The Ministry of Health, Labour and Welfare Ministerial Notification No. 461

In accordance with the provisions of Article 41, Paragraph 1 of the Pharmaceutical Affairs Law (Law No. 145, 1960), we hereby revise a part of the Japanese Pharmacopoeia (Ministerial Notification No. 111, 2001) as follows, and the revised Japanese Pharmacopoeia shall come into effect on January 1, 2005, [including deletion from Official Monographs fro Part II in The Japanese Pharmacopoeia, Fourteenth Edition of the articles of Absorbent Cotton, Purified Absorbent Cotton, Sterile Absorbent Cotton, Sterile Purified Absorbent Cotton and Absorbent Gauze and Sterile Absorbent Gauze (hereinafter referred to as ''sanitary materials'')]. Proviso: With respect to the drugs which are included in the Japanese Pharmacopoeia (hereinafter referred to as ''the old Japanese Pharmacopoeia'') [limited to those included in the Japanese Pharmacopoeia whose standards are changed with this notification published (hereinafter referred to as ''the new Japanese Pharmacopoeia'')] and those which are approved as of January 1, 2005 pursuant to the provisions of Article 14, Paragraph 1 of this Law (including cases where it shall apply *mutatis mutandis* under Article 23 of this Law; the same hereinafter) [including the drugs designated as those exempted from approval (hereinafter referred to as ''the drugs exempted from approval'') among the drugs etc. designated by the Minister of Health, Labour and Welfare as those exempted from manufacturing or import approval pursuant to the provisions of Article 14, Paragraph 1 of the Pharmaceutical Affairs Law (Ministerial Notification No. 104, 1994), the standards established in the old Japanese Pharmacopoeia (limited to the standards for the relevant drugs) shall be recognized, up to June 30, 2006, as the standards established in the new Japanese Pharmacopoeia. With respect the drugs which are included in the new Japanese Pharmacopoeia (excluding those which are included in the old Japanese Pharmacopoeia) and those which are approved as of January 1, 2005 pursuant to the provisions of Article 14, Paragraph 1 of this Law (including the drugs exempted from approval), the drugs may be treated, up to June 30, 2006, as those which are not included in the new Japanese Pharmacopoeia. Further, sanitary materials may be treated, up to September 30, 2006, under the previous regulation.

Hidehisa Otsuji
The Minister of Health, Labour and Welfare

December 28, 2004

(The texts referred to by the term ''as follows'' are omitted here. All of them are made available for public exhibition at the Evaluation and Licensing Division, Pharmaceutical and Medical Safety Bureau, Ministry of Health, Labour and Welfare, at each Regional Bureau of Health and Welfare and at each Prefectural Office in Japan.)

The term ''as follows'' here indicates the contents from Part I to Ultraviolet-visible Reference Spectra in the Supplement II to the Japanese Pharmacopoeia Fourteenth Edition (pp. 1669 – 1866).

CONTENTS

Preface

The Fourteenth Edition of the Japanese Pharmacopoeia was promulgated on March 30, 2001 by Ministerial Notification No. 111 of the Ministry of Health, Labour and Welfare. To keep pace with progress in medical and pharmaceutical sciences, in November 2001, the Council, at a meeting of the Committee on Japanese Pharmacopoeia (JP), established the basic principles for the preparation of the JP Fifteenth Edition, setting out the characteristics and roles of the JP, the definite measures for the revision, the date of the revision, and the organization of the Subcommittee on JP.

At the above meeting, the following ''five pillars'' were established as the basic principles of the JP: Making it more substantial by including all drugs which are important from the viewpoint of health care and medical treatment; Making prompt partial revision as necessary and facilitating smooth administrative operation; Promoting international harmonization; Ensuring transparency regarding the revision and dissemination to the public of the JP; and Positively introducing contemporary analytical tests and developing reference standards. It was decided at the meeting that each panel set up under the Subcommittee on JP should make efforts, on the basis of these principles, to ensure that the JP is used more effectively in the fields of health care and medical treatment by taking appropriate measures, including getting the understanding and cooperation of other parties concerned.

The JP should comprise an official standard being required to assure the quality of drugs in this country in response to the progress in science and technology and clinical demands at the time, it should define the standards for specifications as well as the methods of tests to assure the overall quality of all drugs in principle, and it should have a role in clarifying the criteria for quality of drugs which are recognized to be important from the viewpoint of medical treatment.

At the same time, it was agreed that the JP should be prepared with the aid of the knowledge and experience of many persons involved in the pharmaceuticals, that it should have the characteristics of an official standard, which might be widely used by all parties concerned, that it should provide information and understanding about the quality of drugs to the public, and that it should be conducive to smooth and effective government control of the quality of drugs, and to securing and maintaining international consistency.

It was also agreed that JP articles should cover drugs which are important from the viewpoint of health care and medical treatment, clinical results and frequency of use, as soon as possible after they reach the market.

It was also decided to make a definite rule for selection of articles by clarifying the standards for selection. The rule was shown in the verdict ''What the future Japanese Pharmacopoeia should be'' by the Pharmaceutical Affairs and Food Sanitation Council (PAFSC) on December 2002. The JP Fifteenth Edition was decided to be slated for completion in April 2006.

The panels on JP was reorganized into the following eleven panels in accordance with the recommendation of the PAFSC: Panel on Planning and Revisions; Panel on Nomenclature for Pharmaceuticals; Panel on Excipients; Panel on Physico-chemical Tests; Panel on Medicinal Chemicals; Panel on Biologically Derived Drugs; Panel on Biological Tests; Panel on Antibiotics; Panel on Crude Drugs; Provisional Panel on Planning and Revisions and Panel on Pharmacopoeial Harmonization (PDG), followed by the establishment of new panels: Panel on Water for Pharmaceutical Preparations, Panel on JP Reference Standards, and three working groups under Panel on Medicinal Chemicals to expedite discussion of revision drafts of drug monographs.

In the Committee on Japanese Pharmacopoeia, Mitsuru Uchiyama took the role of chairman from January 2001 to December 2002, Tadao Terao from January to June 2003, and Takao Hayakawa from July 2003 to December 2004.

It was decided that the JP will be revised not only every five years, in line with the basic principles for the preparation of the JP Fifteenth Edition, but also as necessary to take account of recent progress of science and in the interests of international harmonization.

In accordance with the revision principles, the panels continued discussions on selection of articles, and revisions for General Notices, General Rules for Preparations, General Tests, and monographs on drugs, and the supplement I to JP 14 was promulgated on December 2002. After the promulgation of the supplement I, the panels continued discussions to take account of the progress of science and international

harmonization.

Draft revisions covering subjects in General Rules for Crude Drugs, General Tests, and monographs on drugs, for which discussions were finished between March 2002 and December 2003, were prepared for a supplement to the book. They were examined by the Committee on Japanese Pharmacopoeia in September 2004, followed by the PAFSC in December 2004, and then submitted to the Minister of Health, Labour and Welfare.

Numbers of discussions in the panels to prepare supplement drafts were as follows: Panel on Planning and Revisions, 9 times; Panel on Nomenclature for Pharmaceuticals, 10 times; Panel on Excipients, 11 times; Panel on Physico-chemical Tests, 30 times; Panel on Medicinal Chemicals (including the working groups), 24 times; Panel on Biologically Derived Drugs, 11 times; Panel on Biological Tests, 10 times; Panel on Antibiotics, 19 times; Panel on Crude Drugs, 19 times; Provisional Panel on Planning and Revisions, 12 times; Panel on Pharmacopoeial Harmonization (PDG), 9 times; Panel on Water for Pharmaceutical Preparations, 2 times; Panel on JP Reference Standards, 3 times.

It should be noted that in the preparation of the drafts for the Supplement I, generous cooperation was given by the Technical Committee of the Pharmaceutical Manufacturer's Association of Tokyo and of Osaka, the Crude Drugs Association of Tokyo, the Japan Pharmaceutical Excipients Council, the Federation of Crude Drugs Associations of Japan, the Japan Antibiotics Research Association, the Japan Flavor and Fragrance Manufacturer's Association, the Japan Medical Plants Federation, the Japan Pharmaceutical Manufacturer's Association, the Japanese Society of Hospital Pharmacists, the Japan Pharmaceutical Association, and the Japan Oilseed Processors Association.

In consequence of this revision, the JP Fourteenth Edition carries 907 articles in Part I owing to the addition of 27 articles and the deletion of 1 article; and 484 articles in Part II owing to the addition of 12 articles and the deletion of 9 articles.

The principles of description and the salient points of the revision in this volume are as follows:

1. The Supplement II to JP Fourteenth Edition comprises the following items, in order: Notification of the Ministry of Health, Labour and Welfare; Contents; Preface; followed by General Tests, Processes and Apparatus; monographs on drugs in Part I, and General Rules for Crude Drugs; monographs on drugs in Part II, then followed by Ultraviolet-visual Reference Spectra in Part I; Infrared Reference Spectra and Ultraviolet-visible Reference Spectra in Part II; General Information, and as an appendix, a Cumulative Index containing references to the main volume, the supplement I and the supplement II.

2. The articles in General Tests, Processes and Apparatus, Monographs on Drugs, Infrared Reference Spectra and Ultraviolet-visible Reference Spectra are respectively placed in alphabetical order.

3. The following items in each monograph are put in the order shown below, except that unnecessary items are omitted depending on the nature of the drug:

(1) English title
(2) Commonly used name(s)
(3) Latin title (only for Crude Drugs)
(4) Title in Japanese
(5) Structural formula or empirical formula
(6) Molecular formula and molecular mass
(7) Chemical name
(8) Origin
(9) Limits of the content of the ingredient(s) and/or the unit of potency
(10) Labeling requirements
(11) Method of preparation
(12) Description
(13) Identification tests
(14) Specific physical and/or chemical values
(15) Purity tests
(16) Loss on drying, loss on ignition, and/or water
(17) Residue on ignition, total ash, and/or acid-insoluble ash
(18) Tests being required for pharmaceutical preparations and other special tests
(19) Isomer ratio
(20) Assay or the content of the ingredient(s)
(21) Containers and storage
(22) Expiration date
(23) Others

4. In each monograph on a drug, the following physical and chemical values representing the properties and quality of the drug are given in the order indicated below, except that unnecessary items are omitted depending on the nature of the drug:

(1) Alcohol number
(2) Absorbance
(3) Congealing point
(4) Refractive index
(5) Osmolarity
(6) Optical rotation
(7) Viscosity
(8) pH
(9) Specific gravity
(10) Boiling point

(11) Melting point
(12) Acid value
(13) Saponification value
(14) Ester value
(15) Hydroxyl value
(16) Iodine value

5. Identification tests comprise the following items, which are generally put in the order given below:
(1) Coloration reactions
(2) Precipitation reactions
(3) Decomposition reactions
(4) Derivatives
(5) Visible, ultraviolet or infrared spectra
(6) Special reactions
(7) Cations
(8) Anions

6. Purity tests comprise the following items, which are generally put in the order given below, except that unnecessary items are omitted depending on the nature of the drug:
(1) Color
(2) Odor
(3) Clarity and/or color of solution
(4) Acidity or alkalinity
(5) Acid
(6) Alkali
(7) Chloride
(8) Sulfate
(9) Sulfite
(10) Nitrate
(11) Nitrite
(12) Carbonate
(13) Bromide
(14) Iodide
(15) Soluble halide
(16) Thiocyanide
(17) Selenium
(18) Cationic salts
(19) Ammonium
(20) Heavy metals
(21) Iron
(22) Manganese
(23) Chromium
(24) Bismuth
(25) Tin
(26) Aluminum
(27) Zinc
(28) Cadmium
(29) Mercury
(30) Copper
(31) Lead
(32) Silver
(33) Alkaline earth metals
(34) Arsenic
(35) Foreign matter
(36) Related substances
(37) Residual solvent
(38) Other mixtures
(39) Readily carbonizable substances

7. The following items of the General Tests, Processes and Apparatus were partially revised:
Bacterial Endotoxins Test
Nuclear Magnetic Resonance Spectroscopy (^{1}H)
Pyrogen Test
Residue on Ignition Test
Specific Surface Area Determination
Sterility Test

8. The following test was added to the General Tests, Processes and Apparatus:
Powder Particle Density Determination

9. The following Reference Standard was deleted:
Digitalis

10. The following Reference Standards were added:
Azithromycin
Cisplatin
Etoposide
Furosemide
Methylprednisolone Succinate
Nilvadipine
Thiamylal
Tranexamic Acid
Trichlormethiazide

11. English and Latin titles of drugs are derived, in principle, from International Nonproprietary Names (INN) for Pharmaceutical Substances recommended by the World Health Organization. Japanese titles are derived from the Japanese version of this book. The chemical names are based on the rules of the International Union of Pure and Applied Chemistry (IUPAC).

12. Molecular formulas of organic compounds begin with C and then H, followed by other involved elements in the alphabetical order of the symbols of the elements.

13. Structural formulas of drugs represent, as far as possible, steric configurations.

14. Test procedures in monographs in Part I are, in principle, written in full even in corresponding monographs in Part II, and vice versa. The test proce-

dures in monographs for preparations are also written in full even within the same part, except in the monographs for preparations having a corresponding monograph of their principal material substances.

15. The following articles were deleted from Official Monograph

Part I

Santonin Tablets

Part II

Absorbent Cotton
Purified Absorbent Cotton
Sterile Absorbent Cotton
Sterile Purified Absorbent Cotton
Absorbent Gauze
Sterile Absorbent Gauze
Adhesive Plaster
Digitalis
Powdered Digitalis

16. The following articles were newly added to official monographs:

Part I

Alprostadil
Azithromycin Hydrate
Benidipine Hydrochloride
Benidipine Hydrochloride Tablets
Cefepime Dihydrochloride for Injection
Cisplatin
Eperisone Hydrochloride
Etoposide
Flomoxef Sodium for Injection
Flopropione Capsules
Furosemide Tablets
Glutathione
Methylprednisolone Succinate
Metoclopramide Tablets
Nicorandil
Nilvadipine
Nilvadipine Tablets
Oxytocin
Pirenzepine Hydrochloride Hydrate
Piroxicam
Serrapeptase
Tiaramide Hydrochloride Tablets
Tizanidine Hydrochloride
Tranexamic Acid Capsules
Tranexamic Acid Injection
Tranexamic Acid Tablets
Trichlormethiazide Tablets

Part II

Cnidium Monnieri Fruit
Epimedium Herb
Lindera Root
Lonicera Leaf and Stem
Lycium Bark
Lycium Fruit
Processed Aconite Root
Powdered Processed Aconite Root
Processed Ginger
Sappan Wood
Termeric
Tribulus Fruit

17. The following monographs were revised in commonly used name:

Part I

Calcium Folinate

Part II

Cellulose Acetate Phthalate

18. The following monographs were revised in structural formula and in chemical name:

Part I

Cefuroxime Axetil
Mepivacaine Hydrochloride

19. The part of origin of the following monographs were deleted:

Part II

Chorionic Gonadotrophin
Serum Gonadotrophin

20. The following monographs were revised in origin:

Part I

Acetohexamide
Atropine Sulfate Injection
Benzylpenicillin Benzathine
Clarithromycin
Colchicine
Cytarabine
Digoxin Injection
Digoxin Tablets
Dimorpholamine
Dimorpholamine Injection
Ethionamide
Etilefrine Hydrochloride Tablets
Fosfomycin Calcium
Fosfomycin Sodium
Furosemide
dl-Methylephedrine Hydrochloride
Methyltestosterone
Methyltestosterone Tablets
Oxytocin Injection
Pyridoxine Hydrochloride
Pyridoxine Hydrochloride Injection
Sodium Aurothiomalate

Sodium Thiosulfate
Testosterone Propionate Injection
Thiamylal Sodium
Thiamylal Sodium for Injection
Tinidazole
Tranexamic Acid
Trichlormethiazide
Vinblastine Sulfate for Injection

Part II
Benzyl Alcohol
Cellulose Acetate Phthalate
Corn Starch
Opium Alkaloids Hydrochlorides
Powdered Aloe
Powdered Gardenia Fruit
Powdered Peony Root
Powdered Rhubarb
Powdered Scutellaria Root
Rhubarb
Uncaria Thorn

21. The following monographs were revised by addition or change in Method of preparation:
Part I
Oxytocin Injection

Part II
Chorionic Gonadotrophin
Serum Gonadotrophin

22. The following monographs were revised by addition or change in Description and in test(s):
Part I
Acetohexamide
Anhydrous Citric acid
Atropine Sulfate Injection
Benzylpenicillin Benzathine
Cefuroxime Sodium
Ciclosporin
Clarithromycin
Colchicine
Cytarabine
Deferoxamine Mesilate
Digoxin
Digoxin Injection
Digoxin Tablets
Dimorpholamine
Dimorpholamine Injection
Ethionamide
Etilefrine Hydrochloride Tablets
Flavin Adenine Dinucleotide Sodium
Fosfomycin Calcium
Fosfomycin Sodium
Furosemide
Heparin Sodium
Heparin Sodium Injection
Kallidinogenase
D-Mannitol
Meropenem Trihydrate
dl-Methylephedrine Hydrochloride
10% *dl*-Methylephedrine Hydrochloride Powder
Methyltestosterone
Methyltestosterone Tablets
Oxytocin Injection
Pirenoxine
Pyrazinamide
Pyridoxine Hydrochloride
Pyridoxine Hydrochloride Injection
Sodium Aurothiomalate
Sodium Polystyrene Sulfonate
Sodium Thiosulfate
Tegafur
Testosterone Propionate
Testosterone Propionate Injection
Thiamine Hydrochloride
Thiamylal Sodium
Thiamylal Sodium for Injection
Tinidazole
Tranexamic Acid
Trichlormethiazide
Vasopressin Injection
Vinblastine Sulfate
Vinblastine Sulfate for Injection

