-50 parts of talc are usually used for 100 parts of clean crude drugs.

4-2 Frying
(炙 *zhi*). The clean crude drugs are mixed with a liquid excipient until they are moistened, then heated to a specified condition.

4-2-1 Frying with wine
(酒炙 *jiuzhi*). The clean crude drugs are mixed with wine and allowed to stand until they are completely moistened, then placed in a suitable container and gently heated to a specified condition, taken out and allowed to cool.

Yellow rice wine is usually used. 10-20 parts of wine are used for 100 parts of clean crude drugs.

4-2-2 Frying with vinegar
(醋炙 *cuzhi*). The clean crude drugs are mixed with vinegar and allowed to stand until they are completely moistened, then placed in a suitable container and gently heated to a specified condition, taken out and allowed to cool.

Rice vinegar is usually used. 20 parts of vinegar are used for 100 parts of clean crude drugs.

4-2-3 Frying with salt-water
(盐炙 *yanzhi*). The sodium chloride is dissolved in an appropriate quantity of water and the solution is filtered. The clean crude drugs are mixed with salt-water and allowed to stand until they are completely moistened, then placed in a suitable container and gently heated to a specified condition, taken out and allowed to cool.

2 parts of sodium chloride are usually used for 100 parts of clean crude drugs.

4-2-4 Frying with ginger juice
(姜炙 *jiangzhi*). The fresh ginger is crushed to a paste, water is added and juice is obtained by squeezing. The operation is repeated once and the juices combined. The yield of ginger juice to fresh ginger is 1:1 (volume/mass). The clean crude drugs are mixed well with the ginger juice, then gently heated until the ginger juice is absorbed completely, or to a specified condition, taken out and allowed to cool.

10 parts of fresh ginger are usually used for 100 parts of clean crude drugs.

4-2-5 Frying with honey
(蜜炙 *mizhi*). Refined honey is diluted in an appropriate quantity of boiling water. The clean crude drugs are added, mixed and allowed to stand until they are completely moistened, then placed in a suitable container and gently heated to a specified condition, taken out and allowed to cool.

25 parts of refined honey are usually used for 100 parts of clean crude drugs.

4-2-6 Frying with fat
(油炙 *youzhi*). Fat is heated until it melts, the unmelted residue is removed and the clean crude drugs added, mixed well and gently heated until the fat is completely absorbed and their surface is glossy. The drugs are spread out and allowed to cool.

4-3 Carbonising
(制炭 *zhitan*)

4-3-1 Carbonising by dry-frying
(炒炭 *chaotan*). The clean crude drugs are placed in a hot suitable container and dry-fried at a high temperature until the surface of the drugs turns brownish-black and the inner parts turn dark brown, or to a specified condition, sprayed with a small quantity of water, taken out and allowed to cool.

4-3-2 Carbonising by calcining
(煅炭 *duantan*). The clean crude drugs are placed in a calcining pot, tightly closed, heated to a specified condition, allowed to cool and taken out.

4-4 Calcining
(煅 *duan*). The clean crude drugs are calcined until they become crisp and easily crushed.

4-4-1 Calcining openly
(明煅 *mingduan*). The clean crude drugs are fragmented to small pieces, placed in a suitable container and calcined until the drugs become crisp, brittle or red hot. They are taken out, cooled and crushed to a powder.

Inorganic salts containing water of crystallisation are not calcined to red hot, but the water of crystallisation is completely evaporated or the drugs are calcined to honeycomb-like, solid masses.

4-4-2 Calcining and quenching
(煅淬 *duancui*). The clean crude drugs are calcined to red hot and dipped quickly into a specified liquid until they become crisp (the operation is repeated if necessary), taken out, dried, and crushed to a powder.

4-5 Steaming
(蒸 *zheng*). Water or liquid excipient is added to the clean crude drugs, previously classified according to size; they are mixed well and allowed to stand until the drug is moistened. The mixture is placed in a suitable container and steamed to a specified condition. The drugs are taken out, cooled, mixed with the steaming liquid, partly dried in air, cut into slices or sections and allowed to dry.

20-30 parts of water or liquid excipient are usually used for 100 parts of clean crude drugs.

4-6 Boiling
(煮 *zhu*). The clean crude drugs, classified according to size, are boiled with water or liquid excipient until no white colour is observed in the centre of the drugs, taken out, partly dried in air, cut into slices and allowed to dry.

20-30 parts of water or liquid excipient are usually used for 100 parts of clean crude drugs.

4-7 Stewing
(炖 *dun*). Water or liquid excipient is added to the clean crude drugs. They are placed in a suitable container, tightly closed, stewed thoroughly on a water bath or by steaming

until the liquid is entirely absorbed, allowed to cool, taken out, partly dried in air, cut into slices and allowed to dry.

20-30 parts of water or liquid excipient are usually used for 100 parts of clean crude drugs.

4-8 Roasting

(煨 *wei*). The clean crude drugs are wrapped with wet flour or wet paper, or placed regularly between layers of oil-absorbent paper, and heated. Alternatively, the clean crude drug is put together with bran in a suitable container, gently heated to a specified extent and allowed to cool.

50 parts of bran are usually used for 100 parts of clean crude drugs.

5. OTHER TREATMENTS

5-1 Blanching in boiling water

(燀 *chan*). The clean crude drugs are put in boiling water, stirred for a short time and taken out. Some seeds are blanched until the wrinkled testae become extended and smooth and can be removed easily; they are then taken out and soaked in cold water to remove the testae, and then dried in the sun.

5-2 Defatted powder

(制霜 *zhishuang*). The clean crude drugs are usually crushed to a paste, heated gently and pressed to remove some of the oil, to make a fairly dispersible powder.

5-3 Aqueous trituration and flotation

(水飞 *shuifei*). Water is added to the clean crude drugs and they are pulverised to fine particles. More water is added and, after stirring, the supernatant liquid is decanted. The operation is repeated on the sediment several times, the final sediment is removed and the suspensions of fine particles are combined and allowed to settle. Settled particles are separated, dried and reduced to a fine powder.

5-4 Sprouting

(发芽 *faya*). The clean crude drugs are soaked in an appropriate quantity of water, taken out and allowed to sprout to a specified extent at a suitable humidity and temperature, then dried in the sun or at a low temperature.

5-5 Fermenting

(发酵 *faxiao*). The clean crude drugs are mixed with specific excipients, given a specific shape and placed at a suitable humidity and temperature to let micro-organisms grow until the enzyme content has reached a specified range. The drugs are then dried in the sun or at a low temperature.

Supplementary Chapter VIII

Materials for use in the Manufacture of Homoeopathic Preparations

This Supplementary Chapter provides information on Materials for use in the Manufacture of Homoeopathic Preparations.

Introduction

This Supplementary Chapter provides information to help the users of the British Pharmacopoeia gain a greater understanding of the process for elaborating monographs for materials for use in the manufacture of homoeopathic preparations.

Homoeopathic Medicinal Products are classed as medicines under Part 8 of the Human Medicines Regulations 2012, as amended. Homoeopathic Medicinal Products are defined as '*any medicinal product prepared from substances called homeopathic stocks in accordance with a manufacturing procedure described by the European Pharmacopoeia or, in the absence thereof, by the pharmacopoeias currently used officially in the Member States. A homeopathic medicinal product may contain a number of principles.*'. As stated in the British Pharmacopoeia Supplementary Chapter III B concerning the circumstances for selecting materials for monograph development, the British Pharmacopoeia Commission's policy is to support relevant statutory instruments; this in particular relates to parts 6 and 8 of the Human Medicines Regulations 2012, as amended.

The publication of British Pharmacopoeia monographs for materials for use in the manufacture of homoeopathic preparations is intended to provide official, objective and publicly available standards of quality. It is emphasised that, although the monographs provide standards for quality, it is not the role of the British Pharmacopoeia Commission to assess the safety or efficacy of these materials.

To date, preparation of monographs for materials for use in the manufacture of homoeopathic preparations has been based on monographs present in the British Homoeopathic Pharmacopoeia and have been prioritised in accordance with the availability of these materials within the United Kingdom.

The ultimate aim is to ensure that the quality of the monographs for the herbal materials and chemicals for homoeopathic use are of an equivalent standard to those of established monographs for herbal drugs and chemicals.

Authenticated herbal samples should be used for the development of monographs for materials for use in the manufacture of homoeopathic preparations. When a plant species that is not part of the definition in the monograph is known to be a substituent or adulterant, an absence or limit test is included in the monograph where possible.

Monograph Titles

For materials for use in the manufacture of homoeopathic preparations, the title, including any subsidiary titles, includes '... for Homoeopathic Preparations'. The main title of a monograph for a chemical is the English name; the Latin name, where appropriate, is given as the subsidiary title.

Control Methods

In cases where the bulk starting material and any excipients for dilution or trituration are covered by a BP or Ph Eur monograph, the material and/or excipient must comply with the relevant pharmacopoeial monograph. If the material is not covered by an existing BP or Ph Eur monograph, tests to check the quality of the starting material are included in the specific monograph for homoeopathic materials.

Characteristics References to odour are only included where this is highly characteristic. References to taste are not included.

Identification: (a) Herbal drugs Reliance for identification of herbal homoeopathic materials is primarily placed on the macroscopical description. In addition, a microscopical description and thin-layer chromatographic test may be included to strengthen the identification.

The macroscopical description includes those features that can be seen by the unaided eye or by the use of a hand lens.

The microscopical description of the powdered herbal drug gives information on the diagnostic characters.

(b) Chemicals Tests such as specific colour reactions are included unless the raw material is already controlled within the British Pharmacopoeia.

Tests The following tests are applied, where applicable, in addition to the tests or requirements given in the monographs for Homoeopathic Preparations, Herbal Drugs for Homoeopathic Preparations and Mother Tinctures for Homoeopathic Preparations:

(1) tests for absence of any related species;

(2) microbial tests to assure microbial quality;

(3) where appropriate, tests for inorganic impurities, pesticides, aflatoxins and heavy metals;

(4) non-specific purity tests, including extractive tests and sulfated ash.

Monographs for mother tinctures The control of the herbal drug is followed by a separate section entitled 'Mother Tincture' that states the method of production followed by the relevant control tests.

Monographs for chemical stocks The format and layout is similar to that for mother tinctures above, whereby the control of the chemical is followed by a separate heading section entitled 'Production of Stock' that states the method of production followed by the relevant control tests.

Production A cross-reference is made to the method of manufacture given in the monograph entitled Methods of Preparation of Homoeopathic Stocks and Potentisation. If the method is not included, a general statement relating to a suitable method is included in the specific monograph. When a British Pharmacopoeia or European Pharmacopoeia monograph exists for a vehicle or excipient, that vehicle or excipient complies with the monograph.

Tests Identification tests are included for the mother tincture or chemical stocks; vehicles and/or excipients used are also identified. No testing is given for subsequent dilutions or triturations owing to the difficulty in applying analytical methodology to high dilutions. In the case of mother tinctures thin-layer chromatography is usually specified for the identification.

Assay When an assay is given for the bulk material, it is also applied to the relevant mother tincture or chemical stock. Where necessary, the method includes a means of overcoming interference from excipients.

Supplementary Chapter IX

Similar Biological Medicinal Products

This Supplementary Chapter provides a statement on the Pharmacopoeial position in relation to similar biological medicinal products and aspects for consideration concerning preparation of relevant monographs.

Pharmacopoeial position

Introduction

The term 'similar biological medicinal product' is used for a biological product that has been developed to be similar to an authorised biological medicinal product ('reference' product). The term 'biosimilar' has been used interchangeably with 'similar biological medicinal product'. For Great Britain, reference for more information should be made to the applicable EU Guidance on similar biological medicinal products specifically referred to in the Human Medicines Regulations 2012, as amended. For Northern Ireland, reference for more information should be made to the most recent Committee for Medicinal Products for Human Use (CHMP) Guidelines on similar biological medicinal products and other related position papers published by the European Medicines Agency (EMA).

Consistent with the established principles set out in the Human Medicines Regulations 2012, as amended, biological medicinal products, in view of their complexity, differ from conventional medicinal products based on chemically synthesised substances. Similar biological products cannot be regarded as comparable to generic medicinal products. They are likely to be derived from starting materials or manufacturing processes different from those used for the authorised reference biological product. Consequently, even though a 'similar biological medicinal product' is developed to be as close as possible in structure and activity to the reference product, there may be differences in the detailed structure of the active ingredient from that of the 'reference' product. Any difference may result in altered properties that impact on the immunogenicity and clinical performance. Such differences have led to recommendations that similar biological medicinal products should be prescribed by brand only. Additional requirements for proof of safety and efficacy have usually been required when compared to those expected for generic products of chemical origin and before marketing authorisation is granted.

British Pharmacopoeia: Monographs for biological formulated preparations The general policy of the British Pharmacopoeia Commission is that a monograph for a single dosage form should be prepared to cover each available medicinal product of a particular pharmaceutical form containing the same active pharmaceutical ingredient (API). Preparation of a product-specific monograph is avoided where possible. However, similar biological medicinal products present a different situation because, unlike other APIs produced by chemical synthesis, the structures, although very similar, may not be identical. This has an impact on the approach used for the preparation of pharmacopoeial specifications which depend on the degree of similarity or otherwise: whether the differences are translational or post-translational and whether or not test methodology is available to distinguish between similar biological medicinal products and the reference product.

In certain cases a single monograph may be possible for the 'reference' product and any biological medicinal products authorised subsequently whereas in other cases separate monographs may be needed. Consequently the BP Commission has acknowledged that similar biological medicinal products should be considered on a case-by-case basis as to whether a single monograph is sufficient or a group monograph, that covers more than one subsequently authorised product, or several individual monographs are needed.

BP monographs for dosage forms assist in providing an assurance on the quality expected for a medicinal product during its shelf life and provide a framework for use by the manufacturer. However, the analytical tests used are limited in their ability to detect all characteristics in a similar biological medicinal product that may affect clinical efficacy and safety. Aspects such as excipients, pH, buffers, exposure to surfaces, freezing/thawing and mechanical stresses may result in aggregation effects and/or chemical changes during formulation and storage. These may impact on the immunogenicity and clinical performance.

As with other pharmacopoeial monographs, it is recognised that revision of a published monograph for a dosage form may become necessary in order to take account of additional similar biological medicinal products as they become available. Manufacturers are encouraged to inform the BP Secretariat of revisions considered necessary and to provide constructive comments. A list of contact points in the Secretariat is to be found in Supplementary Chapter III A.

Monograph development for similar biological medicinal bulk materials and products

The following aspects are considered when elaborating monographs.

GROUP OR SINGLE MONOGRAPH

If possible, a single monograph is prepared for one or more similar biological medicinal bulk materials and products that have been granted marketing authorisations by comparison with an appropriate reference material. The following points are taken into consideration as to whether one or more monographs are prepared.

1. Active substance with a protein backbone Where the substance is a protein of known structure and amino acid sequence without modification, a single monograph, or modification of an existing monograph, for example to take account of a new method of production, may suffice.

2. Active substance with translational differences Where there is a difference in an amino acid sequence of the protein backbone separate monographs will be needed for the resulting substances whether or not similar products exist e.g. monographs for Insulin Aspart and Insulin Lispro and their relevant injection monographs. Identification methods are needed to distinguish between the structurally related active substances.

3. Active substance with post-translational modifications Substances with identical amino acid sequences in the protein backbone, but with post-translation differences, may be encompassed by one group monograph. The differences may be in the glycosylation, acetylation and/or sulfation. The erythropoietins, a family of closely-related glycoproteins, having the same amino acid sequence in the protein backbone and differences in their isoform compositions, are covered by the bulk material monograph for Erythropoietin Concentrated Solution and a single monograph for the injection.

4. Glycosaminoglycans Separate monographs are usually required e.g. for low molecular weight heparins, there is a group (family) monograph for Low-Molecular-Weight Heparins, that cross-refers to the API monographs for Heparin Sodium and Heparin Calcium and to all the low molecular weight API monographs (such as Dalteparin Sodium, Enoxaparin Sodium and Tinzaparin Sodium). Injection monographs for these three low molecular weight heparins are included in the British Pharmacopoeia.

Specific considerations for BP monographs for formulated preparations

The main considerations needed for similar biological medicinal products are equivalent to those used for other biotechnologically-produced biological formulations.

Monographs for formulated preparations are elaborated taking into account points 1 to 4 above. The monographs are prepared in line with the current BP Commission policy relating to generic products where possible, as explained in Supplementary Chapter III D, and in accord with the following statement given in the Chapter: Formulated preparations: '*The tests applied to the bulk drug substance, including those for impurities arising in manufacture of the bulk drug substance, should be applied, wherever possible - with any necessary modification - in order to demonstrate that material of pharmacopoeial quality has been used in making the formulation.*'. For elaboration of a monograph, information is sought on impurities arising from the manufacture and/or storage of the dosage form.