Part II
Benzyl Alcohol
Carmellose Calsium
Cellulose Acetate Phthalate
Chorionic Gonadotrophin
Chorionic Gonadotrophin for Injection
Chrysanthemum Flower
Corn Starch
Cornus Fruit
Ginger
Opium Alkaloids Hydrochlorides
Potato Starch
Powdered Aloe
Powdered Gardenia Fruit
Powdered Ginger
Powdered Peony Root
Serum Gonadotrophin
Serum Gonadotrophin for Injection
Uncaria Thorn
Wheat Starch

23. The following monographs were revised in Identification by addition or change based on adoption of the Ultraviolet-visible Reference Spectra:

Part I
Alprostadil
Benidipine Hydrochloride
Cisplatin
Colchicine
Cytarabine
Eperisone Hydrochloride
Ethionamide
Etoposide
Furosemide
dl-Methylephedrine Hydrochloride
Methylprednisolone Succinate
Methyltestosterone
Nilvadipine
Oxytocin
Pirenzepine Hydrochloride Hydrate
Piroxicam
Pyridoxine Hydrochloride
Testosterone Propionate
Thiamylal Sodium
Tizanidine Hydrochloride
Trichlormethiazide

24. The following monographs were revised in Identification by addition or change based on adoption of the Infrared Reference Spectra:
Part I
Alprostadil
Azithromycin Hydrate
Benidipine Hydrochloride
Cisplatin
Colchicine
Cytarabine
Digoxin
Dimorpholamine
Eperisone Hydrochloride
Ethionamide
Etoposide
Furosemide
Glutathione
dl-Methylephedrine Hydrochloride
Methylprednisolone Succinate
Methyltestosterone
Nicorandil
Nilvadipine
Pirenzepine Hydrochloride Hydrate
Piroxicam
Pyridoxine Hydrochloride
Testosterone Propionate
Thiamylal Sodium
Tinidazole
Tizanidine Hydrochloride
Tranexamic Acid
Trichlormethiazide
Vinblastine Sulfate

Part II
Benzyl Alcohol

25. The following monographs were revised in Containers and storage:
Part I
Ethionamide
Vinblastine Sulfate
Vinblastine Sulfate for Injection

Part II
Corn Starch
Potato Starch
Wheat Starch

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General Tests, Processes and Apparatus

Change the introduction to read:

General Tests, Processes and Apparatus includes common methods for tests and other articles related to them. Unless otherwise specified, the procedures for absorbance determination, absorbance ratio determination, acid-neutralizing capacity determination of gastrointestinal medicines, alcohol number determination, ammonium determination, arsenic determination, atomic absorption spectrophotometry, test for bacterial endotoxins, boiling point determination, distilling range determination, chloride determination, conductivity measurement, congealing point determination, test for content uniformity, determination of bulk and tapped densities, digestion test, disintegration test, dissolution test, endpoint detection in titrimetry, flame coloration, fluorometry, foreign insoluble matter test for injections, gas chromatography, heavy metals determination, test for glass containers for injections, infrared spectrophotometry, insoluble particulate matter test for injections, insoluble particulate matter test for ophthalmic solutions, iron determination, liquid chromatography, loss on drying determination, loss on ignition determination, mass variation test, melting point determination, test for metal particles in ophthalmic ointments, methanol determination, methoxyl assay, microbial assay for antibiotics, test for microbial limit, test for microbial limit for crude drugs, mineral oil determination, nitrogen determination, nuclear magnetic resonance spectroscopy, optical rotation determination, osmolarity determination, oxygen flask combustion method, paper chromatography, particle size distribution test for preparations, pH determination, test for plastic containers, powder particle density determination, powder particle size determination, test for pyrogen, qualitative test, test for readily carbonizable substances, refractive index determination, residual solvents test, residue on ignition determination, test for rubber closure for aqueous infusions, specific gravity and density determination, specific surface area determination, test for sterility, sulfate determination, thermal analysis, thin-layer chromatography, test for total organic carbon, viscosity determination, vitamin A assay, test for volatile contaminants in ethanol, water determination, and X-ray powder diffraction are performed as directed in the corresponding articles under the General Tests, Processes and Apparatus. The tests for melting point of fats, congealing point of fatty acids, specific gravity, acid value, saponification value, ester value, hydroxyl value, unsaponifiable matter and iodine value of fats and fatty oils are performed as directed in the corresponding items under the Fats and Fatty oils Test, and the tests for foreign matter and loss on drying, total ash, acid-insoluble ash, extract content, essential oil content of crude drugs are performed as directed in the corresponding items under the Crude Drugs Test.

6. Bacterial Endotoxins Test

Change the (ii) Interpretation of (2) Limit test under Gel-clot techniques to read:

Gel-clot techniques

(2) Limit test

(ii) Interpretation

The test is valid when both replicates of solutions B and C are positive and those of solution D are negative.

The sample meets the endotoxin limit requirement of the test when a negative result is found for both replicates of solution A.

Repeat the test in duplicate when the test results are positive for one test but negative for the other one. The sample meets the endotoxin limit requirement of the test when a negative result is found for both replicates of solution A in the repeat test.

The sample does not meet the endotoxin limit requirement of the test when a positive result is found for both replicates of the solution A at a dilution equal to the MVD. If the test is positive for the sample at a dilution less than the MVD, the test may be performed at a dilution not greater than the MVD.

39. Nuclear Magnetic Resonance Spectroscopy (^{1}H)

Change to read:

39. Nuclear Magnetic Resonance Spectroscopy

Nuclear magnetic resonance (NMR) spectroscopy is based on the phenomenon that specific radio frequency radiation is absorbed by magnetic nuclei in a sample placed in a magnetic field; target nuclei are ^{1}H, ^{13}C, ^{15}N, ^{19}F, ^{31}P, etc. These nuclei have intrinsic spin angular momentum, of which the magnitude is given by $I\,(I+1)/\mathrm{h}/2\pi$, where I is the spin quantum number and is integral or half-integral ($I=1/2$ for ^{1}H and ^{13}C). When the magnetic nuclei are placed in a magnetic field, they are oriented in $2I+1$ possible orientations

corresponding to $2I+1$ equally spaced energy levels (two energy levels for ^{1}H and ^{13}C). The transition between two successive quantized energy levels corresponding to adjacent orientations can be induced by electromagnetic radiation with a suitable frequency. The precise relation between the field strength and the resonant frequency ν is given by

$$\nu = \gamma \cdot \frac{H_0}{2\pi}$$

where H_0 is the strength of the applied external magnetic field and γ is the gyromagnetic ratio, a constant characterizing a particular isotope. The absorption of radiation (NMR signal) can occur only when the irradiating radio frequency satisfies the resonance condition. Since the absorption coefficient (the transition probability) does not depend on the environment in which the nuclei are located, the intensity is basically proportional to the number of nuclei. The excess spins shifted to the higher energy levels by the transition process return to the thermal equilibrium state at various rates determined by a characteristic time constant (known as the relaxation time).

A nucleus is shielded from the applied magnetic field by the electrons belonging to its own atom and to the molecule. Therefore nuclei in different environments are shielded to different extents and resonate at different frequencies. The difference in resonance frequencies is defined as chemical shift (δ), which is independent of the strength of the magnetic field, and is given by

$$\delta = \frac{\nu_S - \nu_R}{\nu_R} + \delta_R$$

where,

ν_S: The resonance frequency of the observed signal,

ν_R: The resonance frequency of the reference signal,

ν_R: The chemical shift of the reference signal (in the case of the value not being 0).

The chemical shifts are normally expressed in ppm, a dimensionless unit, by assuming the chemical shift of the reference compound as 0 ppm. When the chemical shift of the reference compound is not assumed to be 0 ppm, chemical shifts of samples are corrected accordingly.

In addition to the shielding due to electrons, the nucleus is subjected to effects due to the spin orientations of other magnetic nuclei through chemical bonds, resulting in an additional splitting of the signal. The spacing between two adjacent components of the signal is known as the spin-spin coupling constant (J). Coupling constants are measured in hertz and are independent of the strength of the external magnetic field. The increased number of interacting nuclei will make the multiplet pattern more complex.

From the NMR spectrum the following four parameters can be obtained: chemical shift, spin-spin coupling constant, resonance intensity (intensities of ^{1}H are proportional to the number of nuclei and those of ^{13}C and others are susceptible to the nuclear Overhauser effect (NOE) and relaxation) and relaxation time. These parameters are useful for structural determination, identification and quantitative analysis of molecules. Spin decoupling, NOE, and two-dimensional NMR techniques are also available for structural analysis.

Spectrometer

There are two types of spectrometers.

(1) Fourier transform NMR (FT-NMR) spectrometers (Figure 1)

Target nuclei are simultaneously excited in all frequency range of the nuclei by means of an intense radio frequency pulse. The FID (free induction decay) after the pulse is detected, which is a time domain signal, is converted to a frequency domain spectrum by Fourier transformation. Number of data points suitable for the spectral range, flip angle, acquisition time, delay time and number of scans should be set appropriately.

Recently FT-NMR is commonly used because of its high sensitivity and various advanced applications.

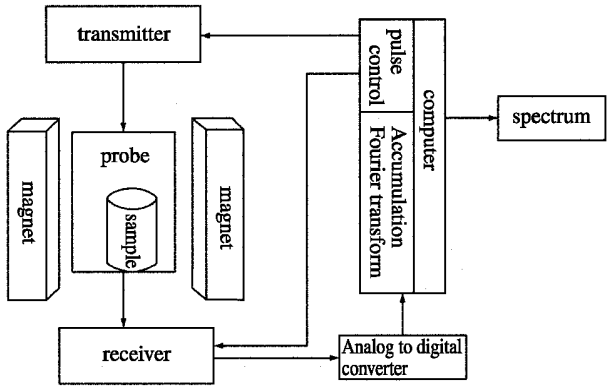


Fig. 1 FT-NMR spectrometer

(2) Continuous wave NMR (CW-NMR) spectrometers (Figure 2)

In the case of the CW method, a spectrum is obtained by sweeping the radio frequency or magnetic field continuously over the frequency range of the nuclei being observed.

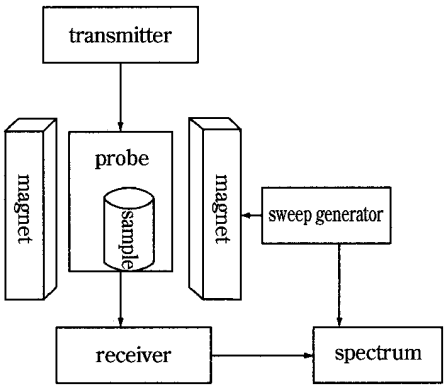


Fig. 2 CW-NMR spectrometer

Measurement

Prior to measurements, the sensitivity and resolution of the instrument must be adjusted to the optimum levels using a standard sample (ethylbenzene, 1,2-dichlorobenzene or acetaldehyde) dissolved in an appropriate NMR solvent.

(1) The sample dissolved in a suitable solvent is trans-

ferred into an NMR tube. The reference compound can be added directly to the sample solution (internal reference), or a sealed capillary tube containing the reference compound can be inserted into the NMR tube (external reference). The sample solutions should be completely homogeneous. In particular, solid contaminants should be removed in order to obtain good spectra. Various deuterated NMR solvents are commonly used for NMR measurement and the following points should be considered in selecting an appropriate solvent: (i) The solvent signals do not overlap with the sample signals. (ii) The sample must be soluble in the solvent selected. (iii) The solvent does not react with the sample. Furthermore, it should be noted that chemical shifts can depend upon the solvent employed, sample concentration and deuterium ion concentration, and that viscous solutions usually give rather broad, poorly resolved spectra.

(2) For the reference standards use the reagents for nuclear magnetic resonance spectroscopy. For ^{1}H and ^{13}C spectra, tetramethylsilane (TMS) is usually used as the reference compound for samples dissolved in organic solvents. For samples dissolved in deuterium oxide, sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) or sodium 3-(trimethylsilyl)propionate (TSP) is used. For other nuclei, nitromethane, trichlorofluoromethane and phosphoric acid are used as reference compounds for ^{15}N, ^{19}F and ^{31}P, respectively. Furthermore, chemical shifts of residual protons in deuterated solvents and ^{13}C in the solvent instead of a reference compound can be used for ^{1}H and ^{13}C NMR.

Record of apparatus and measurement conditions

Type of instrument, frequency, solvent, temperature, sample concentration, reference compound, experimental technique, etc. should be recorded to allow appropriate comparison of spectra, because NMR spectra depend on the measurement conditions.

Identification

The sample solution is prepared and tested by the method directed in each monograph. Usually in the case of ^{1}H NMR, the sample is identified by the following method.

(1) Identification by the use of chemical shift, multiplicity and relative intensity

When chemical shifts, multiplicities and relative intensities of signals are defined, the sample can be identified as being the same substance when all chemical shifts, multiplicities and relative intensities are the same as those prescribed.

(2) Identification by the use of a Reference Standard

Measurement conditions should be the same as those used in the case of the Reference Standard. When the spectra of a sample and the Reference Standard exhibit the same multiplicities and relative intensities of signal at the same chemical shifts, the sample can be identified as being the same substance as the Reference Standard.

Experimental techniques of ^{1}H and ^{13}C NMR spectroscopy

NMR spectroscopy includes one-, two- and multi-dimensional techniques, which are used for various purposes.

Spin decoupling, and NOE are available in one-dimensional ^{1}H spectroscopy. Spin decoupling can assign coupling correlations. As NOE can observe correlations among spatially proximate protons, the configuration and the conformation can be analyzed.

Broadband decoupling, INEPT and DEPT are usually applied in one-dimensional ^{13}C spectroscopy. The broadband decoupling technique simplifies a spectrum and achieves enhancement of sensitivity. INEPT (insensitive nuclei enhanced by polarization transfer) and DEPT (distortionless enhancement of polarization transfer) enhance the sensitivity of ^{13}C by means of polarization transfer from directly bonded ^{1}H with a large magnetic moment. They can be applied to identify primary, secondary, tertiary or quarternary carbon.

Two-dimensional spectroscopy can observe all correlation peaks between nuclei through spin-spin coupling or NOE in a single experiment, and there are many techniques for homonuclear and heteronuclear measurements. Representative techniques are described below.

COSY (2D correlation spectroscopy), HOHAHA (homonuclear Hartmann-Hahn spectroscopy) or TOCSY (total correlation spectroscopy): Correlation between protons through scalar spin-spin coupling is obtained and intramolecular connectivities of hydrogen atoms are revealed.

NOESY (2D nuclear Overhauser enhancement and exchange spectroscopy): NOE is measured by a two-dimensional technique. Approximate distances between spatially proximate hydrogen atoms are obtained to analyze the three-dimensional structure.

INADEQUATE (incredible natural abundance double quantum transfer experiment): Although this technique is insensitive because it involves double quantum transfer by ^{13}C-^{13}C scalar coupling in a sample with natural isotopic abundance, the connectivities of all neighboring ^{13}C nuclei can be obtained to analyze the carbon skeleton.

HMQC (heteronuclear multiple quantum coherence): This technique observes correlations between ^{1}H and ^{13}C with direct spin-spin coupling using ^{1}H detection and reveals intramolecular chemical bonds between hydrogen and carbon atoms.

HMBC (heteronuclear multiple bond connectivity): This technique observes correlations between ^{1}H and ^{13}C with long range spin-spin coupling using ^{1}H detection and reveals intramolecular connectivities of hydrogen and carbon atoms.

There are many other techniques such as DQF-COSY (double quantum filtered COSY) and HSQC (heteronuclear single quantum coherence). Furthermore, multidimensional NMR techniques are used to analyze macromolecules.

47. Pyrogen Test

Change to read:

The Pyrogen Test is a method to test the existence of pyrogens by using rabbits.

Test animals

Use healthy mature rabbits, each weighing not less than 1.5 kg, which have not lost body mass when kept on a constant diet for not less than one week. House the rabbits individually in an area free from disturbances likely to excite them. Keep the temperature of the area constant between 20°C and 27°C for at least 48 hours before and throughout the test. Before using a rabbit that has not previously been used for a pyrogen test, condition it 1 to 3 days prior to the test by conducting a sham test omitting the injection. Do not use a rabbit for pyrogen tests more frequently than once every 48 hours, or after it has been given a test sample that was adjudged pyrogen-positive or that contained an antigen present commonly in the test sample to be examined.

Apparatus, instruments

(1) Thermometer—Use a rectal thermometer or temperature-measuring apparatus with an accuracy of ±0.1°C or less.

(2) Syringe and injection needle—Depyrogenate the syringes and needles in a hot-air oven using a validated process, usually by heating at 250°C for not less than 30 minutes. Sterilized syringes with needles are also available provided that they have been validated to assure that they are free of detectable pyrogens and do not interfere with the test.

Test procedures

(1) Quantity of injection—Unless otherwise specified, inject 10 mL of the sample per kg of body mass of each rabbit.