The requirements of the API monograph are used as a basis for the preparation of the first draft monograph for the formulated preparation. However, not all tests may be applicable, such as in the presence of excipients or when the concentration the API is low. In such situations, alternative procedures and/or methods of detection with increased sensitivity are necessary and validated alternative methods are sought from stakeholders. Any validation studies must take into account the appropriate ICH Guidelines on the validation of analytical procedures.

TEST METHODOLOGY IN MONOGRAPHS

When elaborating monographs for similar biological medicinal products consideration is given to inclusion of the test methods that are specified in the Ph. Eur. monograph for the API which are primarily as follows.

Identification Usually, two identity tests based on the unique characteristics of the product, such as molecular size, charge and structure are included together with a cross-reference to the biological or physico-chemical assay are included in the monograph. The test methodologies used in the current specific monographs for formulated preparations are predominantly reverse-phase liquid chromatography, size-exclusion chromatography, polyacrylamide gel electrophoresis and capillary electrophoresis; their inclusion depends on the characteristics of the molecule under investigation.

Tests Tests for heterogeneity, aggregation, impurities and degradation products are considered for inclusion depending on the characteristics of the API and formulated preparation. As for identification, the methodology used is predominantly reverse-phase liquid chromatography, size-exclusion chromatography, polyacrylamide gel electrophoresis and capillary electrophoresis.

More than one method may be included for the same test in circumstances where interference from an excipient may occur in the drug product. An example of this is in the monograph for Erythropoietin Injection where two methods, A and B, are given in the test for Dimers and related substances of higher molecular weight.

Bacterial endotoxins For new monographs, control is demonstrated by compliance with the requirements in the General Monograph for Substances for Pharmaceutical Use. Alternatively, where validated, a monocyte-activation test or recombinant factor C test may be used. A test is included in a monograph only where a specific method has to be described; in these instances, no limit is specified.
For existing monographs, the test (and limit) may be retained where it is an established and appropriate quality standard.

Assay The assay in the monograph for the medicinal substance is included where possible.

Supplementary Chapter X

Supplementary Chapter on the use of Analytical Quality by Design concepts for analytical procedures

This supplementary chapter is intended to provide non-mandatory information that may be of use to all analytical scientists when applying the principles of Analytical Quality by design to the development of analytical methods.

1. Introduction

This chapter describes some principles for the application of enhanced science- and risk-based approaches to Analytical Methods. These approaches have been described as Analytical Quality by Design (AQbD)[1] and this chapter introduces some practical guidance intended to be helpful to analytical scientists. The chapter covers:

- A discussion of Quality Risk Management (QRM)
  - Risk identification tools: Ishikawa/Fishbone
  - Risk analysis tools: FMEA
  - Risk evaluation and control
- Establishment of method understanding
  - Systematic method development and evaluation
  - Experimental design
  - Statistical approaches to support experimental design
- Analytical control strategy and ongoing trending of analytical methods

Prior to the application of these concepts, the purpose of the analytical procedure should be well defined and understood. The required performance of the method should be defined before undertaking QRM processes and method understanding activities. See Appendix III. *Chromatographic Separation Techniques* for specific guidance on system suitability requirements for a chromatographic procedure.

Application of any of the concepts described in this supplementary chapter can support both the development of validation or qualification of robust analytical methods (pharmacopoeial or *alternative methods*), and also the use of these methods during the product lifecycle to assure the quality of a particular drug substance or its pharmaceutical preparation.

Some of the terms used in this supplementary chapter carry different meanings which depend on their application. Where the following words are stated in this chapter, the intended meaning is as follows:

Robustness Effects of changes to the procedure-related method parameters on the performance of the method

Variable Any aspect of a procedure which may cause variance.

Parameter A Variable specifically related to the analytical method, eg: (HPLC Column temperature, solution concentration etc...)

Procedure Entire process, encompassing the method resulting in a reportable value. This may also detail a study design, a sampling plan, an analytical replication strategy[2], and calculations.

Method The operational components comprised of instrumentation, reagents, standards, sample preparations, calibrations, controls, and suitability criteria.

OFAT (One Factor At a Time) An experimental design process which adjusts a single parameter in a stepwise fashion to learn the effect that this parameter has on the performance of a method or process.

Reportable Value The numerical value/s obtained through following set procedures. The reportable value is the final result of the analytical procedure that is to be compared to the acceptance criteria of the specification

AQbD is an evolving area of analytical science and this supplemental guidance chapter will be updated in future editions of the BP. Additional guidance on the application of the concepts to pharmacopoeial methods and their role within the analytical method lifecycle will be included as the principles become more fully conceptualised.

2. Background

Quality by Design (QbD), as defined in ICH Q8, is a 'systematic approach to development that begins with predefined objectives and emphasises product and process understanding and process control, based on sound science and quality risk management'. Principles described in ICH guidelines Q8[3], Q9[4], Q10[5] and Q11[6] can enhance achieving the desired quality of the drug substances and medicinal product, and application of the more systematic approaches described in these guidelines, including defining a control strategy, could facilitate continual improvement and innovation throughout the product lifecycle.

The development and control of an analytical method across the product lifecycle is a key element of the overall product control strategy. A number of key concepts relating to the application of enhanced science-and risk-based approaches to analytical methods have been described as Analytical Quality by Design. The MHRA and British Pharmacopoeia have explored the practical application of these concepts to a pharmacopoeial assay procedure[7] and are applying the learnings from this study to selected individual finished product monographs where appropriate.

3. Application to the British Pharmacopoeia

In general, analytical method performance, and therefore the results generated by an analytical method, are subject to variability during routine use. Therefore it is important to understand the impact of altering method parameters (e.g., temperature, solvent composition, etc. for an HPLC method) on the results generated by the method, as well as the effect of typical changes in method conditions that can occur over

[1] *https://www.researchgate.net/profile/Joachim_Ermer/publication/282481497_Implications_and_opportunities_of_applying_QbD_principles_to_analytical_measurements/links/5799e3ed08ae29e4fe3947c4/Implications-and-opportunities-of-applying-QbD-principles-to-analytical-measurements.pdf ermer et al,*

[2] *see Ermer, J. and C. Agut, Precision of the reportable result of LC assay. Simultaneous optimisation of number of determinations for sample and reference standard. J. Chromatogr. A, 2014. 1358: p. 859-870*

[3] *ICH Q8(R2) - Pharmaceutical Development: https://database.ich.org/sites/default/files/Q8_R2_Guideline.pdf*

[4] *ICH Q9 - Quality Risk Management: https://database.ich.org/sites/default/files/Q9%20Guideline.pdf*

[5] *ICH Q10 - Pharmaceutical Quality System: https://database.ich.org/sites/default/files/Q10%20Guideline.pdf*

[6] *ICH Q11 - Development and Manufacture of Drug Substances: https://database.ich.org/sites/default/files/Q11%20Guideline.pdf*

[7] *https://www.gov.uk/government/consultations/consultation-on-the-application-of-analytical-quality-by-design-aqbd-principles-to-pharmacopoeial-standards-for-medicines*

time and across laboratories (i.e., instrument type/design, reagent quality, sample shakers, analyst training, etc.).

Pharmacopoeial methods are intended to be applicable to a wide range of available formulations. This process often requires a review of registered methods to ensure they are suitable for pharmacopoeial use

The aim of the MHRA's case study was to demonstrate that AQbD can be used to ensure robust and fit-for-purpose methods are included in the BP. The case study achieved these aims and showed the value of the application of AQbD in building quality and efficiency into monograph development.. The BP is continuing to further investigate the principles to a range of pharmacopoeial procedures.

The principles discussed in sections 4, 5 and 6 are intended as complimentary, supplemental information to that included in Supplementary Chapter I. *Basis of Pharmacopoeial Requirements* and Supplementary Chapter III *C. Monograph Development: Guidance to Manufacturers.*

The concepts elaborated in this chapter may help to verify that a published pharmacopoeial method is fit-for-purpose and can be used for the specific product being examined.

The principles and practices discussed in this Supplementary Chapter can be used alone or in combination where their application will add value to the work of the analyst or organisation.

4. Quality Risk Management for Analytical Procedures

The process of building method understanding begins during method development and continues through the formal validation (in line with conventional ICH Q2: Method Validation)[8], verification, method transfer exercises and routine use, including with pharmacopoeial methods. Historically, the BP has utilised risk management principles to inform the laboratory evaluation of analytical methods.

Users of the pharmacopoeia may not have prior knowledge of a given method, above and beyond what is detailed in the pharmacopoeia. Prior knowledge, such as information contained within monographs, supplementary materials in pharmacopoeias, or information in the literature can be used, if available, as Quality Risk Management (QRM) tools. These tools (See ICH Q9) provide a framework for identifying, studying and understanding the risks to method performance and the results generated by the method. The QRM tools are most effective when initiated during method development or evaluation and applied iteratively. This allows inclusion of the most recent knowledge and reassessment of risks based on any new understanding.

Method development should involve a systematic screening of method conditions, including sample preparation, to identify a set of conditions as a starting point for further evaluation via risk assessments and the QRM process. In the case of a compendial method where formal method development may have been performed by the collaborating manufacturer or by an external laboratory, initial screening of method conditions (for example, screening Design of Experiments (DoEs) – see section 5) can aid in identifying conditions to which the method is sensitive and inform the subsequent risk assessment process. For certain procedures, generic methods may be found suitable and specific method development data may not be available.

4.1. Quality Risk Management

The ICH Q9 'Quality Risk Management' guideline, which reached Step 4 in 2005, noted that while there were some limited examples of the use of quality risk management (QRM) in the pharmaceutical industry, QRM could make a greater contribution and be a valuable component of an effective quality system. One specific area where risk management approaches are being used extensively is in the development of robust control strategies for manufacturing processes. Risk management principles can also be used to develop control strategies for analytical methods. Adopting a risk-based approach ensures that controls applied to analytical methods are focussed on those parameters that are most likely to impact the reliability of the analytical result.

4.2. Risk Identification

The risk process begins by systematically identifying the potential variables associated with the analytical procedure. It is useful to perform a structured, theoretical and practical "walkthrough" of the procedure to identify and record all the steps involved. Process mapping and flow charts tools can be used to provide a pictorial presentation of all of the steps involved (figure 1).

For complicated procedures it can be useful to break the steps down into different "unit operations" eg sampling, measurement, reporting etc.

Based on the process map, each of the steps in the procedure is reviewed and parameters that could potentially vary are identified. Tools such as an Ishikawa/Fish Bone diagram can be used to facilitate categorising the risks. (Figure 2).
A platform approach could also be adopted with the use of templates for different types of analytical methods.

4.3. Risk Analysis

Risk Analysis involves estimating the risk associated with each of the variables identified in the previous step.
It considers both the likelihood that the input may vary (probability) and the impact any variation is likely to have on the reportable result (severity). Numerous tools and approaches can be used to facilitate the risk analysis step (see ICH Q9 appendices for a range of examples).

A commonly used tool for performing a quantitative analysis is Failure Mode Effects Analysis (FMEA) – an extract from an example of which is shown in Table 1.

Table 1: Example FMEA extract

Variable	Severity (1 = Low, 5 = High)	Probability of variation (1 = Low, 5 = High)	Risk score
% Acetonitrile in dissolving solvent	3	4	12
Sonication Time (mins)	4	3	12
% Acetonitrile in the mobile phase	3	4	12
Wavelength	4	2	8
% Humidity	1	5	5
Equilibration time	1	2	2

[8] *ICH Q2(R1) - Validation of Analytical Procedures: https://database.ich.org/sites/default/files/Q2%28R1%29%20Guideline.pdf*

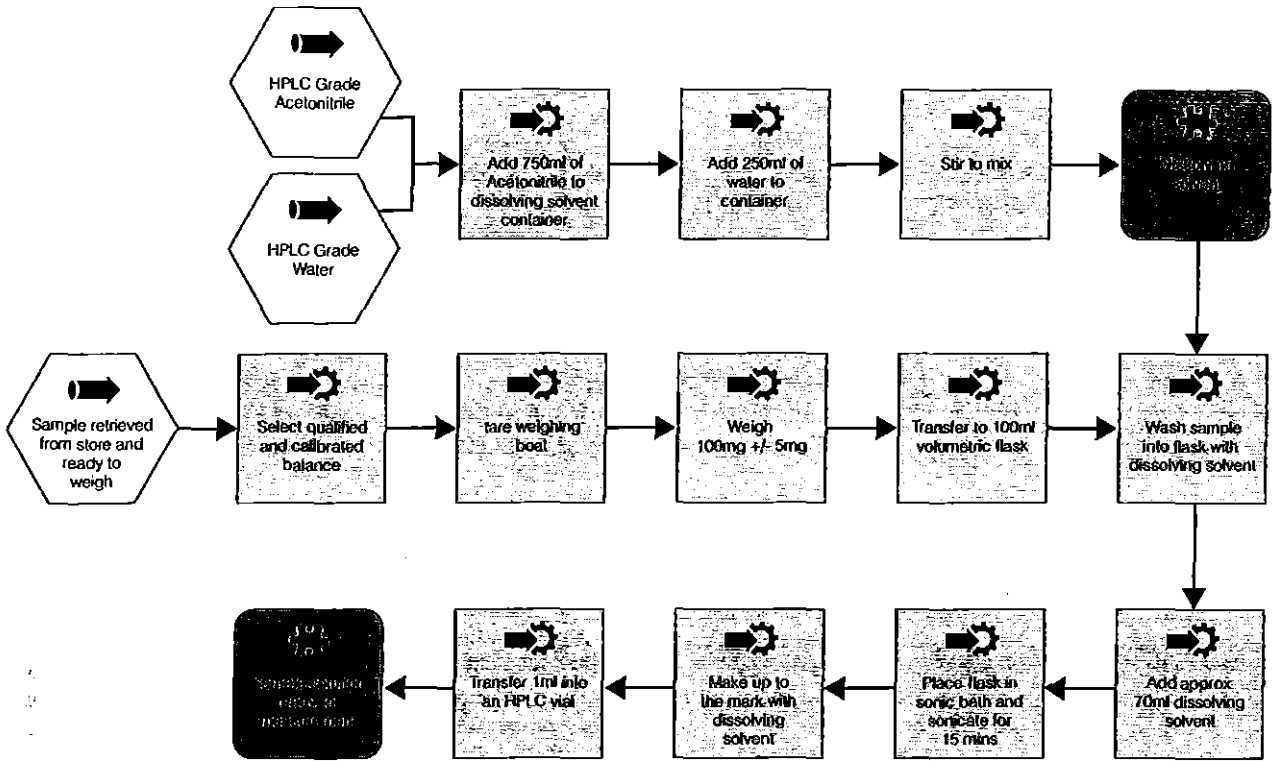


Figure 1[9] – Example of process map for an HPLC analysis

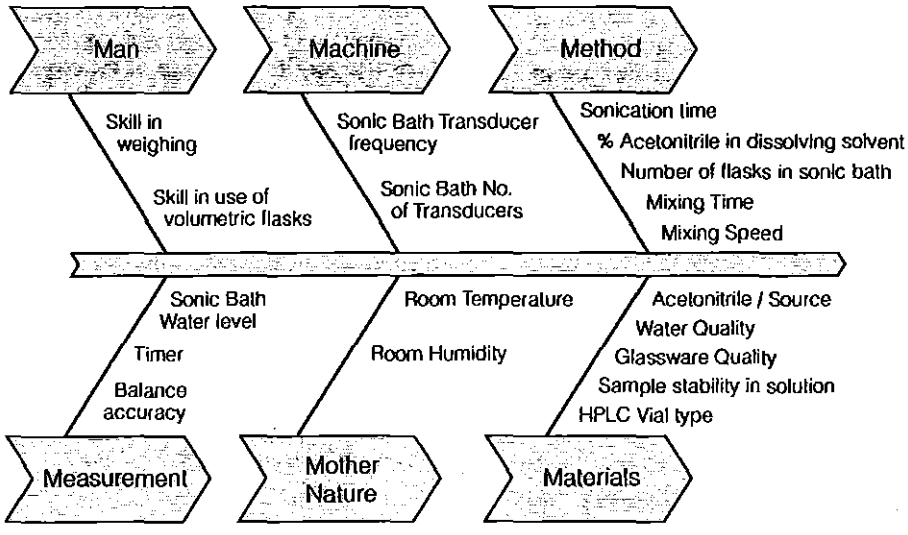


Figure 2[10] – Example of Ishikawa/Fish Bone diagram for sample preparation for HPLC Analysis

FMEA estimates are made for each input variable with respect to the probability (likelihood) of variation and the impact (severity) on the reportable result. As with identifying potential variables, the effectiveness of the risk assessment step is heavily dependent on the knowledge available to support the estimates made. Where the knowledge of the impact of a variable may have on the accuracy and/or precision of the reportable result is limited, experimental studies should be performed to provide this understanding. Design of Experiments (DoE) is an approach that can be used to identify effects of variables (separately and in combination) and minimise the experimental work involved in obtaining the necessary knowledge. When using an FMEA approach it is useful to treat it as an iterative process and update the assessment as more knowledge and understanding becomes available.