(2) Procedure—Perform the test in a separate area at an environmental temperature similar to that of the room wherein the animals were housed and free from disturbances likely to excite them. Withhold food from the rabbits for several hours before the first record of the temperature and throughout the testing period. The test animals are usually restrained with loosely fitting neck stocks that allow the rabbits to assume a natural resting posture. Determine the temperature of each rabbit by inserting the thermometer or temperature-measuring probe into the rectum of the test animal to a constant depth within the range of 60 mm to 90 mm. The ''control temperature'' of each rabbit is the mean of two temperature readings recorded for that rabbit at an interval of 30 min in the 40 min immediately preceding the injection of the sample to be examined. Rabbits showing a temperature variation greater than 0.2°C between the two successive temperature readings or rabbits having an initial temperature higher than 39.8°C are withdrawn from the test.

Warm the test solution to a temperature of 37±2°C before injection, and inject the solution slowly into the marginal vein of the ear of each rabbit over a period not exceeding 10 min. Hypotonic test sample may be made isotonic by the addition of pyrogen-free sodium chloride. Record the temperature of each rabbit during a period of 3 hours after the injection, taking the measurements at intervals of not more than 30 min. The difference between the control temperature and the maximum temperature of each rabbit is taken to be the rise in body temperature. Consider any temperature decreases as zero rise.

Interpretation of results

The test is carried out on a group of three rabbits and the result is judged on the basis of the sum of the three temperature rises. Repeat if necessary on further groups of three rabbits to a total of three groups, depending on the results obtained. If the summed response of the first group does not exceed 1.3°C, the sample is judged to be pyrogen-negative. If the summed response exceeds 2.5°C, the sample is judged to be pyrogen-positive. If the summed response exceed 1.3°C but does not exceed 2.5°C, repeat the test on another group of three rabbits. If the summed response of the first and second group does not exceed 3.0°C, the sample is judged to be pyrogen-negative. If the summed response of the 6 rabbits exceeds 4.2°C, the sample is judged to be pyrogen-positive. If the summed response exceeds 3.0°C but does not exceed 4.2°C, repeat the test on one more group of three rabbits. If the summed response of the 9 rabbits does not exceed 5.0°C, the sample is judged to be pyrogen-negative. If the summed response exceeds 5.0°C, the sample is judged to be pyrogen-positive.

When the test sample is judged to be pyrogen-negative, the sample passes the pyrogen test.

52. Residue on Ignition Test

Change the Procedure to read:

Procedure

Previously ignite a suitable crucible (silica, platinum, quartz or porcelain) at 600±50°C for 30 minutes, and weigh accurately after cooling in a desiccator (silica gel or other suitable dessicant).

Take the sample of the amount directed in the monograph, transfer into the ignited crucible, and weigh accurately. When the quantity of the sample to be taken is indicated in a volume, pipet exactly the amount directed in the monograph and transfer into the above crucible. When directed as ''after evaporating,'' heat properly to evaporate the solution.

Moisten the sample with a small amount of sulfuric acid, usually 1 mL, then heat slowly at a temperature as low as practicable until the sample is completely carbonized, and cool. Moisten again with a small amount (usually 1 mL) of sulfuric acid, 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. Cool the crucible in a desiccator (silica gel or other suitable dessicant), and reweigh accurately to calculate the percentage of residue.

Unless otherwise specified, if the amount of the residue so obtained exceeds the limit specified in the individual monograph, repeat the moistening with sulfuric acid, heating and ignition as before until constant mass is attained or until the percentage of residue complies with the limit in the individual monograph.

53. Specific Surface Area Determination

Change to read the text up to the paragraph headed "Method 1 Dynamic Flow Method" as follows:

Specific Surface Area Determination is a method to determine the specific surface area (the total surface area of powder per an unit mass) of a powdered pharmaceutical preparation by using the gas adsorption method. The gas adsorption method is a method for measuring the amount of gas adsorbed on the surface of a powder sample as a function of the pressure of the adsorbate gas, and the measurements are usually performed at the boiling point of liquid nitrogen (−196°C).

When the gas is physically adsorbed by the powder sample, the following relationship holds when P/P_0 is in the range of 0.05 to 0.30 for pressure P of the adsorbate gas in equilibrium for the volume of gas adsorbed, V_a.

$$\frac{1}{V_a\left(\frac{P_0}{P}-1\right)} = \frac{(C-1)}{V_m C} \times \frac{P}{P_0} + \frac{1}{V_m C}$$

P: Partial vapor pressure of adsorbate gas in equilibrium (kPa)

P_0: Saturated vapor pressure of the adsorbate gas at −196°C (kPa)

V_a: Volume of gas adsorbed at equilibrium (mL)

V_m: Volume of gas adsorbed in a monolayer (mL)

C: Dimensionless constant relating to the enthalpy of adsorption and condensation of the adsorbate gas

The specific surface area, S, is determined from V_m, the volume of gas adsorbed in a monolayer on the sample.

$$S = \frac{V_m \times N \times a}{m \times 22400}$$

S: Specific surface area (m^2/g)

N: Avogadro constant 6.022×10^{23}/mol

a: Effective cross-sectional area of one adsorbate molecule (m^2)

N_2: 0.162×10^{-18}

Kr: 0.195×10^{-18}

m: Mass of the test powder (g)

Specific surface area is generally expressed in unit of m^2/g.

Either of the methods described below can be used to measure the gas adsorption.

54. Sterility Test

Change to read:

Test for sterility is the method to establish the presence or absence of viable microorganisms (bacteria and fungi) using the defined culturing method. Unless otherwise specified, the test is carried out by I. Membrane filtration method or II. Direct inoculation method. Water, reagents, test solutions, equipment, materials and all other requisites for the test should be pre-sterilized. 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 microorganisms 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.

Media and rinsing fluids

Fluid thioglycolate medium, soybean-casein digest medium are used, unless otherwise specified. When it is difficult to use fluid thioglycolate medium due to turbidity or viscosity of samples, alternative thioglycolate medium can be used, provided it is heated on a water bath just prior to use and incubated under anaerobic conditions. Other products of suitable quality yielding similar formulations may be used according to the indications on the label.

(1) Fluid thioglycolate medium

L-Cystine	0.5 g
Agar	0.75 g
Sodium chloride	2.5 g
Glucose, monohydrate/anhydrate	5.5/5.0 g
Yeast extract (water-soluble)	5.0 g
Pancreatic digest of casein	15.0 g
Sodium thioglycolate or	0.5 g
Thioglycolic acid	0.3 mL
Resazurin sodium solution (1 in 1000), freshly prepared	1.0 mL
Water	1000 mL

(pH after sterilization 7.1 ± 0.2)

Mix the L-cystine, agar, sodium chloride, glucose, water-soluble yeast extract and pancreatic digest of casein with the water, and heat until solution is effected. Dissolve the sodium thioglycolate or thioglycolic acid in the solution and, if necessary, add sodium hydroxide TS so that, after sterilization, 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 ration of surface to depth of medium such that not more than the upper half of the medium has undergone a color change indicative of oxygen uptake at the end of the incubation period. Sterilize using a validated process. Store the medium at a temperature between 2 – 25°C. If more than the upper one-third of the medium has acquired a pink color, the medium may be restored once by

heating the containers in a water-bath or in free-flowing steam until the pink color disappears and cooling quickly, taking care to prevent the introduction of non-sterile air into the container.

(2) Alternative thioglycolate medium

L-Cystine	0.5 g
Sodium chloride	2.5 g
Glucose, monohydrate/anhydrate	5.5/5.0 g
Yeast extract (water-soluble)	5.0 g
Pancreatic digest of casein	15.0 g
Sodium thioglycolate or	0.5 g
Thioglycolic acid	0.3 mL
Water	1000 mL

(pH after sterilization 7.1 ± 0.2)

The methods for preparation follow those of fluid thioglycolate medium.

(3) Soybean-casein digest medium

Casein peptone	17.0 g
Soybean peptone	3.0 g
Sodium chloride	5.0 g
Dipotassium hydrogen phosphate	2.5 g
Glucose, monohydrate/anhydrous	2.5/2.3 g
Water	1000 mL

(pH after sterilization 7.3 ± 0.2)

Mix all the ingredients and heat until solution is effected. If necessary, add sodium hydroxide TS so that, after sterilization, the solution will have a pH of 7.3 ± 0.2. Filter, if necessary, to clarify, distribute into suitable vessels and sterilize using a validated process. Store at a temperature between 2 – 25°C in a sterile container.

(4) Rinsing fluids

Meat or casein peptone	1.0 g
Water	1000 mL

(pH after sterilization 7.1 ± 0.2)

Dissolve animal tissue or casein peptone in water and adjust the pH of the solution so that, after sterilization, it will show 7.1 ± 0.2. Filter, if necessary, to clarify, distribute into suitable vessels and sterilize using a validated process. Store at a temperature between 2 – 25°C in a sterile container.

To rinsing fluid to be used for antibiotics or pharmaceutical products containing an antimicrobial agent, a suitable neutralizer or inactive agent at concentration shown to be appropriate in the validation of the test can be added. To rinsing fluid to be used for oils, oily solutions, ointments or creams, suitable emulsifying agent at a concentration shown to be appropriate in the validation of the test, for example polysorbate 80 at a concentration of 10 g/L can be added.

Suitability of media

The media used comply with the following tests, carried out before or in parallel with the test on the product to be examined.

(1) Sterility of media

Confirm the sterility of each sterilized batch of medium by incubating a portion of the media at the specified incubation temperature for 14 days. No growth of microorganisms occurs.

(2) Growth promotion test

Test each batch of ready-prepared medium and each batch (lot)of medium prepared either from dehydrated medium or from ingredients[1]. Inoculate a small number (not more than 100 CFU) of microorganism listed in Table 1 or other strains considered to be equivalent to these strains in containers of each medium. Each of the test organisms should show clearly visible growth in all inoculated media within 3 days for bacteria and within 5 days for fungi.

Table 1. Microorganisms for growth promotion test and the validation test

Medium	Test microorganisms	Incubation conditions
Fluid thioglycolate medium	*Staphylococcus aureus* (ATCC 6538, NBRC13276, CIP 4.83, NCTC 10788, NCIMB 9518) *Pseudomonas aeruginosa* (ATCC 9027, NBRC 13275, NCIMB 8626, CIP 82.118) *Clostridium sporogenes* (ATCC 19404, CIP 79.3, NCTC 532, or ATCC 11437, NBRC 14293)	Aerobic
Alternative thioglycolate medium	*Clostridium sporogenes* (ATCC 19404, CIP 79.3, NCTC 532, or ATCC 11437, NBRC 14293)	Anaerobic
Soybean-casein digest medium	*Bacillus subtilis* (ATCC 6633, NBRC 3134, CIP 52.62, NCIMB 8054) *Candida albicans* (ATCC 10231, NBRC 1594, IP 48.72, NCPF 3179) *Aspergillus niger* (ATCC 16404, NBRC 9455, IP 1431.83, IMI 149007)	Aerobic

Seed lot culture maintenance techniques (seed-lot systems) are used so that the viable microorganisms used for inoculation are not more than five passages removed from the original master seed-lot.

Effective period of media

If prepared media are stored in unsealed containers, they can be used for one month, provided that they are tested for growth promotion within two weeks of the time of use and that color indicator requirements are met. If stored in tight containers, the media can be used for one year, provided that they are tested for growth promotion within 3 months of the time of use and that the color indicator requirements are met.

Validation test

The validation may be performed simultaneously with the Test for sterility of the product to be examined in the following cases.

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.

Carry out the 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 content 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 contents of the container or containers to be tested 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*. 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 validation test.

In the membrane filtration, the antimicrobial activity should be suppressed by suitable means such as replacement of the membrane filters with less adsorptive ones, increase of the amount of rinsing fluid, or addition of a suitable inactivating agent to the rinsing fluid. Do not exceed a washing cycle of 5 times 100 mL per filter, even if during validation it has been demonstrated that such a cycle does not fully eliminate the antimicrobial activity.

In the direct inoculation, use a suitable inactivating agent which does not affect the growth of microorganisms or increase the volume of medium irrespective of the prescription in II-2 so that no antimicrobial activity remains.

Test for sterility of the products to be examined

Number of articles to be tested

Items to be used for the test are taken from the lot according to an appropriate sampling plan prepared by referring to the numbers specified in Table 2.

Table 2. Number of items to be taken from the lot

Number of items in the lot	Minimum number of items to be tested for each medium*
Injections	
Not more than 100 containers	10% or 4 containers, whichever is greater
More than 100 but not more than 500 containers	10 containers
More than 500 containers	2% or 20 containers, whichever is less
For large-volume products (More than 100 mL)	2% or 10 containers, whichever is less
Ophthalmic and other non-injectable products	
Not more than 200 containers	5% or 2 containers, whichever is 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 use	
Bulk solid products	
Up to 4 containers	Each container
More than 5 containers but not more than 50 containers	20% or 4 containers, whichever is greater
More than 50 containers	2% or 10 containers, whichever is greater
Antibiotic Solids	
Pharmacy bulk packages (<5 g)	20 containers
Pharmacy bulk packages (≥ 5 g)	6 containers

*If the contents of one container are enough to inoculate the two media, this column gives the number of containers needed for both the media together.

Testing methods

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.

I. Membrane filtration

By this method, a test article is filtered through a membrane filter, and the filter is rinsed and incubated by being transferred to a medium or by adding a medium to the filtration apparatus. Use membrane filter made from suitable material having a nominal pore size of 0.45 μm or smaller. Use a filter funnel sterilizable by the moist heat method or other methods and free from any leakage or back flow when filtration is performed with the membrane in place. 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.

I-1. Preparation of sample solution

a) Liquid medicine: Use as it is, as the sample solution.

b) Solid medicine: In the case of a solid medicine, to be administered after dissolving or suspending, the sample solution is prepared with the provided solvent, isotonic sodium chloride solution or water to give the concentration of use.

c) Oils and oily solutions: 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.

d) Ointments and creams: Ointments in a fatty base and emulsions of the water-in-oil type may be diluted by using sterile isopropyl myristate that has previously been filtered through a sterilizing membrane filter or by using other solvents not affecting the growth of microorganisms. Heat the sample preparation, if necessary, to not more than 40°C. In exceptional cases it may be necessary to heat to not more than 44°C.

I-2. Quantities of sample solution to be tested

Use for each medium not less than quantity of the product prescribed in Table 3, unless otherwise specified. If the contents of one container are insufficient to inoculate the two media, twice or more containers shown in Table 2 are used. 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 3, diluting where necessary to about 100 mL with a suitable sterile rinsing fluid.

Table 3. Minimum quantity to be used for each medium

Quantity per container	Minimum quantity to be used for each medium
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% of the contents of the container but not less than 20 mL
Antibiotic liquids	1 mL
Other preparations soluble in water or in isopropyl myristate	The whole contents of each container to provide not less than 200 mg
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 – 5 g	150 mg
Greater than 5 g	500 mg

I-3. Procedures

Usually complete the filtration of the sample solution with one or two separate filter funnels. Transfer the contents of the container or containers to be tested to the membrane or membranes. If the sample solution is not readily filterable, it may be further diluted with rinsing fluid and thereafter filtered. Rinse the membrane(s) with each 100-mL of rinsing fluid per filter for established cycles in the validation test. Provided the sample does not have antimicrobial activity, the rinsing procedure can be omitted. Employ either of the two methods described below for incubation of the membrane(s). Use the same volume of each medium as in the validation test.

(1) The processed membrane is aseptically transferred from the apparatus and cut into two equal parts, or half the volume of sample solution is filtered into an entire membrane. Transfer each half of the cut membrane, or each whole membrane into the medium.

(2) After filtration of sample solution into the apparatus to which the membrane filters are fitted, each medium is added to the apparatus itself.

II. Direct inoculation of the culture medium

This is the method by which the entire content or a portion of the content of a sample container is transferred directly to the culture medium and incubated. Usually, this method is applied for medicines to which the membrane filtration method cannot be applied or for which the application of the direct transfer method, rather than the membrane filtration

method, is rational.

For products containing a mercurial preservative that cannot be tested by the membrane-filtration method, fluid thioglycolate medium incubated at 20 – 25°C may be used instead of Soybean-casein digest medium.

II-1. Preparation of sample solution

Usually, proceed as directed for the membrane filtration method. In the case of an insoluble medicine, the product is suspended or crushed in a suitable manner and used as a sample.

a) Oily liquids. Use media to which have been added a suitable emulsifying agent at a concentration shown to be appropriate in the validation of the test, for example polysorbate 80 at a concentration of 10 g/L.
b) 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.

II-2. Quantities of sample solution to be tested

Transfer the quantity of the preparation to be examined prescribed in Table 3, by using pipette, syringe or other suitable inoculation devices, directly into the culture medium so that the volume of the product is not more than 10% of the volume of the medium, unless otherwise prescribed. Shake cultures containing oily products gently each observation day. However when thioglycolate medium is used for the detection of anaerobic microorganisms keep shaking or mixing to a minimum in order to maintain anaerobic conditions.

Cultivation and observation

Fluid thioglycolate medium and Alternative thioglycolate medium are to be incubated at 30 – 35°C and Soybean-casein digest medium is to be incubated at 20 – 25°C for not less than 14 days. Observe the cultures several times during the incubation period. 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 suitable portions of the medium to fresh vessels of the same medium and then incubate the original and transfer vessels for not less than 4 days.

Observation and interpretation of results

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 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. If no evidence of microbial growth is found in the repeat test the product complies with the Sterility Test. If microbial growth is found in the repeat test the product does not comply with the Sterility Test.