4.4. Risk Evaluation and control

Once the risk associated with each input variable is understood, an evaluation of the required controls is performed. These controls should focus on the variables which are most likely to impact the reportable result and reduce the variability and/or mitigate the impact of any variation. This is the first step in building an analytical control strategy for a method (see section 5.1). When using

[9] *see figure 6 of USP 42(5) Stimuli to the revision process: Analytical control strategy: https://www.usp.org/sites/default/files/usp/document/get-involved/stakeholder-forums/stimuli-articles-analytical-control-strategy-analytical-target-profile-2016-10-13.pdf*

[10] *see figure 7 of USP 42(5) Stimuli to the revision process: Analytical control strategy: https://www.usp.org/sites/default/files/usp/document/get-involved/stakeholder-forums/stimuli-articles-analytical-control-strategy-analytical-target-profile-2016-10-13.pdf*

an FMEA approach this means identifying steps that will reduce the probability score so that the overall risk is reduced. Once such steps have been identified, the risk score should be reassessed with suitable controls in place.

Where the probability of variation cannot be reduced significantly, consideration should be given to whether there are opportunities to detect unacceptable variation before the reportable result is generated, such that it can be invalidated. For example, if batch-to-batch variation in chromatographic packing material cannot be eliminated, a resolution check may be introduced to detect potential impact on the ability of the method to resolve and accurately quantify analytes (see section 6.2 for trending concepts). Ideally "system suitability" tests can help to mitigate known risks by assuring that the method is performing as designed.

5. Establishing method understanding

An analytical control strategy is a planned set of controls to ensure the reportable value obtained from procedures are fit-for-purpose for the product, derived from an understanding of the analytical procedure and the management of risk.

The effectiveness of a risk assessment, and ultimately the analytical control strategy for a given method, is related to the level of understanding of the relationship between method parameters and the method output, i.e. the result for the specific product being tested. Section 4 sets out approaches to ensuring method understanding that can be adopted throughout the product lifecycle.

The MHRA and British Pharmacopoeia case study has been used to illustrate how the Pharmacopoeia may apply AQbD concepts when assessing a method's suitability for compendial use. It is not expected that all of the concepts are used for assessment of the suitability of every method.

5.1. Systematic method development and evaluation

In support of risk assessments, experimental studies are conducted to understand the impact of method parameters and environment on method performance. These experimental studies are usually designed to evaluate:

- the influences of deliberate variations in procedure-related method parameters (solvent strength, pH, sample concentration, etc.)
- the influences of 'noise' factors (analyst, column batch etc.) which typically cannot be, or are preferred not to be controlled.

When investigating deliberate variations in procedure-related method parameters multifactor empirical modelling (e.g., DoE's) is generally preferred compared to One factor at a time (OFAT) experiments, though OFAT may be appropriate in some cases. Mechanistic modelling, or combined mechanistic and empirical modelling, have the potential to reduce the experimental burden and can be employed when demonstrated to be appropriate. The effect of method changes on the results generated by the method can be considered in addition to other key method attributes (e.g. resolution, sensitivity, accuracy, etc.) to understand method performance and to optimize method conditions.

'Noise' factors can often be the root cause of issues arising through the routine use of analytical methods. It is possible that not all of these factors can be studied experimentally, however through the systematic investigation of those that can be studied, the risk of method failure may be reduced. Investigations of noise factors typically require designs applicable to parameters with discrete values (e.g. column temperature) and are used to challenge the method and understand their effect on precision in the longer term.

Identification of a noise factor having a large effect on the variation of test results may indicate that control of additional method parameters is needed.

The case study also investigated noise factors as it involved the investigation of multiple product suppliers, which is mainly applicable to pharmacopoeial use. A more typical example of the study of noise factors in provided in section 5.2. By necessity, the case study explored these concepts extensively and it would not necessarily be appropriate or effective to uniformly apply for all methods.

5.2. Utilising method development tools and statistical analysis

Analytical method parameters are factors related to the operation of the method which can be specified within a continuous range or at controllable, unique levels. Experimentation which uses fractional factorial designs (DoEs) to develop general purpose (linear) models describing the effect of changes in analytical method parameters is outlined below. These models are used to identify and/or confirm (typically) a setpoint and ranges for analytical method parameters, which in combination assure robustness. It is possible to define a wider operating region throughout which the method may be used but this is not typically done.

As part of the practical application of AQbD to the pharmacopoeial Assay procedure, DoEs were performed to investigate sample extraction, chromatography and solution stability. The two-level fractional factorial design for the investigation of four mobile phase factors is illustrated in Figure 3. A full two-level factorial design consists of all the combinations of chosen low and high levels of the parameters as represented by the corners of the cubes. A fractional factorial design uses a specific set of points (fraction) - as shown by the solid circles in this example. Here it is a half fraction because the design uses 8 out of the possible 16 (2^4) combinations of low and high levels for the four factors. In addition, centre points (mid value between low and high and often the planned setpoint for the method) are used to estimate variability at the same conditions and assess whether a linear model in the parameters is adequate. Fractional factorial designs are very effective due to hidden replication (half of the factorial points are performed at a level of a parameter rather than just one for OFAT experiments) and it is not necessary to run all factorial combinations (an adequate model is usually obtained from the effects of single factors or interactions between two).

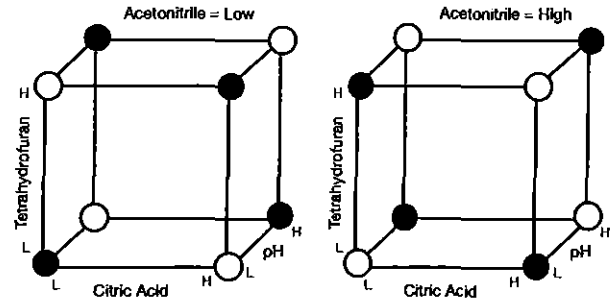


Figure 3: Fractional factorial design (solid circles) for four factor mobile phase experiment

Whilst Figure 3 illustrates the concept, a design listing (see Table 2) is used to describe the design. It is useful for any number of parameters to be investigated and describes the

Table 2: Design Listing for Mobile Phase Composition DoE

		Factor A	Factor B	Factor C	Factor D	Response 1
Run Order	Space Type	Citric Acid (g)	pH	Tetrahydrofuran (mL)	Acetonitrile (mL)	Atorvastatin Tailing Factor
1	Factorial	8.66	3.8	130	175	1.12
2	Factorial	8.66	3.8	70	95	1.05
3	Centre	9.62	4	100	135	1.07
4	Factorial	10.58	3.8	130	95	1.06
5	Factorial	10.58	4.2	70	95	1.06
6	Factorial	8.66	4.2	130	95	1.1
7	Factorial	8.66	4.2	70	175	1.09
8	Centre	9.62	4	100	135	1.1
9	Centre	9.62	4	100	135	1.08
10	Factorial	10.58	4.2	70	175	1.06
11	Factorial	10.58	4.2	130	175	1.17
12	Centre	10.58	4	100	175	1.08

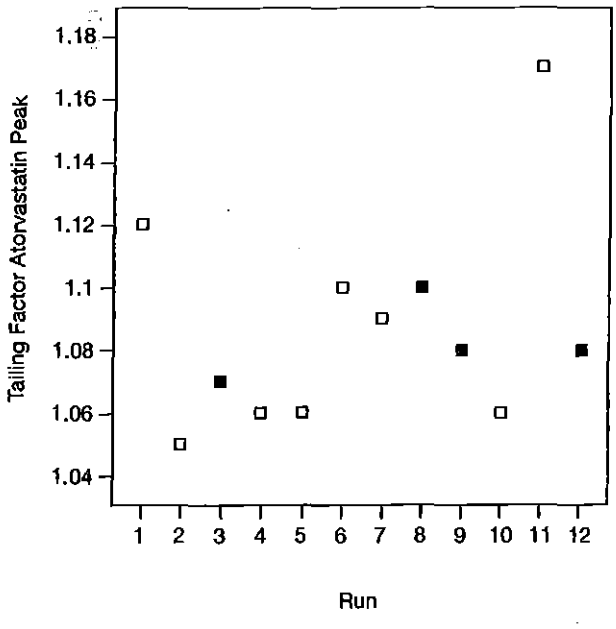


Figure 4: Tailing factor for the Atorvastatin peak plotted against run (centre points are shaded)

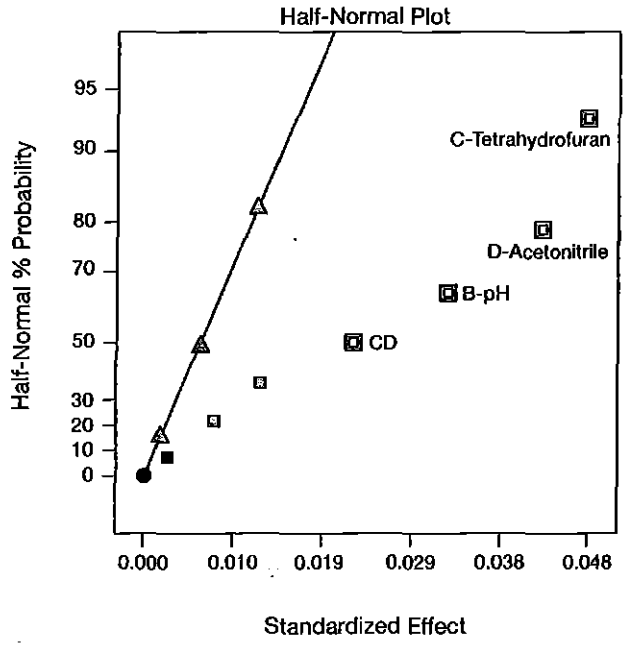


Figure 5: Half-normal plot for model terms for Tailing Factor for Atorvastatin peak

random order in which the design is to be run. The method is operated under the condition for each run in turn and the results recorded. For illustration, the tailing factor response is listed in Table 2 and plotted in Figure 4.

The acceptance criteria for the tailing factor of the Atorvastatin peak is ≤ 1.2. If all the results from the DoE runs had been far below the acceptance criteria a statistical analysis may not be required, though if there is a wide range between the factorial points it is useful to understand which parameters may cause this. Here it is seen that one run had a value of 1.17. Although this meets the criteria it is possible that a combination of factor settings not explored in the design may exceed 1.2. Also, it is noted that repeating the same conditions will give variable results (see variation in the centre points). Thus, a statistical analysis is desired.

The first step in the statistical analysis is to identify which are the major parameter effects. Figure 5 illustrates one tool – a half-normal plot. Model terms are selected (represented by small squares) which are greater than background noise (to the right of the line through the triangles which represent the background variability estimated from the four centre points). Parameters Tetrahydrofuran (C), Acetonitrile (D) and pH (B) have the largest effects. The fourth term selected is an interaction between two parameters. Statistically it is not possible from the design to know whether this is the interaction between A and B or C and D. Since C and D are the largest effects and this makes analytical sense the CD interaction was also included in the model. The next term was not included in the model. It was not statistically significant, it is small and the design cannot distinguish between the two possible interactions, BC and AD.

A statistical analysis will typically assess statistical significance of terms identified for inclusion in the model and assess

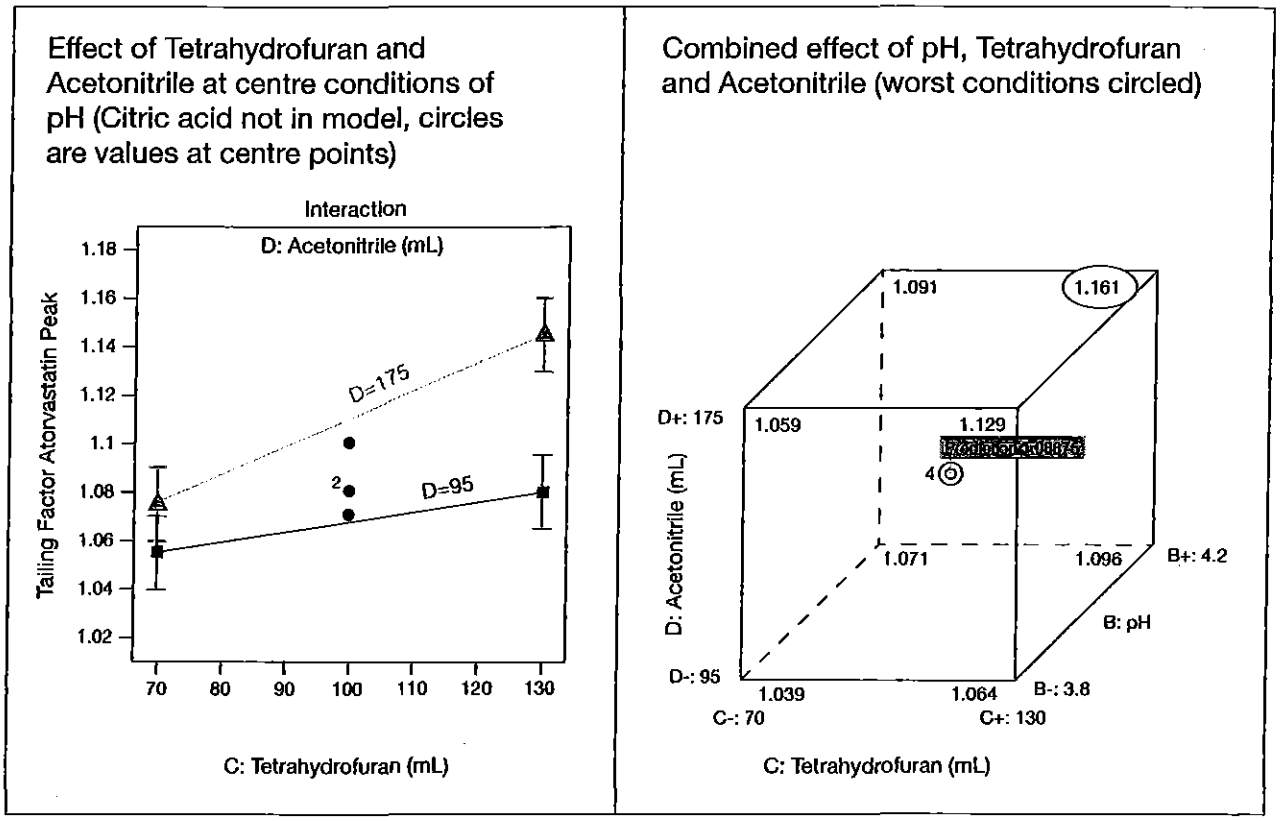


Figure 6: Effect of Parameters on Tailing Factor.

assumptions used in the modelling (linear model is adequate, residuals are normally distributed and no outliers are observed). The model is then used to predict the effect of method parameters. Figure 6 shows the effect of Tetrahydrofuran, Acetonitrile and pH on the mean tailing factor.

Various system suitability responses were examined (tailing factors, efficiencies, resolution, retention times). In addition, the effect of method parameters on %Assay may be examined. The %RSD of %Assay over the combinations investigated may be useful in addition to a similar analysis to that applied to the tailing factor.

If the model only contains linear terms for parameter effects and any interactions then it is sufficient to establish that results are acceptable at the limits of the parameter ranges (in combination). If the model is more complex than linear e.g. non-linear, or a mechanistic model, then evaluation within the parameter ranges may be required. If a model with linear terms is insufficient, response surface designs may be used to include quadratic terms to model curvature. If the results are not acceptable at the limits of the parameter ranges, it may be possible to use the model to predict ranges within those investigated, in which the method could be satisfactorily operated or further experimentation may be required to identify an acceptable region. Where mechanistic models are available (e.g. chromatographic in-silico modelling systems), designs appropriate to the model should be applied (and in some cases little experimentation may be required).

Examples in literature[11] provide a very comprehensive explanation of full and fractional factorial designs and the application of experimental design to analytical methods (focused on chromatography)[12]. The design should be carefully chosen to meet the purpose of the study – is the design intended to identify parameters having an effect or optimise a method over a wide parameter region, or to assess robustness around the setpoint. A limited evaluation of the data could be gained from plotting the data and calculating means at the low and high levels for each factor to establish which has the largest effect. However, to understand interactions between factors, assess statistical significance (i.e. that effects seen are likely not to be due to chance) and produce predictions, software that can fit general purpose models with linear and quadratic terms in the parameters is likely to be required. Software which combines functionality for designing factorial type experimentation with corresponding statistical analysis is particularly helpful. The software may be integrated with a chromatographic system, focused on designed experiments (either entirely or a specific module) or more general statistical software.