70. Reference Standards; Reagents, Test Solutions; Standard Solutions for Volumetric Analysis; Standard Solutions; Matching Fluids for Color; Optical Filters for Wavelength and Transmission Rate Calibration; and Measuring Instruments, Appliances

(1) Reference Standards

Add the following:

Azithromycin, Cisplatin, Etoposide, Furosemide, Methylprednisolone Succinate, Nilvadipine, Thiamylal, Tranexamic Acid, Trichlormethiazide

(2) Reagents, Test Solutions

Delete the following:

Cyclohexylamine for thin-layer chromatography
Dicyclohexylurea for thin-layer chromatography

Change the following:

Calcium hydroxide for pH determination Calcium hydroxide prepared for pH determination.

[6]-Gingerol for thin-layer chromatography $C_{17}H_{26}O_4$ A yellow-white to yellow, liquid or solid. Freely soluble in methanol, in ethanol (99.5) and in diethyl ether, and practically insoluble in water.

Purity Related substances—Dissolve 1.0 mg of [6]-gingerol for thin-layer chromatography in exactly 2 mL of methanol. Perform the test with 10 μL of this solution as directed in the Identification under Ginger: any spot other than the principal spot at the *R*f value of about 0.3 does not appear.

Piperidine hydrochloride $C_5H_{11}N.HCl$ A white crystalline powder. Dissolves in water and in methanol. The pH of the aqueous solution (1 in 20) is between 3.0 and 5.0.

Melting point: 247 – 252°C

Purity Clarity and color of solution—Dissolve 1.0 g of piperidine hydrochloride in 20 mL of water: the solution is clear and colorless.

Residue on ignition: not more than 0.10% (1 g).

Content: not less than 99.0%. Assay—Dissolve about 0.25 g of piperidine hydrochloride, accurately weighed, in 50 mL of water, add 5 mL of diluted nitric acid (1 in 3), and titrate with 0.1 mol/L silver nitrate VS (potentiometric titration). Perform a blank determination in the same manner,

and make any necessary correction.

Each mL of 0.1 mol/L silver nitrate VS
= 12.16 mg of $C_5H_{11}N.HCl$

Sodium 1-decanesulfonate $C_{10}H_{21}NaO_3S$ A white powder.

Purity Clarity and color of solution—Dissolve 1.0 g in 20 mL of water: the solution is clear and colorless.

Loss on drying: not more than 3.0% (1 g, 105°C, 3 hours).

Content: not less than 98.0%. Assay—Weigh accurately about 0.45 g of sodium 1-decanesulfonate, dissolve in 50 mL of water, and pass through a column, about 1.2 cm in inside diameter and about 25 cm in length, packed with about 20 mL of strongly acidic ion-exchange resin (0.3 to 1.0 mm, H type) at a flow rate of about 4 mL per minute. Wash with 150 mL of water at a flow rate of about 4 mL per minute. Combine the washing and the elute, and titrate with 0.1 mol/L sodium hydroxide VS (potentiometric titration). Perform a blank determination in the same manner, and make any necessary correction.

Each mL of 0.1 mol/L sodium hydroxide VS
= 24.43 mg of $C_{10}H_{21}NaO_3S$

Thioglycolate medium I for sterility test See fluid thioglycolate medium.

Thioglycolate medium II for sterility test See alternative thioglycolate medium.

Turmeric paper Macerate 20 g of powdered dried rhizome of *Curcuma longa* Linné with four 100 mL-portions of cold water, decant the supernatant liquid each time, and discard it. Dry the residue at a temperature not over 100°C. Macerate the dried residue with 100 mL of ethanol (95) for several days, and filter. Immerse filter paper in this ethanol decoction, and allow the ethanol (95) to evaporate spontaneously in clean air.

Sensitivity—Dip a strip of turmeric paper, about 1.5 cm length, in a solution of 1 mg of boric acid in a mixture of 1 mL of hydrochloric acid and 4 mL of water, after 1 minute remove the paper from the liquid, and allow it to dry spontaneouly: the yellow color changes to brown. When the strip is moistened with ammonia TS, the color of the strip changes to greenish black.

L-Tyrosine $C_9H_{11}NO_3$ White, crystals or crystalline powder. Odorless and tasteless. Freely soluble in formic acid, very slightly soluble in water, and practically insoluble in ethanol (95) and in diethyl ether. It dissolves in dilute hydrochloric acid and in dilute nitric acid.

Optical rotation $[\alpha]_D^{20}$: -10.5 – $-12.5°$ (after drying, 2.5 g, 1 mol/L hydrochloric acid TS, 50 mL, 100 mm).

Loss on drying: not more than 0.30% (1 g, 105°C, 3 hours).

Content: not less than 99.0%. Assay—Weigh accurately about 0.3 g of L-tyrosine, previously dried, dissolve in 6 mL of formic acid, add 50 mL of acetic acid (100), and titrate with 0.1 mol/L perchloric acid VS (potentiometric titration). Perform a blank determination, and make any necessary correction.

Each mL of 0.1 mol/L perchloric acid VS
= 18.12 mg of $C_9H_{11}NO_3$

Add the following:

0.05 mol/L Acetic acid-sodium acetate buffer solution, pH 4.6 Dissolve 6.6 g of sodium acetate trihydrate in 900 mL of water, and add 3 mL of acetic acid and water to make 1000 mL.

0.25 mol/L Acetic acid TS To 3 g of acetic acid (100) add water to make 200 mL.

Aconitine for purity $C_{34}H_{47}NO_{11}$ White, crystals or crystalline powder. Sparingly soluble in acetonitrile and in ethanol (99.5), slightly soluble in diethyl ether, and practically insoluble in water. Melting point: about 185°C (with decomposition).

Identification—Determine the infrared absorption spectrum of aconitine for purity as directed in the potassium bromide disk method under the Infrared Spectrophotometry: it exhibits absorption at the wave numbers of about 3500 cm^{-1}, 1718 cm^{-1}, 1278 cm^{-1}, 1111 cm^{-1}, 1097 cm^{-1} and 717 cm^{-1}.

Absorbance $E_{1\,cm}^{1\%}$ (230 nm): 211 – 243 [5 mg dried for not less than 12 hours in a desiccator (reduced pressure not exceeding 0.67 kPa, phosphorus (V) oxide, 40°C), ethanol (99.5), 200 mL].

Purity Related substances—

(1) Dissolve 5.0 mg of aconitine for purity in 2 mL of acetonitrile, and use as the sample solution. Pipet 1 mL of the sample solution, add acetonitrile to make exactly 50 mL, and use as the standard solution. Perform the test with these solutions as directed under the Thin-layer Chromatography. Spot 20 μL each of the sample solution and the standard solution on a plate of silica gel for thin-layer chromatography, and proceed the test as directed in the Identification in Processed Aconite Root: the spot other than the principal spot obtained with the sample solution is not more intense than the spot with the standard solution.

(2) Dissolve 5.0 mg of aconitine for purity in 5 mL of acetonitrile, and use as the sample solution. Pipet 1 mL of the sample solution, add acetonitrile to make exactly 50 mL, and use as the standard solution. Perform the test with exactly 10 μL each of the sample solution and the standard solution as directed under the Liquid Chromatography according to the following conditions, and determine each peak area by the automatic integration method: the total area of the peaks other than the peaks of aconitine and the solvent obtained with the sample solution is not larger than the peak area of aconitine with the standard solution.

Operating conditions

Detector, column, and column temperature: Proceed as directed in the operating conditions in the Purity under Processed Aconitine Root.

Mobile phase: A mixture of phosphate buffer solution for processed aconite root and tetrahydrofuran (9:1).

Flow rate: Adjust the flow rate so that the retention time

of aconitine is about 26 minutes.

Time span of measurement: About 3 times as long as the retention time of aconitine.

System suitability

Test for required detectability: Pipet 1 mL of the standard solution, and add acetonitrile to make exactly 20 mL. Confirm that the peak area of aconitine obtained from 10 μL of this solution is equivalent to 3.5 to 6.5% of that obtained from 10 μL of the standard solution.

System performance: Dissolve 1 mg each of aconitine for purity, hypaconitine for purity and mesaconitine for purity, and 8 mg of jesaconitine for purity in 200 mL of acetonitrile. When the procedure is run with 10 μL of this solution under the above operating conditions, mesaconitine, hypaconitine, aconitine and jesaconitine are eluted in this order, and each resolution between these peaks is not less than 1.5, respectively.

System repeatability: When the test is repeated 6 times with 10 μL of the standard solution under the above operating conditions, the relative standard deviation of the peak area of aconitine is not more than 1.5%.

Water: not more than 1.0% [5 mg dried for not less than 12 hours in a desiccator (reduced pressure not exceeding 0.67 kPa, phosphorus (V) oxide, 40°C), coulometric titration].

Aconitum diester alkaloids standard solution for purity It is a solution containing 10 mg of aconitine for purity, 10 mg of jesaconitine for purity, 30 mg of hypaconitine for purity and 20 mg of mesaconitine for purity in 1000 mL of a mixture of phosphate buffer solution for processed aconite root and acetonitrile (1:1). When proceed the test with 20 μL of this solution as directed in the Purity under Processed Aconite Root, using the detection wavelength, 231 nm, the peaks of aconitine, jesaconitine, hypaconitine and mesaconitine are observed, and the ratio of their peak heights is about 10:1:35:30. When proceed the test using the detection wavelength, 254 nm, the peaks of aconitine, jesaconitine, hypaconitine and mesaconitine are observed, and the ratio of their peak heights is about 2:8:7:6.

Alternative thioglycolate medium See the Sterility Test under the General Tests, Processes and Apparatus.

4-(Aminomethyl)benzoic acid $C_8H_9NO_2$ A white powder.

Purity—Dissolve 10 mg of 4-(aminomethyl)benzoic acid in 100 mL of water, and use this as the sample solution. Pipet 1 mL of the sample solution, add water to make exactly 20 mL, and use this solution as the standard solution. Perform the test with exactly 20 μL each of the sample solution and the standard solution as directed under the Liquid Chromatography according to the operating conditions as directed in the Purity (5) under Tranexamic Acid, and determine each peak area by the automatic integration method: each area of the peak other than 4-(aminomethyl)benzoic acid obtained from the sample solution is not more than the peak area of 4-(aminomethyl)benzoic acid from the standard solution.

Ammonium amminetrichloroplatinate for liquid chromatography $Cl_3H_7N_2Pt$ To 20 g of cisplatin add 600 mL of 6 mol/L hydrochloric acid TS, and heat under a reflux condenser for 4 – 6 hours to boil while stirring. After cooling, evaporate the solvent, and dry the orange residue at room temperature under reduced pressure. To the residue so obtained add 300 mL of methanol, and heat at about 50°C to dissolve. Filter, separate insoluble yellow solids, and wash the solids with 10 mL of methanol. Combine the filtrate and the washing, heat at about 50°C, and add slowly 100 mL of ethyl acetate while stirring. Cool the mixture to room temperature avoiding exposure to light, and allow to stand at −10°C for 1 hour. Filter the mixture to take off the formed crystals, wash the crystals with 100 mL of acetone, combine the washing to the filtrate, and evaporate to dryness to obtain orange crystals. If necessary, repeat the purification procedure described above to take off the insoluble crystals. To the orange crystals obtained add 300 to 500 mL of a mixture of acetone and methanol (5:1), and heat at about 50°C while stirring to dissolve. Filter while hot to take off the insoluble crystals, wash the crystals with the mixture, and combine the filtrate and washing. Repeat the procedure several times, and evaporate to dryness. Suspense the crystals so obtained in 50 mL of acetone, filter, wash the crystals with 20 mL of acetone, and dry the crystals at room temperature under reduced pressure. It is a yellow-brown crystalline powder.

Identification—Determine the infrared absorption spectrum of the substance to be examined, previously dried at 80°C for 3 hours, as directed in the potassium bromide disk method under the Infrared Spectrophotometry: it exhibits absorption at the wave numbers of about 3480 cm^{-1}, 3220 cm^{-1}, 1622 cm^{-1}, 1408 cm^{-1} and 1321 cm^{-1}.

Related substances—Cisplatin Conduct this procedure using light-resistant vessels. Dissolve 10 mg in *N*,*N*-dimethylformamide to make exactly 10 mL, and use this solution as the sample solution. Separately, dissolve 10 mg of Cisplatin in *N*,*N*-dimethylformamide to make exactly 50 mL. Pipet 5 mL of this solution, add *N*,*N*-dimethylformamide to make exactly 20 mL, and use this solution as the standard solution. Perform the test with exactly 40 μL each of the sample solution and the standard solution as directed under the Liquid Chromatography according to the following conditions, and determine the peak area of cisplatin by the automatic integration method: the peak area from the sample solution is not more than that from the standard solution.

Operating conditions

Proceed as directed in the operating conditions in the Assay under Cisplatin.

System suitability

System performance: When the procedure is run with 40 μL of the standard solution under the above operating conditions, the number of theoretical plates and the symmetry factor of the peak of cisplatin are not less than 2500 and not more than 2.0, respectively.

System repeatability: When the test is repeated 6 times with 40 μL of the standard solution under the above

operating conditions, the relative standard deviation of the peak area of cisplatin is not more than 5.0%.

Benidipine hydrochloride $C_{28}H_{31}N_3O_6.HCl$ [Same as the namesake monograph]

Benidipine hydrochloride for assay $C_{28}H_{31}N_3O_6.HCl$ [Same as the monograph Benidipine Hydrochloride. When dried, it contains not less than 99.5% of benidipine hydrochloride ($C_{28}H_{31}N_3O_6.HCl$)]

Benzoin $C_6H_5CH(OH)COC_6H_5$ White to pale yellow, crystals or powder.

Melting point: 132 – 137°C

***N*-Benzoyl-L-isoleucyl-L-glutamyl(γ-OR)-glycyl-L-arginyl-*p*-nitroanilide hydrochloride** An equal amount mixture of two components, R = H and R = CH_3. A white powder. Slightly soluble in water.

Absorbance $E^{1\%}_{1\,cm}$ (316 nm): 166 – 184 (10 mg, water, 300 mL).

Benzoylmesaconine hydrochloride for thin-layer chromatography $C_{31}H_{43}NO_{10}.HCl.xH_2O$ White, crystals or crystalline powder. Soluble in water and in ethanol (99.5). Melting point: about 250°C (with decomposition).

Purity Related substances—Dissolve 1.0 mg of benzoylmesaconine hydrochloride for thin-layer chromatography in exactly 10 mL of ethanol (99.5), and use this solution as the sample solution. Perform the test with this solution as directed under the Thin-layer Chromatography. Spot 10 μL of the sample solution on a plate of silica gel for thin-layer chromatography. Proceed the test as directed in the Identification under Processed Aconite Root: no spot other than the principal spot at around *R*f 0.4 appears.

4,4′-Bis(diethylamino)benzophenone $(C_2H_5)_2NC_6H_4]_2CO$ Light yellow crystals.

Content: not less than 98%. Assay—Weigh accurately 0.25 g of 4,4′-bis(diethylamino)benzophenone, dissolve in 50 mL of acetic acid (100), and titrate with 0.1 mol/L perchloric acid VS (potentiometric titration). Perform a blank titration in the same manner, and make any necessary correction.

Each mL of 0.1 mol/L perchloric acid VS
= 16.22 mg of $C_{21}H_{28}N_2O$

Borate-hydrochloric acid buffer solution, pH 9.0 Dissolve 19.0 g of sodium borate in 900 mL of water, adjust the pH to exactly 9.0 with 1 mol/L hydrochloric acid TS, and add water to make 1000 mL.

Bovine activated blood coagulation factor X A protein obtained from bovine plasma. It has an activity to decompose prothrombin specifically and limitedly and produce thrombin. It does not contain thrombin and plasmin. It contains not less than 500 Units per mg protein. One unit indicates an amount of the factor X which hydrolyzes 1 μmol of *N*-benzoyl-L-isoleucyl-L-glutamyl(γ-OR)-glycyl-L-arginyl-*p*-nitroanilide in 1 minute at 25°C.

Calibration ball for particle density measurement Calibration ball with a known volume prepared for measurement of particle density. The volume of the calibration ball must be accurately determined to the nearest 0.001 cm^3.

Caprylic acid $CH_3(CH_2)_6COOH$ A clear and colorless oily liquid, having a slight unpleasant odor. Freely soluble in ethanol (95) and in chloroform, and very slightly soluble in water.

Refractive index: n_D^{20} 1.426 – 1.430

Specific gravity d_4^{20}: 0.908 – 0.912

Distilling range 238 – 242°C, not less than 95 vol%.

Chlorobutanol $C_4H_7Cl_3O$ [Same as the namesake monograph in Part II]

Chlorogenic acid for thin-layer chromatography $C_{16}H_{18}O_9.xH_2O$ A white powder. Freely soluble in methanol and in ethanol (99.5), and sparingly soluble in water. Melting point: about 205°C (with decomposition).

Purity Related substances—Dissolve 1.0 mg of chlorogenic acid for thin-layer chromatography in 2 mL of methanol, and use this solution as the sample solution. Perform the test with this solution as directed under the Thin-layer Chromatography. Spot 10 μL of the sample solution on a plate of silica gel for thin-layer chromatography, develop the plate with a mixture of ethyl acetate, water and formic acid (6:1:1) to a distance of about 10 cm, and air-dry the plate. Examine under ultraviolet light (main wavelength: 365 nm): no spot other than the principal spot at around *R*f 0.5 appears.

Cisplatin [Same as the namesake monograph]

Cyclohexylamine $C_6H_{11}NH_2$ A clear and colorless liquid, having a characteristic amine-like odor. Miscible with water, with *N*,*N*-dimethylformamide and with acetone.