5.3. Assessment of variation in method operating conditions

The variations in method operating conditions considered here are typical changes in method conditions that can occur over time and across laboratories, (i.e., instrument type, reagent quality, sample shakers, analyst training, etc.). These are changes which are not specified or controlled as method parameters- "noise factors". Studies are designed with the purpose of challenging the method and to understand their effect on precision in the longer term. Noise factors having a large effect on the precision can be identified and further investigated with the aim of improvement and/or control

[11] *https://www.springer.com/gp/book/9780387891026 - Mee, Robert. A comprehensive guide to factorial two-level experimentation. Springer Science & Business Media, 2009.*

[12] *https://pubmed.ncbi.nlm.nih.gov/22333438/ - Hibbert, D. Brynn. "Experimental design in chromatography: a tutorial review." Journal of chromatography B 910 (2012): 2-13.*

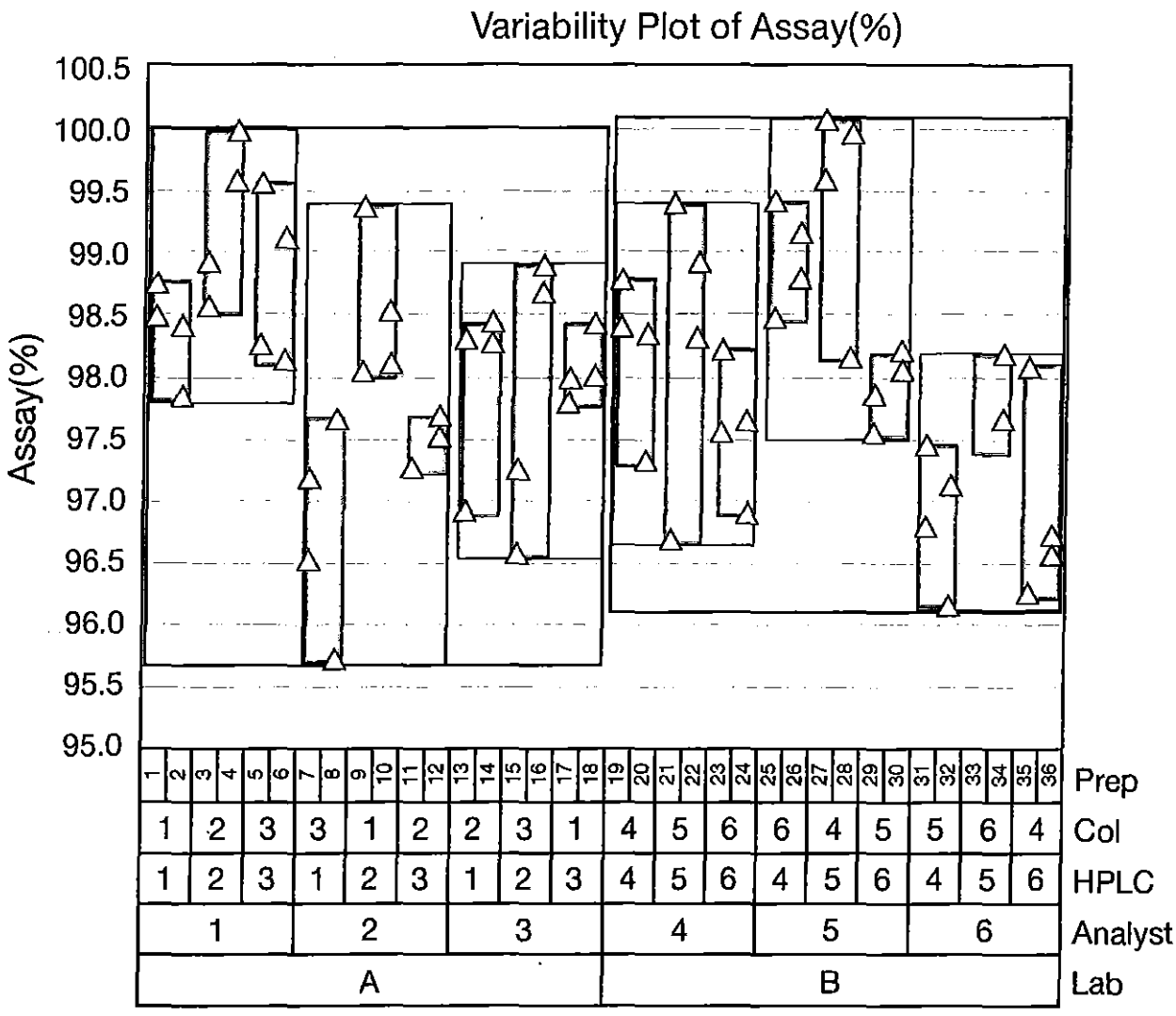


Figure 7: Plot of assay results from a study of potential noise factors

where possible. In contrast to the designs for assessing the effect of analytical method parameters which predominantly evaluate continuous factors, the focus of these studies is on factors with discrete levels, uncontrolled in the method description e.g. analyst. It is often useful to perform such a study as part of a method transfer as the two activities can be combined saving resource and the use of two labs provides more challenge to the method.

Figure 7 shows an example study – the design should be chosen based on the risk assessment and the practicalities which apply. The factors and chosen levels can be seen at the bottom of the plot. Factors may be "crossed" or "nested" in such a design. In the example analyst, HPLC and column (col) are nested within lab, i.e. an analyst only occurs at one site. Preparation level is nested within each analyst, HPLC and column combination and two injections are performed for each prep. However, analyst, HPLC and column are crossed with each other i.e. an analyst uses more than one HPLC equipment. It is desirable to use a reasonable number of levels for all factors in the design (not just preps and injections) in order to better allow any effects/variation due to the factor to manifest itself during the study (acknowledging that there will be practical constraints).

The statistical analysis typically consists of visualising the data and fitting models to estimate the variation due to each noise source or their interactions (variance components). This is usually a variance components or mixed model analysis.

Figure 8 shows the results of a statistical analysis with the estimated variance components shown for the various factors. It is seen that the injection variation and analyst variation contribute most to the variation. Some variability due to HPLC is also seen but the column and preparation contribute very little variability.

Figures 7 and 8 show the clear benefits of using data visualisation to identify the cause/s of variation. Figure 5 appears to show variation at all levels other than between laboratories. This indicates a systematic issue with the methodology, which is being replicated from Lab A to Lab B. Digging deeper, figure 6 shows that the main proportion of variance is from injection to injection and analyst to analyst, rather than preparation, column or HPLC instrument. This type of analysis can be key to both identifying issues with procedures during validation as well as during a root cause analysis on existing procedures, where an issue may have occurred.

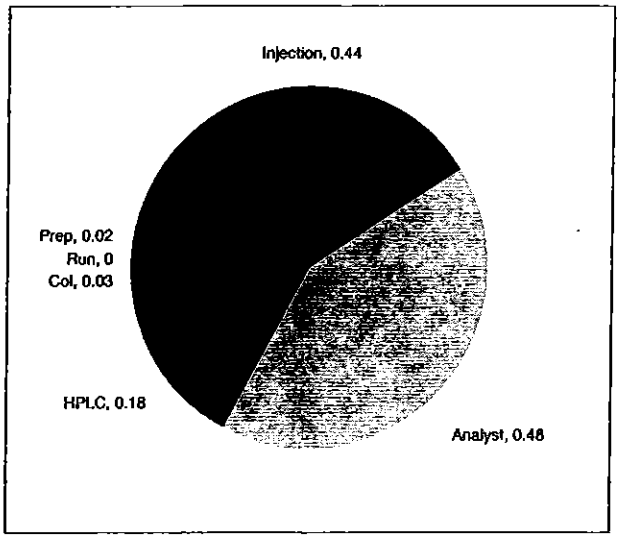


Figure 8: Variance components from various noise factors

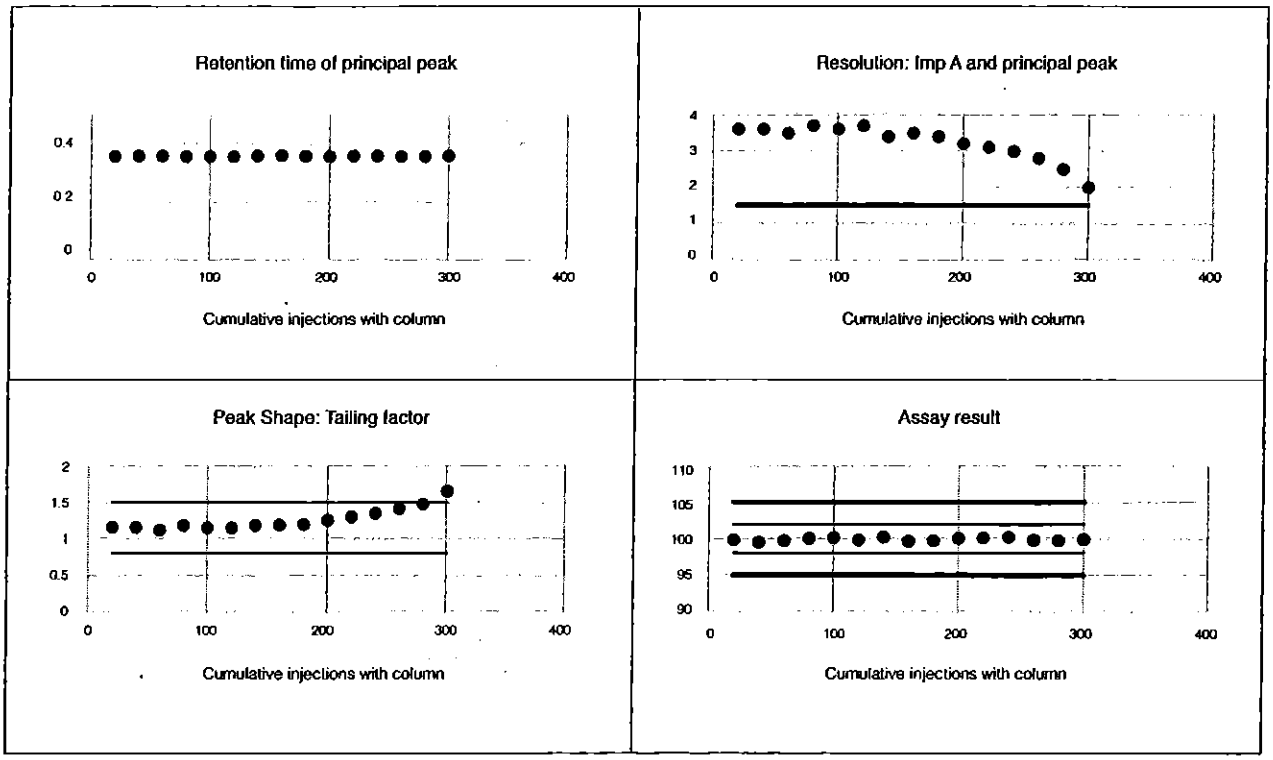


Figure 9 – Example of control charts monitoring different aspects of ongoing method performance

The precision of the analytical measurement can be calculated by summing the variance estimates, taking into account any replication used in providing the reported result. For example, the intermediate precision (variance) for a reported result from the mean of two preparations would be found by summing the variance components for between analytical run sources of variability (Analyst, HPLC, Column, Run) and half of the preparation and injection components of variance. Thus:

$$SD = \sqrt{0.48 + 0.18 + 0.03 + 0 + \frac{0.02}{2} + \frac{0.44}{2}} = 0.96$$

The overall mean of the results was 98.0 giving:

$$\%RSD = 100 \times \frac{0.96}{98.0} = 0.98\%$$

Additional literature sources provide further details and examples of such studies[13]. Performing the study and plotting the data can be useful in its own right. However, to perform the statistical analysis to gain most information from the study, software that can fit mixed models is likely to be required (usually Restricted Maximum Likelihood – REML is used).

[13] *https://pubmed.ncbi.nlm.nih.gov/21889624/ - Borman, Phil J., Marion J. Chatfield, Ivana Damjanov, and Patrick Jackson. "Method ruggedness studies incorporating a risk-based approach: a tutorial." Analytica chimica acta 703, no. 2 (2011): 101-113.*

6. The analytical control strategy and its role in ongoing monitoring of method performance.

6.1. Analytical Control Strategy

ICHQ10 defines a control strategy as: *a planned set of controls derived from product and process understanding, that assures process performance and product quality. The controls can include parameters and attributes related to drug substance and drug product materials and components, facility and equipment operating conditions, in process controls, finished product specifications and the associated methods and frequency of monitoring and control.*

Clearly this definition applies to manufacturing processes however the idea of a control strategy for analytical methods mirrors the above if the output of the process is regarded as the reportable result rather than a drug product. The key point is that the control strategy should be comprehensive and consider all of the materials used, operating conditions, system suitability checks, etc that are required to assure the quality of the reportable result.

Sections 4 and 5 detail the concepts one may adopt to produce an analytical control strategy built on deep method and product understanding. The MHRA case study confirmed that to complete a suitable control strategy for the atorvastatin tablets pharmacopoeial Assay method was to follow system suitability criteria in the form of a minimum resolution between a critical pair (Atorvastatin and close eluting related substance).

For a pharmacopoeial method, the chromatographic conditions and permissible adjustments set out in Appendix III Chromatographic Separation Techniques are complementary to the analytical control strategy developed in line with the processes described in this supplementary chapter.

6.2. Ongoing monitoring of methods

Monitoring of the results of analytical methods helps to build process capability insights. However, additional trending of a range of different method outputs is another strategy used to identify areas of risk and alert users to take action before method failure.

Monitoring an analytical method is helpful since it can alert users to trends. There are different ways to conduct monitoring – trending results, control sample results, performance attributes (e.g. retention time of specific analytes, peak shape of principal peaks and more). Monitoring of measurements for reference or standard material repeatably analysed over time may also be useful.

Figure 9 is a simple model of additional parameters that may require trending through the lifetime of a method (or in this case, an individual column). Here you can see an inverse relationship between the resolution of critical pair and the peak shape of the drug substance. In this instance, an increase in peak tailing is causing a loss in separation between the drug substance and a major impurity. Ultimately, the Assay value here is unaffected, however the resolution is heading towards a failure of system suitability criteria, as is the peak shape.

The BP appreciates that the modelling and subsequent decision point to change a column, detailed above, is a small and distinct use for the monitoring of an analytical method. More detailed monitoring and change is likely to add benefit to users[14] and may be built on in future iterations of this supplementary chapter. It is noted that statistical techniques may also be useful in monitoring the performance of system suitability and/or reference measurements over time.

[14] *see Ermer, J., et al., Lifecycle management in pharmaceutical analysis: How to establish an efficient and relevant continued performance monitoring program. J Pharm Biomed Anal, 2020. 181: p. 113051*

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Zinc Stearate, II-1336
Zinc Sulfate, V-A160
Zinc Sulfate Capsules, III-1516
Zinc Sulfate Eye Drops, III-1516
Zinc Sulfate Heptahydrate, II-1337
Zinc Sulfate Heptahydrate, *see Zinc Sulfate*
Zinc Sulfate Hexahydrate, II-1337
Zinc Sulfate Injection, III-1517
Zinc Sulfate Lotion, III-1517
Zinc Sulfate Monohydrate, II-1338
Zinc Sulfate Tablets, III-1517
0.1M Zinc Sulfate VS, V-A168
Zinc Sulphate, *see Zinc Sulfate*
Zinc Sulphate Capsules, *see Zinc Sulfate Capsules*
Zinc Sulphate Eye Drops, *see Zinc Sulfate Eye Drops*
Zinc Sulphate Injection, *see Zinc Sulfate Injection*
Zinc Sulphate Lotion, *see Zinc Sulfate Lotion*
Zinc Sulphate Tablets, *see Zinc Sulfate Tablets*
Zinc Undecenoate, II-1338
Zinc Undercylenate, *see Zinc Undecenoate*
Zincum Sulfuricum, V-A705
Ziprasidone Hydrochloride Monohydrate, II-1339
Ziprasidone Mesilate Trihydrate, II-1341
Zirconium Standard Solution (1 g/L Zr), V-A173
Zirconyl Chloride, V-A160
Zirconyl Nitrate, V-A160
Zirconyl Nitrate Solution, V-A160
(*Z*)-Ligustilide, V-A92
Z-Ligustilide CRS, V-A181
Zoledronic Acid Monohydrate, II-1343
Zolmitriptan, II-1345
Zolpidem Tablets, III-1519
Zolpidem Tartrate, II-1347
Zopiclone, 151, II-1349
Zopiclone Tablets, III-1520
Zuclopenthixol Acetate, 152, II-1350
Zuclopenthixol Acetate Injection, III-1522
Zuclopenthixol Decanoate, II-1352
Zuclopenthixol Decanoate Injection, III-1523
Zuclopenthixol Hydrochloride, 152, II-1353
Zuclopenthixol Tablets, III-1524