Purity Related substances—Use cyclohexylamine as the sample solution. Separately, pipet 1 mL of cyclohexylamine, add hexane to make exactly 100 mL, and use this as the standard solution. Perform the test as directed in the Thin-layer Chromatography. Spot 5 μL each of the sample solution and the standard solution on a plate of silica gel for thin-layer chromatography, develop the plate with a mixture of ethyl acetate, methanol, ammonia water (28) and cyclohexane (6:2:1:1) to a distance of about 10 cm, and air-dry the plate. Allow the plate to stand in iodine vapor: the spot other than the principal spot obtained with the sample solution is not more intense than the spot with the standard solution.

Cyclohexylmethanol $C_7H_{14}O$ A liquid having slight camphor odor. Soluble in ethanol (99.5).

Refractive index n_D^{20}: about 1.464

Bioling point: about 185°C

***N,N′*-Dibenzylethylenediamine diacetate** A white to slightly pale yellow crystalline powder.

Identification—Determine the infrared absorption spectrum of the substance to be examined as directed in the potassium bromide disk method under the Infrared Spectrophotometry: it exhibits absorption at the wave numbers of about 1530 cm^{-1}, 1490 cm^{-1}, 1460 cm^{-1}, 1400 cm^{-1} and

1290 cm^{-1}.

Content: not less than 99.0%. Assay—Weigh accurately about 25 mg of *N*,*N*′-dibenzylethylenediamine diacetate, dissolve in 25 mL of methanol, and add a solution containing 1.02 g of disodium hydrogen phosphate and 6.80 g of potassium dihydrogen phosphate in 1000 mL of water to make exactly 50 mL. Pipet 5 mL of this solution, add a mixture of the solution containing 1.02 g of disodium hydrogen phosphate and 6.80 g of potassium dihydrogen phosphate in 1000 mL of water and methanol (1:1) to make exactly 20 mL, and use this solution as the sample solution. Separately, weigh accurately about 8 mg of acetic acid (100), add 25 mL of methanol, and add the solution containing 1.02 g of disodium hydrogen phosphate and 6.80 g of potassium dihydrogen phosphate in 1000 mL of water to make exactly 50 mL. Pipet 5 mL of this solution, add a mixture of the solution containing 1.02 g of disodium hydrogen phosphate and 6.80 g of potassium dihydrogen phosphate in 1000 mL of water and methanol (1:1) to make exactly 20 mL, and use this solution as the control solution. Perform the test with exactly 20 μL each of the sample solution and the control solution as directed under the Liquid Chromatography according to the following conditions, and determine each peak area by the automatic integration method. After making correction for the peak areas based on the valiance of the base-line and the peak of acetic acid on the chromatogram obtained with the sample solution, calculate the amount of *N*,*N*′-dibenzylethylenediamine by the area percentage method.

Operating conditions

Detector: An ultraviolet absorption photometer (wavelength: 220 nm).

Column: A stainless steel column 4.6 mm in inside diameter and 25 cm in length, packed with octadecylsilanized silica gel for liquid chromatography (5 μm in particle diameter).

Column temperature: A constant temperature of about 40°C.

Mobile phase: A mixture of water, methanol and 0.25 mol/L potassium dihydrogen phosphate TS, pH 3.5 (11:7:2).

Flow rate: Adjust the flow rate so that the retention time of *N*,*N*′-dibenzylethylenediamine is about 4 minutes.

Time span of measurement: About 5 times as long as the retention time of *N*,*N*′-dibenzylethylenediamine.

System suitability

System performance: Dissolve an amount of Benzylpenicillin Benzathine, equivalent to about 85,000 Units, in 25 mL of methanol, add a solution containing 1.02 g of disodium hydrogen phosphate and 6.80 g of potassium dihydrogen phosphate in 1000 mL of water to make exactly 50 mL. Pipet 5 mL of this solution, add a mixture of the solution containing 1.02 g of disodium hydrogen phosphate and 6.80 g of potassium dihydrogen phosphate in 1000 mL of water and methanol (1:1) to make exactly 20 mL. When the procedure is run with 20 μL of this solution under the above operating conditions, *N*,*N*′-dibenzylethylenediamine and benzylpenicillin are eluted in this order with the resolution between these peaks being not less than 20.

System repeatability: When the test is repeated 6 times with 20 μL of the standard solution under the above operating conditions, the relative standard deviation of the peak area of *N*,*N*′-dibenzylethylenediamine is not more than 2.0%.

1,2-Dichlorobenzene $C_6H_4Cl_2$ A colorless liquid.
Specific gravity d_4^{20}: 1.306
Boiling point: 180 – 181°C

2,6-Dichlorophenol $C_6H_4Cl_2O$ White to purplish white crystals.
Melting point: 65 – 67°C

Dicyclohexyl $C_{12}H_{22}$
Specific gravity d_{20}^{20}: about 0.864
Boiling point: about 227°C
Melting point: about 4°C

Dicyclohexylurea $C_6H_{11}NHCONHC_6H_{11}$ A white crystalline powder, having no odor.

Purity Related substances—Dissolve 50 mg of dicyclohexylurea in methanol to make 100 mL. Pipet 10 mL of this solution, and add methanol to make 100 mL. Pipet 20 mL of this solution, add 10 mL of 0.5 mol/L sodium hydroxide TS, shake, then add 5 mL of diluted hydrochloric acid (1 in 10), and shake. Perform the test with 50 μL of this solution as directed under the Liquid Chromatography according to the following conditions, determine the area of each peak by the automatic integration method, and calculate the amount by the area percentage method: the total amount of the peaks other than dicyclohexylurea is not more than 3.0%.

Operating conditions

Detector, column, column temperature, mobile phase, and flow rate: Proceed as directed in the operating conditions in the Purity (5) (ii) under Acetohexamide.

Time span of measurement: About 5 times as long as the retention time of dicyclohexylurea after the solvent peak.

System suitability

Test for required detectability: To exactly 5 mL of the standard solution add water to make exactly 200 mL. Confirm that the peak area of dicyclohexylurea obtained with 50 μL of this solution is equivalent to 1.8 to 3.3% of that with 50 μL of the standard solution.

System performance, and system repeatability: Proceed as directed in the system suitability in the Purity (5) (ii) under Acetohexamide.

Digoxin $C_{41}H_{64}O_{14}$ [Same as the namesake monograph]

0.04 mol/L Disodium dihydrogen ethylenediamine tetraacetate TS Dissolve 14.890 g of disodium dihydrogen ethylenediamine tetraacetate dihydrate in water to make 1000 mL.

Ethylbenzene $C_6H_5C_2H_5$ A colorless liquid. Freely soluble in acetone and in ethanol (99.5), and practically insoluble in water.
Specific gravity d_4^{20}: 0.862 ~ 0.872

Boiling point: about 135°C

Flopropione [Same as the namesake monograph]

Flopropione for assay [Same as the monograph Flopropione. It contains not less than 99.0% of flopropione ($C_9H_{10}O_4$), calculated on the anhydrous basis.]

Fluid thioglycolate medium See the Sterility Test under the General Tests, Processes and Apparatus.

85% Glycerin $C_3H_8O_3$ [Same as the monograph Glycerin]

Human antithrombin III Serine protease inhibition factor obtained from normal plasma of health human. It is a protein, which inhibits the activities of thrombin and activated blood coagulation factor X. It contains not less than 300 Units per mg protein. One unit indicates an amount of the antithrombin III which inhibits 1 unit of thrombin at 25°C under the existence of heparin.

Dried human normal plasma powder Freeze-dried normal plasma obtained from healthy human.

Hydrogen peroxide-sodium hydroxide TS To a mixture of water and hydrogen peroxide (30) (9:1) add 3 drops of bromophenol blue TS, and then add 0.01 mol/L sodium hydroxide TS until a purple-blue color develops. Prepare before use.

***N*-(2-Hydroxyethyl)isonicotinamide nitric ester** $C_8H_9N_3O_4$ A white crystalline powder.

Identification—Determine the infrared absorption spectrum of *N*-(2-hydroxyethyl)isonicotinamide nitric ester as directed in the potassium bromide disk method under the Infrared Spectrophotometry: it exhibits absorption at the wave numbers of about 3270 cm^{-1}, 1653 cm^{-1}, 1546 cm^{-1} and 1283 cm^{-1}.

Hypaconitine for purity $C_{33}H_{45}NO_{10}$ White, crystals or crystalline powder. Soluble in acetonitrile, sparingly soluble in ethanol (99.5) and in diethyl ether, and practically insoluble in water. Melting point: about 175°C (with decomposition).

Identification—Determine the infrared absorption spectrum of hypaconitine for purity as directed in the potassium bromide disk method under the Infrared Spectrophotometry: it exhibits absorption at the wave numbers of about 3500 cm^{-1}, 1728 cm^{-1}, 1712 cm^{-1}, 1278 cm^{-1}, 1118 cm^{-1}, 1099 cm^{-1} and 714 cm^{-1}.

Absorbance $E_{1\,cm}^{1\%}$ (230 nm): 217 – 252 [5 mg dried for not less than 12 hours in a desiccator (reduced pressure not exceeding 0.67 kPa, phosphorus (V) oxide, 40°C), ethanol (99.5), 200 mL].

Purity Related substances—(1) Dissolve 5.0 mg of hypaconitine for purity in 2 mL of acetonitrile, and use as the sample solution. Pipet 1 mL of the sample solution, add acetonitrile to make exactly 50 mL, and use as the standard solution. Perform the test with these solutions as directed under the Thin-layer Chromatography. Spot 20 μL each of the sample solution and the standard solution on a plate of silica gel for thin-layer chromatography, and proceed the test as directed in the Identification in Processed Aconite Root: the spot other than the principal spot obtained with the sample solution is not more intense than the spot with the standard solution.

(2) Dissolve 5.0 mg of hypaconitine for purity in 5 mL of acetonitrile, and use as the sample solution. Pipet 1 mL of the sample solution, add acetonitrile to make exactly 50 mL, and use as the standard solution. Perform the test with exactly 10 μL each of the sample solution and the standard solution as directed under the Liquid Chromatography according to the following conditions, and determine each peak area by the automatic integration method: the total area of the peaks other than the peaks of hypaconitine and the solvent obtained with the sample solution is not larger than the peak area of hypaconitine with the standard solution.

Operating conditions

Detector, column, and column temperature: Proceed as directed in the operating conditions in the Purity under Processed Aconite Root.

Mobile phase: A mixture of phosphate buffer solution for processed aconite root and tetrahydrofuran (9:1).

Flow rate: Adjust the flow rate so that the retention time of hypaconitine is about 23 minutes.

Time span of measurement: About 3 times as long as the retention time of hypaconitine.

System suitability

Test for required detectability: Pipet 1 mL of the standard solution, and add acetonitrile to make exactly 20 mL. Confirm that the peak area of hypaconitine obtained from 10 μL of this solution is equivalent to 3.5 to 6.5% of that obtained from 10 μL of the standard solution.

System performance: Dissolve 1 mg each of aconitine for purity, hypaconitine for purity and mesaconitine for purity, and 8 mg of jesaconitine for purity in 200 mL of acetonitrile. When the procedure is run with 10 μL of this solution under the above operating conditions, mesaconitine, hypaconitine, aconitine and jesaconitine are eluted in this order, and each resolution between these peaks is not less than 1.5, respectively.

System repeatability: When the test is repeated 6 times with 10 μL of the standard solution under the above operating conditions, the relative standard deviation of the peak area of hypaconitine is not more than 1.5%.

Water: not more than 1.0% [5 mg dried for not less than 12 hours in a desiccator (reduced pressure not exceeding 0.67 kPa, phosphorus (V) oxide, 40°C), coulometric titration].

Icariin for thin-layer chromatography $C_{33}H_{40}O_{15}$ Light yellow crystals. Very slightly soluble in methanol and in ethanol (99.5), and practically insoluble in water. Melting point: about 234°C (with decomposition).

Purity Related substances—Dissolve 1.0 mg of icariin for thin-layer chromatography in 1 mL of methanol. Perform the test with 10 μL of this solution as directed in the Identification under Epimedium Herb: no spot other than the principal spot having *R*f value about 0.4 appears.

L-Isoleucine $C_6H_{13}NO_2$ [Same as the namesake monograph]

Jesaconitine for purity $C_{35}H_{49}NO_{12}$ A white powder. Freely soluble in acetonitrile, in ethanol (99.5) and in diethyl ether, and practically insoluble in water.

Identification—Determine the infrared absorption spectrum of jesaconitine for purity as directed in the potassium bromide disk method under the Infrared Spectrophotometry: it exhibits absorption at the wave numbers of about 3500 cm^{-1}, 1715 cm^{-1}, 1607 cm^{-1}, 1281 cm^{-1}, 1259 cm^{-1}, 1099 cm^{-1} and 772 cm^{-1}.

Absorbance $E^{1\%}_{1cm}$ (258 nm): 270 – 291 [5 mg dried for not less than 12 hours in a desiccator (reduced pressure not exceeding 0.67 kPa, phosphorus (V) oxide, 40°C), ethanol (99.5), 200 mL].

Purity Related substances—(1) Dissolve 5.0 mg of jesaconitine for purity in 2 mL of acetonitrile, and use as the sample solution. Pipet 1 mL of the sample solution, add acetonitrile to make exactly 50 mL, and use as the standard solution. Perform the test with these solutions as directed under the Thin-layer Chromatography. Spot 20 μL each of the sample solution and the standard solution on a plate of silica gel for thin-layer chromatography, and proceed the test as directed in the Identification in Processed Aconite Root: the spot other than the principal spot is not more intense than the spot with the standard solution.

(2) Dissolve 5.0 mg of jesaconitine for purity in 5 mL of acetonitrile, and use as the sample solution. Pipet 1 mL of the sample solution, add acetonitrile to make exactly 50 mL, and use as the standard solution. Perform the test with exactly 10 μL each of the sample solution and the standard solution as directed under the Liquid Chromatography according to the following conditions, and determine each peak area by the automatic integration method: the total area of the peaks other than the peaks of jesaconitine and the solvent is not larger than the peak area of jesaconitine with the standard solution.

Operating conditions

Detector, column, and column temperature: Proceed as directed in the operating conditions in the Purity under Processed Aconite Root.

Mobile phase: A mixture of phosphate buffer solution for processed aconite root and tetrahydrofuran (9:1).

Flow rate: Adjust the flow rate so that the retention time of jesaconitine is about 36 minutes.

Time span of measurement: About 3 times as long as the retention time of jesaconitine.

System suitability

Test for required detectability: Pipet 1 mL of the standard solution, and add acetonitrile to make exactly 20 mL. Confirm that the peak area of jesaconitine obtained from 10 μL of this solution is equivalent to 3.5 to 6.5% of that obtained from 10 μL of the standard solution.

System performance: Dissolve 5 mg each of aconitine for purity, hypaconitine for purity and mesaconitine for purity, and 1 mg of jesaconitine for purity in 200 mL of acetonitrile. When the procedure is run with 10 μL of this solution under the above operating conditions, mesaconitine, hypaconitine, aconitine and jesaconitine are eluted in this order, and each resolution between these peaks is not less than 1.5, respectively.

System repeatability: When the test is repeated 6 times with 10 μL of the standard solution under the above operating conditions, the relative standard deviation of the peak area of jesaconitine is not more than 1.5%.

Water: not more than 1.0% [5 mg dried for not less than 12 hours in a desiccator (reduced pressure not exceeding 0.67 kPa, phosphorus (V) oxide, 40°C), coulometric titration].

Loganin for thin-layer chromatography $C_{17}H_{26}O_{10}$ White, crystals or crystalline powder. Soluble in water, sparingly soluble in methanol, and very slightly soluble in ethanol (99.5). Melting point: 221 – 227°C.

Purity Related substances—Dissolve 1.0 mg of loganin for thin-layer chromatography in 2 mL of methanol. Perform the test with 10 μL of this solution as directed in the Identification under Cornus Fruit: any spot other than the principal spot at the *R*f value of about 0.4 does not appear.

Mesaconitine for purity $C_{33}H_{45}NO_{11}$ White, crystals or crystalline powder. Slightly soluble in acetonitrile and in ethanol (99.5), very slightly soluble in diethyl ether, and practically insoluble in water. Melting point: about 190°C (with decomposition).

Identification—Determine the infrared absorption spectrum of mesaconitine for purity as directed in the potassium bromide disk method under the Infrared Spectrophotometry: it exhibits absorption at the wave numbers of about 3510 cm^{-1}, 1713 cm^{-1}, 1277 cm^{-1}, 1116 cm^{-1}, 1098 cm^{-1} and 717 cm^{-1}.

Absorbance $E^{1\%}_{1cm}$ (230 nm): 211 – 247 [5 mg dried for not less than 12 hours in a desiccator (reduced pressure not exceeding 0.67 kPa, phosphorus (V) oxide, 40°C), ethanol (99.5), 200 mL].

Purity Related substances—(1) Dissolve 5.0 mg of mesaconitine for purity in 2 mL of acetonitrile, and use as the sample solution. Pipet 1 mL of the sample solution, add acetonitrile to make exactly 50 mL, and use as the standard solution. Perform the test with these solutions as directed under the Thin-layer Chromatography. Spot 20 μL each of the sample solution and the standard solution on a plate of silica gel for thin-layer chromatography, and proceed the test as directed in the Identification under Processed Aconite Root: the spot other than the principal spot is not more intense than the spot with the standard solution.

(2) Dissolve 5.0 mg of mesaconitine for purity in 5 mL of acetonitrile, and use as the sample solution. Pipet 1 mL of the sample solution, add acetonitrile to make exactly 50 mL, and use as the standard solution. Perform the test with exactly 10 μL each of the sample solution and the standard solution as directed under the Liquid Chromatography according to the following conditions, and determine each peak area by the automatic integration method: the total area of the peaks other than the peaks of mesaconitine and the solvent is not larger than the peak area of mesaconitine with the

standard solution.

Operating conditions

Detector, column, and column temperature: Proceed as directed in the operating conditions in the Purity under Processed Aconite Root.

Mobile phase: A mixture of phosphate buffer solution for processed aconite root and tetrahydrofuran (9:1).

Flow rate: Adjust the flow rate so that the retention time of mesaconitine is about 19 minutes.

Time span of measurement: About 3 times as long as the retention time of mesaconitine.

System suitability

Test for required detectability: Pipet 1 mL of the standard solution, and add acetonitrile to make exactly 20 mL. Confirm that the peak area of mesaconitine obtained from 10 μL of this solution is equivalent to 3.5 to 6.5% of that obtained from 10 μL of the standard solution.

System performance: Dissolve 1 mg each of aconitine for purity, hypaconitine for purity and mesaconitine for purity, and 8 mg of jesaconitine for purity in 200 mL of acetonitrile. When the procedure is run with 10 μL of this solution under the above operating conditions, mesaconitine, hypaconitine, aconitine and jesaconitine are eluted in this order, and each resolution between these peaks is not less than 1.5, respectively.

System repeatability: When the test is repeated 6 times with 10 μL of the standard solution under the above operating conditions, the relative standard deviation of the peak area of mesaconitine is not more than 1.5%.

Water: not more than 1.0% [5 mg dried for not less than 12 hours in a desiccator (reduced pressure not exceeding 0.67 kPa, phosphorus (V) oxide, 40°C), coulometric titration].

Methanol for liquid chromatography CH_3OH A clear, colorless liquid. Mixable with water.

Purity Ultraviolet-absorbing substances—Perform the test as directed in the Ultraviolet-visible Spectrophotometry using water as the blank: the absorbances at 210 nm, at 220 nm, at 230 nm, at 240 nm and at 254 nm are not more than 0.70, 0.30, 0.15, 0.07 and 0.02, respectively.

Metoclopramide for assay [Same as the monograph Metoclopramide. When dried, it contains not less than 99.0% of metoclopramide ($C_{14}H_{22}ClN_3O_2$).]

1,3-Naphthalenediol $C_{10}H_8O_2$ Red-brown crystals or gray-brown powder. Freely soluble in water, in methanol and in ethanol (99.5). Melting point: about 124°C.

1,3-Naphthalenediol TS Dissolve 50 mg of 1,3-naphthalenediol in 25 mL of ethanol (99.5), and add 2.5 mL of phosphoric acid.

3-Nitrophenol $C_6H_5NO_3$ A light yellow crystalline powder.

Melting point: 96 – 99°C

Osthole for thin-layer chromatography $C_{15}H_{16}O_3$ A white crystalline powder, having no odor. Freely soluble in methanol and in ethyl acetate, soluble in ethanol (99.5), and practically insoluble in water. Melting point: 83 – 84°C.

Purity Related substances—Dissolve 1.0 mg of osthole for thin-layer chromatography in 1 mL of methanol. Perform the test with 10 μL of this solution as directed in the Identification under Cnidium Monnieri Fruit: on spot appears other than the principal spot at around *R*f 0.3.

Oxytocin $C_{43}H_{66}N_{12}O_{12}S_2$ [Same as the namesake monograph]

D-Phenylglycine $C_8H_9NO_2$ White, crystals or crystalline powder. Slightly soluble in water.

Loss on drying: not more than 0.5% (1 g, 105°C, 3 hours).

Content: not less than 98.5%. Assay—Weigh accurately about 0.3 g of D-phenylglycine, previously dried, dissolve in 3 mL of formic acid, add 50 mL of acetic acid (100), and titrate with 0.1 mol/L perchloric acid VS (potentiometric titration). Perform a blank determination in the same manner, and make any necessary correction.

Each mL of 0.1 mol/L perchloric acid VS
= 15.12 mg of $C_8H_9NO_2$

Phenylpiperazine hydrochloride $C_{10}H_{14}N_2.HCl$ A white powder.

Melting point: about 247°C (with decomposition).

Phenylsilanized silica gel for liquid chromatography Prepared for liquid chromatography.

0.05 mol/L Phosphate buffer solution, pH 3.5 To 1000 mL of 0.05 mol/L potassium dihydrogen phosphate TS add a suitable amount of a solution of phosphoric acid (49 in 10,000) to make a solution having pH 3.5.

Phosphate buffer solution for processed aconite root Dissolve 19.3 g of disodium hydrogen phosphate 12-water in 3660 mL of water, and add 12.7 g of phosphoric acid.

[6]-Shogaol for thin-layer chromatography $C_{17}H_{24}O_3$ A pale yellow oil. Miscible with methanol, ethanol (99.5) and with diethyl ether, and practically insoluble in water.

Purity Related substances—Dissolve 1.0 mg of [6]-shogaol for thin-layer chromatography in 2 mL of methanol, and perform the test with this solution as directed under the Thin-layer chromatography. Spot 10 μL on a plate of silica gel for thin-layer chromatography, develop the plate with a mixture of ethyl acetate and hexane (1:1) to a distance of about 10 cm, and air-dry the plate. Spray evenly 4-dimethylaminobenzaldehyde TS on the plate, heat at 105°C for 5 minutes, and allow to cool: no spot other than the principal spot at around *R*f 0.5 appears.

0.0375 mol/L Sodium 1-decanesulfonate TS Dissolve 3.665 g of sodium 1-decanesulfonate in 400 mL of water.

Sodium dihydrogen phosphate NaH_2PO_4 A white, powder or crystalline powder. Freely soluble in water, and very slightly soluble in ethanol (99.5). It has a hygroscopic property.

A solution is acidic.

Soybean-casein digest medium See the Sterility Test under the General Tests, Processes and Apparatus.

Thioacetamide C_2H_5NS A white crystalline powder or colorless crystals. Freely soluble in water and in ethanol (99.5). Melting point: 112 – 115°C

Thioacetamide TS To 0.2 mL of a solution of thioacetamide (1 in 25) add 1 mL of a mixture of 15 mL of sodium hydroxide TS, 5 mL of water and 20 mL of 85% glycerin, and heat in a water bath for 20 seconds. Prepare before use.

Tiaramide hydrochloride for assay [Same as the monograph Tiaramide Hydrochloride. When dried, it contains not less than 99.0% of tiaramide hydrochloride ($C_{15}H_{18}ClN_3O_3S.HCl$).]

Trichloroacetic acid TS for serrapeptase Dissolve 1.80 g of trichloroacetic acid and 1.80 g of anhydrous sodium acetate in 5.5 mL of 6 mol/L acetic acid TS and water to make 100 mL.

Trichlorofluoromethane CCl_3F A colorless liquid or gas.

Specific gravity $d_4^{17.2}$: 1.494

Boiling point: 23.7°C

Vasopressin $C_{46}H_{65}N_{15}O_{12}S_2$ A white powder.

Constituent amino acids—Perform the test as directed in the Constituent amino acids under Oxytocin, and calculate the respective molar ratios with respect to glycine: 0.9 – 1.1 for aspartic acid, 0.9 – 1.1 for glutamic acid, 0.9 – 1.1 for proline, 0.8 – 1.1 for tyrosine, 0.9 – 1.1 for phenylalanine, 0.9 – 1.1 for arginine and 0.8 – 1.1 for cystine, and not more than 0.03 for other amino acids.

0.04 mol/L Zinc chloride TS Dissolve 5.452 g of zinc chloride in water to make 1000 mL.

(3) Standard Solutions for Volumetric Analysis

Change the following:

Iodine, 0.05 mol/L

1000 mL of this solution contains 12.690 g of iodine (I: 126.90).

Preparation—Dissolve 13 g of iodine in 100 mL of a solution of potassium iodide (2 in 5), add 1 mL of dilute hydrochloric acid and water to make 1000 mL, and standardize the solution as follows:

Standardization—Measure exactly 15 mL of the iodine solution, and titrate with 0.1 mol/L sodium thiosulfate VS (Indicator method: starch TS; or potentiometric titration: platinum electrode). In the indicator method, when the solution assumes a pale yellow color as the end point is approached, add 3 mL of starch TS, and continue the titration until the blue color disappears. Calculate the molarity factor.

Note: Store protected from light. This solution, if stored for a long period, should be restandardized before use.

Add the following:

Sodium Thiosulfate, 0.002 mol/L

1000 mL of this solution contains 0.4964 g of sodium thiosulfate pentahydrate ($Na_2S_2O_3.5H_2O$: 248.19).

Preparation—Before use, dilute 0.1 mol/L sodium thiosulfate VS with freshly boiled and cooled water to make exactly 50 times the initial volume.

Add the following:

74. Powder Particle Density Determination

Powder Particle Density Determination is a method to determine particle density of powdered pharmaceutical drugs or raw materials of drugs, and the gas displacement pycnometer is generally used. The powder density by this method is determined with an assumption that the volume of the gas displaced by the powder in a closed system is equal to the volume of the powder. The bulk density at loose packing or the tapped density at tapping express the apparent densities of the powder, since interparticulate void volume of the powder is assumed to contribute a part of the volume of the powder. On the contrary, the pycnometric particle density expresses the powder density nearly equal to the crystal density, since the volume of the powder, that is deducted with void volume of open pores accessible to gas, is counted.

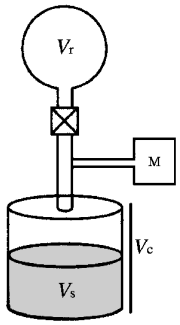
Powder particle density is expressed in mass per unit volume (kg/m^3), and generally expressed in g/cm^3.

Apparatus

The schematic diagram of particle density apparatus for gas displacement pycnometric measurement is shown in Figure 1. The apparatus consists of a test cell in which the sample is placed, a reference cell and a manometer.

Generally, helium is used as the measurement gas. The apparatus has to be equipped with a system capable of pressuring the test cell to the defined pressure through the manometer.

Calibration of apparatus The volumes of the test cell (V_c) and the reference cell (V_r) must be accurately determined to the nearest 0.001 cm^3, and to assure accuracy of the results of volume obtained, calibration of the apparatus is carried out as follows using a calibration ball of known volume for particle density measurement. The final pressures (P_f) are determined for the initial empty test cell followed by the test cell placed with the calibration ball for particle density measurement in accordance with the procedures, and V_c and V_r are calculated using the equation described in the section of Procedure. Calculation can be made taking into account that the sample volume (V_s) is zero in the first run.



V_r: Reference cell volume (cm^3)
V_c: Test cell volume (cm^3)
V_s: Sample volume (cm^3)
M: Manometer

Figure 1. Schematic diagram of a gas displacement pycnometer

Procedure

The measurement of the particle density is carried out between 15 and 30°C, and temperature must not vary by more than 2°C during the course of measurement.

Firstly, weigh the mass of the test cell and record it. After weighing out the amount of the sample as described in the individual monograph and placing it in the test cell, seal the cell in the pycnometer. Secondly, introduce the measurement gas (helium) into the test cell, and remove volatile contaminants in the powder. If necessary, keep the sample powder under reduced pressure to remove the volatile contaminants in advance and use it as the test sample for measurement.

Open the valve which connects the reference cell with the test cell, confirm with the manometer that the pressure inside the system is stable, and then read the system reference pressure (P_r). Secondly, close the valve that connects to the two cells, and introduce the measurement gas into the test cell to achieve positive pressure. Confirm with the manometer that the pressure inside the system is stable, and then read the initial pressure (P_i). Open the valve to connect the test cell with the reference cell. After confirming that the indicator of the manometer is stable, read the final pressure (P_f), and calculate the sample volume (V_s) with the following equation.

$$V_s = V_c - \frac{V_r}{\dfrac{P_i - P_r}{P_f - P_r} - 1}$$

V_r: Reference cell volume (cm^3)
V_c: Test cell volume (cm^3)
V_s: Sample volume (cm^3)
P_i: Initial pressure (kPa)
P_f: Final pressure (kPa)
P_r: Reference pressure (kPa)

Repeat the measurement sequence for the same powder sample until consecutive measurements of the sample volume agree to within 0.5%, and calculate the mean of sample volumes (V_s). Finally, unload the test cell, weigh the mass of test cell, and calculate the final sample mass by deducting the empty cell mass from the test cell mass. The powder particle density ρ is calculated by the following equation.

$$\rho = \frac{m}{V_s}$$

ρ: Powder particle density (g/cm^3)
m: Final sample mass (g)
V_s: Sample volume (cm^3)

Official Monographs for Part I

Acetohexamide

アセトヘキサミド

Change the origin/limits of content to read:

Acetohexamide, when dried, contains not less than 98.0% and not more than 101.0% of $C_{15}H_{20}N_2O_4S$.

Change the Description to read:

Description Acetohexamide occurs as a white to yellowish white powder.

It is freely soluble in *N*,*N*-dimethylformamide, sparingly soluble in acetone, slightly soluble in methanol and in ethanol (99.5), and practically insoluble in water.

Melting point: about 185°C (with decomposition).

Change the Purity (5) to read:

Purity

(5) Related substances (i) Cyclohexylamine—Dissolve exactly 1.0 g of Acetohexamide in exactly 30 mL of 0.5 mol/L sodium hydroxide TS, add exactly 5 mL of hexane, shake vigorously for 60 minutes, allow to stand for 5 minutes, and use the upper layer as the sample solution. Separately, dissolve exactly 50 mg of cyclohexylamine in 0.5 mol/L sodium hydroxide TS to make exactly 50 mL. Pipet 2 mL of this solution, and add 0.5 mol/L sodium hydroxide TS to make exactly 300 mL. Pipet 30 mL of this solution, add exactly 5 mL of hexane, shake vigorously for 60 minutes, allow to stand for 5 minutes, and use the upper layer as the standard solution. Perform the test with exactly 2 μL each of the sample solution and the standard solution as directed under the Gas Chromatography according to the following conditions, and determine the peak area of cyclohexylamine by the automatic integration method: the peak area of cyclohexylamine is not more than that with the standard solution.

Operating conditions—

Detector: A hydrogen flame-ionization detector.

Column: A fused-silica column 0.53 mm in inside diameter and 30 m in length, coated the inner surface with methylsilicone polymer for gas chromatography 1.5 μm in thickness.

Column temperature: A constant temperature of about 90°C.

Injection port temperature: A constant temperature of about 150°C.

Detector temperature: A constant temperature of about 210°C.

Carrier gas: Helium

Flow rate: Adjust the flow rate so that the retention time of cyclohexylamine is about 4 minutes.

Split ratio: 1:1

System suitability—

System performance: When the procedure is run with 2 μL of the standard solution under the above operating conditions, the number of theoretical plates of the peak of cyclohexylamine is not less than 8000.

System repeatability: When the test is repeated 6 times with 2 μL of the standard solution under the above operating conditions, the relative standard deviation of the peak area of cyclohexylamine is not more than 5%.

(ii) Dicyclohexylurea—Dissolve exactly 1.0 g of Acetohexamide in exactly 10 mL of 0.5 mol/L sodium hydroxide TS, add exactly 20 mL of methanol, shake, then add exactly 5 mL of diluted hydrochloric acid (1 in 10), shake vigorously for 15 minutes, and centrifuge. Filter 10 mL or more of the supernatant liquid through a membrane filter with pore size of not larger than 0.5 μm. Discard the first 5 mL of the filtrate, and use the subsequent filtrate as the sample solution. Separately, dissolve exactly 50 mg of dicyclohexylurea in methanol to make exactly 100 mL. Pipet 2 mL of this solution, and add methanol to make exactly 100 mL. Pipet 20 mL of this solution, add exactly 10 mL of 0.5 mol/L sodium hydroxide TS, shake, then add exactly 5 mL of diluted hydrochloric acid (1 in 10), shake, and use this solution as the standard solution. Perform the test with exactly 50 μL each of the sample solution and the standard solution as directed under the Liquid Chromatography according to the following conditions, and determine the peak area of dicyclohexylurea by the automatic integration method: the peak area of dicyclohexylurea is not more than that with the standard solution.

Operating conditions—

Detector: An ultraviolet absorption photometer (wavelength: 210 nm).

Column: A stainless steel column 4.6 mm in inside diameter and 25 cm in length, packed with octadecylsilanized silica gel for liquid chromatography (5 μm in particle diameter).

Column temperature: A constant temperature of about 25°C.

Mobile phase: Dissolve 0.5 g of sodium hydroxide in 1000 mL of 0.05 mol/L sodium dihydrogen phosphate TS, and adjust the pH to 6.5 with 0.5 mol/L sodium hydroxide TS. To 500 mL of this solution add 500 mL of acetonitrile.

Flow rate: Adjust the flow rate so that the retention time

of dicyclohexylurea is about 10 minutes.

System suitability—

System performance: When the procedure is run with 50 μL of the standard solution under the above operating conditions, the number of theoretical plates of the peak of dicyclohexylurea is not less than 10,000.

System repeatability: When the test is repeated 6 times with 50 μL of the standard solution under the above operating conditions, the relative standard deviation of the peak area of dicyclohexylurea is not more than 2.0%.

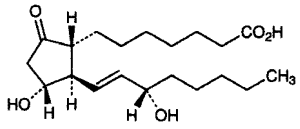
(iii) Other related substances—Dissolve 0.10 g of Acetohexamide in 10 mL of acetone, and use this solution as the sample solution. Pipet 1 mL of the sample solution, and add acetone to make exactly 20 mL. Pipet two 1-mL portions of this solution, add acetone to make exactly 10 mL and 25 mL, respectively, and use these solutions as the standard solution (1) and the standard solution (2). Perform the test with these solutions as directed under the Thin-layer Chromatography. Spot 10 μL each of the sample solution, the standard solution (1) and the standard solution (2) on a plate of silica gel with fluorescent indicator for thin-layer chromatography. Develop the plate with a mixture of ethyl acetate, methanol, ammonia water (28) and cyclohexane (6:2:1:1) to a distance of about 10 cm, and air-dry the plate. Examine under ultraviolet light (main wavelength: 254 nm): the spots other than the principal spot from the sample solution are not more intense than spot from the standard solution (1), and the number of them which are more intense than the spot from the standard solution (2) is not more than 4.

Add the following:

Alprostadil

アルプロスタジル

Prostaglandin E_1



$C_{20}H_{34}O_5$: 354.48

7-{(1*R*,2*R*,3*R*)-3-Hydroxy-2-[(1*E*,3*S*)-3-hydroxyoct-1-en-1-yl]-5-oxocyclopentyl}heptanoic acid [745-65-3]

Alprostadil, when dried, contains not less than 97.0% and not more than 103.0% of $C_{20}H_{34}O_5$.

Description Alprostadil occurs as white crystals or crystalline powder.

It is freely soluble in ethanol (99.5) and in tetrahydrofuran, slightly soluble in acetonitrile, and practically insoluble in water.

Identification (1) The absorption spectrum of a solution of Alprostadil in ethanol (99.5) (1 in 100,000) determined as directed under the Ultraviolet-visible Spectrophotometry shows no absorption between 210 nm and 350 nm. Separately, to 10 mL of this solution add 1 mL of potassium hydroxide-ethanol TS, allow to stand for 15 minutes, and determine the absorption spectrum in the same way. Compare the spectrum so obtained with the Reference Spectrum or the spectrum of a solution of Alprostadil Reference Standard prepared in the same manner as the sample solution: both spectra exhibit similar intensities of absorption at the same wavelengths.

(2) Determine the infrared absorption spectrum of Alprostadil, previously dried, as directed in the potassium bromide disk method under the Infrared Spectrophotometry, and compare the spectrum with the Reference Spectrum or the spectrum of previously dried Alprostadil Reference Standard: both spectra exhibit similar intensities of absorption at the same wave numbers.

Optical rotation $[\alpha]_D^{20}$: −53 – −61° (after drying, 25 mg, tetrahydrofuran, 5 mL, 100 mm).

Melting point 114 – 118°C

Purity Related substances—Dissolve 4 mg of Alprostadil in 2 mL of a mixture of acetonitrile for liquid chromatography and water (9:1), and use this solution as the sample solution. Pipet 0.5 mL of the sample solution, and add the mixture of acetonitrile for liquid chromatography and water (9:1) to make exactly 10 mL. Pipet 2 mL of this solution, add the mixture of acetonitrile for liquid chromatography and water (9:1) to make exactly 10 mL, and use this solution as the standard solution. Perform the test with exactly 5 μL each of the sample solution and the standard solution as directed under the Liquid Chromatography according to the following conditions, and determine each peak area by the automatic integration method: the area of the peaks, having the relative retention time of about 0.70 and 1.26 with respect to alprostadil, is not larger than 1/2 times the peak area of alprostadil with the standard solution, the area of the peaks, having the relative retention time of about 0.88 and 1.18 with respect to alprostadil, is not larger than the peak area of alprostadil with the standard solution, the area of the peaks other than alprostadil and the peaks mentioned above is not larger than 1/10 times the peak area of alprostadil with the standard solution and the total area of the peaks other than alprostadil is not larger than 2 times the peak area of alprostadil with the standard solution.

Operating conditions—

Detector, column, column temperature, mobile phase, and flow rate: Proceed as directed in the operating conditions in the Assay.

Time span of measurement: About 5 times as long as the retention time of alprostadil after the solvent peak.

System suitability—

Test for required detectability: Measure exactly 2 mL of the standard solution, add the mixture of acetonitrile for liquid chromatography and water (9:1) to make exactly 20 mL. Confirm that the peak area of alprostadil obtained with 5 μL of this solution is equivalent to 7 to 13% of that with 5 μL of the standard solution.

System performance: Proceed as directed in the system suitability in the Assay.

System repeatability: When the test is repeated 6 times with 5 μL of the standard solution under the above operating conditions, the relative standard deviation of the peak area of alprostadil is not more than 1.5%.

Loss on drying Not more than 1.0% (0.1 g, in vacuum, phosphorus (V) oxide, 4 hours).

Assay Weigh accurately about 5 mg each of Alprostadil and Alprostadil Reference Standard, previously dried, dissolve in exactly 5 mL of the internal standard solution, add a mixture of acetonitrile for liquid chromatography and water (9:1) to make 50 mL, and use these solutions as the sample solution and the standard solution, respectively. Perform the test with 5 μL each of the sample solution and the standard solution as directed under the Liquid Chromatography according to the following conditions, and determine the ratios, Q_T and Q_S, of the peak area of alprostadil to that of the internal standard.

$$\text{Amount (mg) of } C_{20}H_{34}O_5 = W_S \times \frac{Q_T}{Q_S}$$

W_S: Amount (mg) of Alprostadil Reference Standard

Internal standard solution—A solution of dimethyl phthalate in the mixture of acetonitrile for liquid chromatography and water (9:1) (1 in 10,000).

Operating conditions—

Detector: An ultraviolet absorption photometer (wavelength: 196 nm).

Column: A stainless steel column 4.6 mm in inside diameter and 15 cm in length, packed with octadecylsilanized silica gel for liquid chromatography (5 μm in particle diameter).

Column temperature: A constant temperature of about 40°C.

Mobile phase: Dissolve 9.07 g of potassium dihydrogen phosphate in water to make 1000 mL. Adjust the pH to 6.3 with a solution prepared by dissolving 9.46 g of disodium hydrogen phosphate in water to make 1000 mL, and dilute to 10 times its volume with water. To 360 mL of this solution add 110 mL of acetonitrile for liquid chromatography and 30 mL of methanol for liquid chromatography.

Flow rate: Adjust the flow rate so that the retention time of alprostadil is about 10 minutes.

System suitability—

System performance: When the procedure is run with 5 μL of the standard solution under the above operating conditions, alprostadil and the internal standard are eluted in this order with the resolution between these peaks being not less than 9.

System repeatability: When the test is repeated 6 times with 5 μL of the standard solution under the above operating conditions, the relative standard deviation of the ratio of the peak area of alprostadil to that of the internal standard is not more than 1.0%.

Containers and storage Containers—Tight containers.

Storage—Light-resistant, and at a temperature not exceeding 5°C.

Atropine Sulfate Injection

硫酸アトロピン注射液

Change the origin/limits of content to read:

Atropine Sulfate Injection is an aqueous solution for injection.

It contains not less than 93.0% and not more than 107.0% of the labeled amount of atropine sulfate [$(C_{17}H_{23}NO_3)_2.H_2SO_4.H_2O$: 694.83].

Change the Identification (2) to read:

Identification

(2) Evaporate an exactly measured volume of Atropine Sulfate Injection, equivalent to 5 mg of Atropine Sulfate according to the labeled amount, on a water bath to dryness. After cooling, dissolve the residue in 1 mL of ethanol (95), and use this solution as the sample solution. If insoluble substance remains, crush it, allow to stand, and use the supernatant liquid as the sample solution. Separately, dissolve 10 mg of Atropine Sulfate Reference Standard in 2 mL of ethanol (95), and use this solution as the standard solution. Perform the test with these solutions as directed under the Thin-layer Chromatography. Spot 5 μL each of the sample solution and the standard solution on a plate of silica gel for thin-layer chromatography. Develop the plate with a mixture of acetone, water and ammonia water (28) (90:7:3) to a distance of about 10 cm, and dry the plate at 80°C for 10 minutes. After cooling, spray evenly Dragendorff's TS for spraying on the plate: the spots obtained from the sample solution and the standard solution show an orange color and the same *R*f value.

Add the following next to Identification:

Bacterial endotoxins Less than 75 EU/mg.

Actual volume It meets the requirements of Injections.

Foreign insoluble matter Perform the test according to Method 1: it meets the requirement of the Foreign Insoluble Matter Test for Injections.

Insoluble particulate matter Perform the test according to Method 1: it meets the requirement of the Insoluble Particulate Matter Test for Injections.

Sterility Perform the test: it meets the requirement of the Sterility Test.

Change the Assay to read:

Assay To an exactly measured volume of Atropine Sulfate Injection, equivalent to about 5 mg of atropine sulfate [$(C_{17}H_{23}NO_3)_2.H_2SO_4.H_2O$], add exactly 3 mL of the internal standard solution and water to make 50 mL, and use this

solution as the sample solution. Separately, weigh accurately about 25 mg of Atropine Sulfate Reference Standard, separately determine its loss on drying in the same conditions as for Atropine Sulfate, and dissolve in water to make exactly 50 mL. Pipet 10 mL of this solution, add exactly 3 mL of the internal standard solution and water to make 50 mL, and use this solution as the standard solution. Perform the test with 20 μL each of the sample solution and the standard solution as directed under the Liquid Chromatography according to the following conditions, and determine the ratios, Q_T and Q_S, of the peak area of atropine sulfate to that of the internal standard.

$$\text{Amount (mg) of atropine sulfate } [(C_{17}H_{23}NO_3)_2.H_2SO_4.H_2O] = W_S \times \frac{Q_T}{Q_S} \times \frac{1}{5} \times 1.027$$

W_S: Amount (mg) of Atropine Reference Standard, calculated based on the dried basis

Internal standard solution—A solution of etilefrine hydrochloride (1 in 1000).

Operating conditions—

Detector: An ultraviolet absorption photometer (wavelength: 210 nm).

Column: A stainless steel column 4.6 mm in inside diameter and 15 cm in length, packed with octadecylsilanized silica gel for liquid chromatography (5 μm in particle diameter).

Column temperature: A constant temperature of about 40°C.

Mobile phase: To 0.4 g of sodium lauryl sulfate add 500 mL of diluted phosphoric acid (1 in 1000) to dissolve, and adjust the pH to 3.0 with sodium hydroxide TS. To 240 mL of this solution add 70 mL of tetrahydrofuran, and mix.

Flow rate: Adjust the flow rate so that the retention time of atropine sulfate is about 16 minutes.

System suitability—

System performance: When the procedure is run with 20 μL of the standard solution under the above operating conditions, the internal standard and atropine sulfate are eluted in this order with the resolution between these peaks being not less than 3.

System repeatability: When the test is repeated 6 times with 20 μL of the standard solution under the above operating conditions, the relative standard deviation of the ratio of the peak area of atropine sulfate to that of the internal standard is not more than 1.5%.

Add the following:

Azithromycin Hydrate

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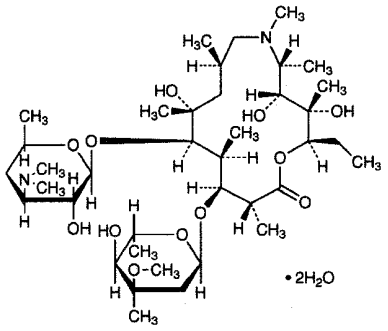

$C_{38}H_{72}N_2O_{12}.2H_2O$: 785.02

(2*R*,3*S*,4*S*,5*R*,6*R*,8*R*,11*R*,12*R*,13*S*,14*R*)-5-(3,4,6-Trideoxy-3-dimethylamino-β-D-*xylo*-hexopyranosyloxy)-3-(2,6-dideoxy-3-*C*-methyl-3-*O*-methyl-α-L-ribo-hexopyranosyloxy)-10-aza-6,12,13-trihydroxy-2,4,6,8,10,11,13-heptamethylhexadecan-14-olide dihydrate [*117772-70-0*]

Azithromycin Hydrate is the derivative of erythromycin.

It contains not less than 945 μg (potency) and not more than 1030 μg (potency) per mg, calculated on the anhydrous basis. The potency of Azithromycin Hydrate is expressed as mass (potency) of azithromycin ($C_{38}H_{72}N_2O_{12}$: 748.98).

Description Azithromycin Hydrate occurs as a white crystalline powder.

It is freely soluble in methanol and in ethanol (99.5), and practically insoluble in water.

Identification Determine the infrared absorption spectrum of Azithromycin Hydrate as directed in the potassium bromide disk method under the Infrared Spectrophotometry, and compare the spectrum with the Reference Spectrum or the spectrum of Azithromycin Reference Standard: both spectra exhibit similar intensities of absorption at the same wave numbers.

Optical rotation $[\alpha]_D^{20}$: -45 – $-49°$ (400 mg calculated on the anhydrous basis, ethanol (99.5), 20 mL, 100 mm).

Purity **(1)** Heavy metals—Proceed with 1.0 g of Azithromycin Hydrate according to Method 2, and perform the test. Prepare the control solution with 1.0 mL of Standard Lead Solution (not more than 10 ppm).

(2) Related substances—Being specified separately.

(3) Residual solvent—Being specified separately.

Water Not less than 4.0% and not more than 5.0% (0.4 g, volumetric titration, direct titration).

Residue on ignition Not more than 0.1% (1 g).

Assay Weigh accurately an amount of Azithromycin

Hydrate and Azithromycin Reference Standard, equivalent to about 50 mg (potency), dissolve each in an adequate amount of a mixture of acetonitrile and water (3:2), add exactly 2 mL of the internal standard solution and the mixture of acetonitrile and water (3:2) to make 50 mL, and use these solutions as the sample solution and the standard solution. Perform the test with 5 μL each of the sample solution and the standard solution as directed under the Liquid Chromatography according to the following conditions, and determine the ratios, Q_T and Q_S, of the peak area of azithromycin to that of the internal standard.

Amount [μg (potency)] of azithromycin ($C_{38}H_{72}N_2O_{12}$)

$$= W_S \times \frac{Q_R}{Q_S} \times 1000$$

W_S: Amount [mg (potency)] of Azithromycin Reference Standard

Internal standard solution—A solution of 4,4′-bis(diethylamino)benzophenone in acetonitrile (3 in 4000).

Operating conditions—

Detector: An ultraviolet absorption photometer (wavelength: 215 nm).

Column: A stainless steel column 4.6 mm in inside diameter and 25 cm in length, packed with octadecylsilanized polyvinyl alcohol gel polymer for liquid chromatography (5 μm in particle diameter).

Column temperature: A constant temperature of about 40°C.

Mobile phase: Dissolve 6.97 g of dipotassium hydrogen phosphate in about 750 mL of water, adjust the pH to 11.0 with potassium hydroxide TS, and add water to make 1000 mL. To 400 mL of this solution add 600 mL of acetonitrile for liquid chromatography.

Flow rate: Adjust the flow rate so that the retention time of azithromycin is about 10 minutes.

System suitability—

System performance: When the procedure is run with 5 μL of the standard solution under the above operating conditions, azithromycin and the internal standard are eluted in this order with the resolution between these peaks being not less than 2.0.

System repeatability: When the test is repeated 6 times with 5 μL of the standard solution under the above operating conditions, the relative standard deviation of the ratios of the peak area of azithromycin to that of the internal standard is not more than 1.0%.

Containers and storage Containers—Tight containers.

Add the following:

Benidipine Hydrochloride

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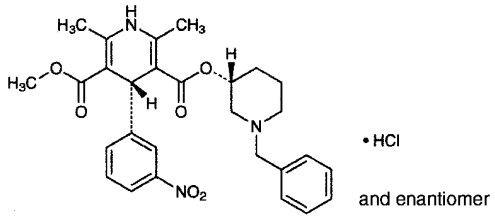

$C_{28}H_{31}N_3O_6.HCl$: 542.02

3-[(3*RS*)-1-Benzylpiperidin-3-yl] 5-methyl (4*RS*)-1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)pyridine-3,5-dicarboxylate monohydrochloride [*91599-74-5*]

Benidipine Hydrochloride, when dried, contains not less than 99.0% and not more than 101.0% of $C_{28}H_{31}N_3O_6.HCl$.

Description Benidipine Hydrochloride occurs as a yellow crystalline powder.

It is very soluble in formic acid, soluble in methanol, sparingly soluble in ethanol (99.5), and practically insoluble in water.

A solution of Benidipine Hydrochloride in methanol (1 in 100) shows no optical rotation.

Melting point: about 200°C (with decomposition).

Identification **(1)** Determine the absorption spectrum of a solution of Benidipine Hydrochloride in methanol (1 in 100,000) as directed under the Ultraviolet-visible Spectrophotometry, and compare the spectrum with the Reference Spectrum: both spectra exhibit similar intensities of absorption at the same wavelengths.

(2) Determine the infrared absorption spectrum of Benidipine Hydrochloride, previously dried, as directed in the potassium chloride disk method under the Infrared Spectrophotometry, and compare the spectrum with the Reference Spectrum: both spectra exhibit similar intensities of absorption at the same wave numbers.

(3) To 5 mL of a solution of Benidipine Hydrochloride (1 in 10) add 5 mL of ammonia TS, heat on a water bath for 5 minutes, cool, and filter. The filtrate, which is acidified with dilute nitric acid, responds to the Qualitative Tests (2) for chloride.

Purity **(1)** Heavy metals—Proceed with 1.0 g of Benidipine Hydrochloride according to Method 2, and perform the test. Prepare the control solution with 2.0 mL of Standard Lead Solution (not more than 20 ppm).

(2) Related substances—Dissolve 20 mg of Benidipine Hydrochloride in 100 mL of a mixture of water and methanol (1:1), and use this solution as the sample solution. Pipet 1 mL of the sample solution, add the mixture of water and methanol (1:1) to make exactly 500 mL, and use this solution as the standard solution. Perform the test with exactly 10 μL each of the sample solution and the standard so-

lution as directed under the Liquid Chromatography according to the following conditions, and determine each peak area by the automatic integration method: the peak areas of bisbenzylpiperidyl ester having the relative retention time of about 0.35 with respect to benidipine, dehydro derivative having the relative retention time of about 0.75 and other related substances are not larger than 1/2 times the peak area of benidipine with the standard solution, and the total area of the peaks other than benidipine is not larger than the peak area of benidipine with the standard solution. For this calculation, use the peak areas of bisbenzylpiperidyl ester and dehydro derivative after multiplying by their response factors, 1.6, respectively.

Operating conditions—

Detector: An ultraviolet absorption photometer (wavelength: 237 nm).

Column: A stainless steel column 4.6 mm in inside diameter and 10 cm in length, packed with octadecylsilanized silica gel for liquid chromatography (3 μm in particle diameter).

Column temperature: A constant temperature of about 25°C.

Mobile phase: A mixture of 0.05 mol/L potassium dihydrogen phosphate TS, pH 3.0, methanol and tetrahydrofuran (65:27:8).

Flow rate: Adjust the flow rate so that the retention time of benidipine is about 20 minutes.

Time span of measurement: About 2 times as long as the retention time of benidipine after the solvent peak.

System suitability—

Test for required detectability: Measure exactly 5 mL of the standard solution, and add the mixture of water and methanol (1:1) to make exactly 20 mL. Confirm that the peak area of benidipine obtained with 10 μL of this solution is equivalent to 18 to 32% of that with 10 μL of the standard solution.

System performance: Dissolve 6 mg of Benidipine Hydrochloride and 5 mg of benzoin in 200 mL of the mixture of water and methanol (1:1). When the procedure is run with 10 μL of this solution under the above operating conditions, benzoin and benidipine are eluted in this order with the resolution between these peaks being not less than 8.

System repeatability: When the test is repeated 6 times with 10 μL of the standard solution under the above operating conditions, the relative standard deviation of the peak area of benidipine is not more than 3.5%.

Loss on drying Not more than 0.5% (0.5 g, 105°C, 2 hours).

Residue on ignition Not more than 0.10% (1 g).

Assay Weigh accurately about 0.7 g of Benidipine Hydrochloride, previously dried, dissolve in 10 mL of formic acid, add 70 mL of acetic anhydride, and titrate with 0.1 mol/L perchloric acid VS (potentiometric titration). Perform a blank determination in the same manner, and make any necessary correction.

Each mL of 0.1 mol/L perchloric acid VS
= 54.20 mg of $C_{28}H_{31}N_3O_6$.HCl

Containers and storage Containers—Tight containers.

Add the following:

Benidipine Hydrochloride Tablets

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Benidipine Hydrochloride Tablets contain not less than 95.0% and not more than 105.0% of the labeled amount of benidipine hydrochloride ($C_{28}H_{31}N_3O_6$.HCl: 542.02).

Method of preparation Prepare as directed under Tablets, with Benidipine Hydrochloride.

Identification Shake well a quantity of powdered Benidipine Hydrochloride Tablets, equivalent to 10 mg of Benidipine Hydrochloride according to the labeled amount, with 100 mL of methanol, and centrifuge. To 10 mL of the supernatant liquid add methanol to make 100 mL, and use this solution as the sample solution. Determine the absorption spectrum of the sample solution as directed under the Ultraviolet-visible Spectrophotometry: it exhibits maxima between 235 nm and 239 nm, and between 350 nm and 360 nm.

Purity Dehydro derivative—Weigh accurately the mass of not less than 20 Benidipine Hydrochloride Tablets, and powder in an agate mortar. To an amount of the powder, equivalent to 20 mg of Benidipine Hydrochloride, add about 80 mL of a mixture of diluted phosphoric acid (1 in 500) and methanol (1:1), shake well, and add the mixture of diluted phosphoric acid (1 in 500) and methanol (1:1) to make exactly 100 mL. Filter through a membrane filter with pore size of 0.45 μm, and use the filtrate as the sample solution. Separately, dissolve 20 mg of benidipine hydrochloride for assay in the mixture of diluted phosphoric acid (1 in 500) and methanol (1:1) to make exactly 100 mL. Pipet 1 mL of this solution, add the mixture of diluted phosphoric acid (1 in 500) and methanol (1:1) to make exactly 100 mL, and use this solution as the standard solution. Perform the test with exactly 10 μL each of the sample solution and the standard solution as directed under the Liquid Chromatography according to the following conditions, and determine each peak area by the automatic integration method: the peak area of dehydro derivative having the relative retention time of about 0.75 with respect to benidipine is not larger than 1/2 times the peak area of benidipine with the standard solution. For this calculation, use the peak area of dehydro derivative after multiplying by the relative response factor, 1.6.

Operating conditions—

Perform as directed in the operating conditions in the Assay.

System suitability—

Test for required detectability: Measure exactly 2 mL of the standard solution, and add the mixture of diluted phosphoric acid (1 in 500) and methanol (1:1) to make exactly 20 mL. Confirm that the peak area of benidipine obtained with 10 μL of this solution is equivalent to 7 to 13% of that with 10 μL of the standard solution.

System performance: Dissolve 6 mg of benidipine hydrochloride and 5 mg of benzoin in 200 mL of a mixture of water and methanol (1:1). When the procedure is run with 10 μL of this solution under the above operating conditions, benzoin and benidipine are eluted in this order with the resolution between these peaks being not less than 8.

System repeatability: When the test is repeated 6 times with 10 μL of the standard solution under the above operating conditions, the relative standard deviation of the peak area of benidipine is not more than 2.0%.

Content uniformity Perform the test as directed in the Assay, and determine the content: it meets the requirements of the Content Uniformity Test.

Dissolution Perform the test with 1 tablet of Benidipine Hydrochloride Tablets at 50 revolutions per minute according to Method 2 under the Dissolution Test using the sinker, using 900 mL of the 1st fluid under the Disintegration Test as the dissolution medium. Withdraw 20 mL or more of the dissolution medium 30 minutes after starting the test for a 2-mg and a 4-mg tablet or 45 minutes after starting the test for a 8-mg tablet, and filter through a membrane filter with pore size of not more than 0.45 μm. Discard the first 10 mL of the filtrate, pipet the subsequent V mL, and add the 1st fluid to make exactly V' mL so that each mL contains about 2.2 μg of benidipine hydrochloride ($C_{28}H_{31}N_3O_6$.HCl) according to the labeled amount. Pipet 5 mL of this solution, add exactly 5 mL of the mobile phase, and use this solution as the sample solution. Separately, weigh accurately about 22 mg of benidipine hydrochloride for assay, previously dried at 105°C for 2 hours, and dissolve in the mobile phase to make exactly 100 mL. Pipet 2 mL of this solution, and add the mobile phase to make exactly 50 mL. Pipet 5 mL of this solution, and add the mobile phase to make exactly 20 mL. Pipet 5 mL of this solution, add exactly 5 mL of the 1st fluid, and use this solution as the standard solution. Perform the test with exactly 50 μL each of the sample solution and the standard solution as directed under the Liquid Chromatography according to the following conditions, and determine the peak areas, A_T and A_S, of benidipine: the dissolution rates for 2-mg or 4-mg tablet in 30 minutes and for 8-mg tablet in 45 minutes are not less than 80% and not less than 85%, respectively.

Dissolution rate (%) with respect to the labeled amount of benidipine hydrochloride ($C_{28}H_{31}N_3O_6$.HCl)

$$= W_S \times \frac{A_T}{A_S} \times \frac{V'}{V} \times \frac{1}{C} \times 9$$

W_S: Amount (mg) of benidipine hydrochloride for assay
C: Labeled amount (mg) of benidipine hydrochloride ($C_{28}H_{31}N_3O_6$.HCl) in 1 tablet.

Operating conditions—

Detector: An ultraviolet absorption photometer (wavelength: 237 nm).

Column: A stainless steel column 4.6 mm in inside diameter and 15 cm in length, packed with octadecylsilanized silica gel for liquid chromatography (5 μm in particle diameter).

Column temperature: A constant temperature of about 25°C.

Mobile phase: A mixture of 0.05 mol/L potassium dihydrogen phosphate TS, pH 3.0 and acetonitrile (11:9).

Flow rate: Adjust the flow rate so that the retention time of benidipine is about 5 minutes.

System suitability—

System performance: When the procedure is run with 50 μL of the standard solution under the above operating conditions, the number of theoretical plates and the symmetry factor of the peak of benidipine are not less than 3000 and not more than 2.0, respectively.

System repeatability: When the test is repeated 6 times with 50 μL of the standard solution under the above operating conditions, the relative standard deviation of the peak area of benidipine is not more than 1.5%.

Assay Shake 1 tablet of Benidipine Hydrochloride Tablets with 40 mL of a mixture of diluted phosphoric acid (1 in 500) and methanol (1:1) to disintegrate, add the mixture of diluted phosphoric acid (1 in 500) and methanol (1:1) to make exactly V mL so that each mL contains 40 μg of benidipine hydrochloride ($C_{28}H_{31}N_3O_6$.HCl), and centrifuge. Pipet 20 mL of the supernatant liquid, add exactly 10 mL of the internal standard solution and the mixture of diluted phosphoric acid (1 in 500) and methanol (1:1) to make 50 mL, and use this solution as the sample solution. Separately, weigh accurately about 40 mg of benidipine hydrochloride for assay, previously dried at 105°C for 2 hours, and dissolve in the mixture of diluted phosphoric acid (1 in 500) and methanol (1:1) to make exactly 100 mL. Pipet 2 mL of this solution, add exactly 10 mL of the internal standard solution and the mixture of diluted phosphoric acid (1 in 500) and methanol (1:1) to make 50 mL, and use this solution as the standard solution. Perform the test with 10 μL each of the sample solution and the standard solution as directed under the Liquid Chromatography according to the following conditions, and determine the ratios, Q_T and Q_S, of the peak area of benidipine to that of the internal standard. Perform the test described above with not less than 10 tablets, and calculate the average of these results.

Amount (mg) of benidipine hydrochloride ($C_{28}H_{31}N_3O_6$.HCl)

$$= W_S \times \frac{Q_T}{Q_S} \times \frac{V}{1000}$$

W_S: Amount (mg) of benidipine hydrochloride for assay

Internal standard solution—A solution of benzoin in a mixture of water and methanol (1:1) (13 in 200,000).

Operating conditions—

Detector: An ultraviolet absorption photometer (wavelength: 237 nm).

Column: A stainless steel column 4.6 mm in inside diameter and 10 cm in length, packed with octadecylsilanized silica gel for liquid chromatography (3 μm in particle diameter).

Column temperature: A constant temperature of about 25°C.

Mobile phase: A mixture of 0.05 mol/L potassium dihydrogen phosphate TS, pH 3.0, methanol and tetrahydrofuran (65:27:8).

Flow rate: Adjust the flow rate so that the retention time of benidipine is about 20 minutes.

System suitability—

System performance: When the procedure is run with 10 μL of the standard solution under the above operating conditions, the internal standard and benidipine are eluted in this order with the resolution between these peaks being not less than 8.

System repeatability: When the test is repeated 6 times with 10 μL of the standard solution under the above operating conditions, the relative standard deviation of the ratio of the peak area of benidipine to that of the internal standard is not more than 1.0%.

Containers and storage Containers—Well-closed containers.

Benzylpenicillin Benzathine

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Change the origin/limits of content to read:

Benzylpenicillin Benzathine is the *N*,*N*′-dibenzylethylenediamine salt of a penicillin compound having antibacterial activity produced by the growth of Penicillium species.

It contains not less than 1152 Units and not more than 1272 Units per mg, calculated on the anhydrous basis. The potency of Benzylpenicillin Benzathine is expressed as unit calculated from the amount of benzylpenicillin sodium ($C_{16}H_{17}N_2NaO_4S$: 356.37). 1 Unit of Benzylpenicillin Benzathine is equivalent to 0.6 μg of benzylpenicillin sodium ($C_{16}H_{17}N_2NaO_4S$). It contains not less than 24.0% and not more than 27.0% of *N*,*N*′-dibenzylethylenediamine ($C_{16}H_{20}N_2$: 240.34), calculated on the anhydrous basis.

Change the Purity (3) to read:

Purity

(3) Related substances—Dissolve 70 mg of Benzylpenicillin Benzathine in 25 mL of methanol, add a solution prepared by dissolving 1.02 g of disodium hydrogen phosphate and 6.80 g of potassium dihydrogen phosphate in water to make 1000 mL to make 50 mL, and use this solution as the sample solution. Pipet 1 mL of the sample solution, add the mobile phase A to make exactly 100 mL, and use this solution as the standard solution. Perform the test with exactly 20 μL each of the sample solution and the standard solution as directed under the Liquid Chromatography according to the following conditions, and determine each peak area by the automatic integration method: the area of the peak having the relative retention time of about 2.4 with respect to benzylpenicillin is not more than 2 times the total area of the peaks of benzylpenicillin and *N*,*N*′-dibenzylethylenediamine obtained from the standard solution, and the area of the peak other than benzylpenicillin, *N*,*N*′-dibenzylethylenediamine and the peak having the relative retention time of about 2.4 is not more than the total area of the peaks of benzylpenicillin and *N*,*N*′-dibenzylethylenediamine obtained from the standard solution.

Operating conditions—

Detector: An ultraviolet absorption photometer (wavelength: 220 nm).

Column: A stainless steel column 4.0 mm in inside diameter and 25 cm in length, packed with octadecylsilanized silica gel for liquid chromatography (5 μm in particle diameter).

Column temperature: A constant temperature of about 40°C.

Mobile phase A: A mixture of water, methanol and 0.25 mol/L potassium dihydrogen phosphate TS, pH 3.5 (6:3:1).

Mobile phase B: A mixture of methanol, water and 0.25 mol/L potassium dihydrogen phosphate TS, pH 3.5 (6:3:1).

Flowing of the mobile phase: Control the gradient by mixing the mobile phases A and B as directed in the following table.

Time after injection of sample (min)	Mobile phase A (%)	Mobile phase B (%)
0 – 10	75	25
10 – 20	75 → 0	25 → 100
20 – 55	0	100

Flow rate: 1.0 mL/min

Time span of measurement: About 3 times as long as the retention time of benzylpenicillin after the solvent peak.

System suitability—

Test for required detectability: To exactly 1 mL of the standard solution add the mobile phase A to make exactly 20 mL. Confirm that the peak area of benzylpenicillin obtained from 20 μL of this solution is equivalent to 3.5 to 6.5% of that from the standard solution.

System performance: When the procedure is run with 20 μL of the standard solution under the above operating conditions, *N*,*N*′-dibenzylethylenediamine and benzylpenicillin are eluted in this order with the resolution between these peaks being not less than 25.

System repeatability: When the test is repeated 3 times with 20 μL of the standard solution under the above operating conditions, the relative standard deviation of the peak

areas of benzylpenicillin is not more than 2.0%.

Change the Assay to read:

Assay (1) Benzylpenicillin—Weigh accurately an amount of Benzylpenicillin Benzathine, equivalent to about 85,000 Units, dissolve in 25 mL of methanol, and add a solution containing 1.02 g of disodium hydrogen phosphate and 6.80 g of potassium dihydrogen phosphate in 1000 mL of water to make exactly 50 mL. Pipet 5 mL of this solution, add a mixture of the solution containing 1.02 g of disodium hydrogen phosphate and 6.80 g of potassium dihydrogen phosphate in 1000 mL of water and methanol (1:1) to make exactly 20 mL, and use this solution as the sample solution. Separately, weigh accurately an amount of Benzylpenicillin Potassium Reference Standard, equivalent to about 85,000 Units, and about 25 mg of *N*,*N*′-dibenzylethylenediamine diacetate, dissolve in 25 mL of methanol, and add the solution containing 1.02 g of disodium hydrogen phosphate and 6.80 g of potassium dihydrogen phosphate in 1000 mL of water to make exactly 50 mL. Pipet 5 mL of this solution, add a mixture of the solution containing 1.02 g of disodium hydrogen phosphate and 6.80 g of potassium dihydrogen phosphate in 1000 mL of water and methanol (1:1) to make exactly 20 mL, and use this solution as the standard solution. Perform the test with exactly 20 μL each of the sample solution and the standard solution as directed under the Liquid Chromatography according to the following conditions, and determine the peak areas, A_T ands A_S, of benzylpenicillin.

Amount (unit) of benzylpenicillin sodium ($C_{16}H_{17}N_2NaO_4S$)

$$= W_S \times \frac{A_T}{A_S}$$

W_S: Amount (unit) of Benzylpenicillin Potassium Reference Standard

Operating conditions—

Detector: An ultraviolet absorption photometer (wavelength: 220 nm).

Column: A stainless steel column 4.6 mm in inside diameter and 25 cm in length, packed with octadecylsilanized silica gel for liquid chromatography (5 μm in particle diameter).

Column temperature: A constant temperature of about 40°C.

Mobile phase: A mixture of water, methanol and 0.25 mol/L potassium dihydrogen phosphate TS, pH 3.5 (11:7:2).

Flow rate: Adjust the flow rate so that the retention time of benzylpenicillin is about 18 minutes.

System suitability—

System performance: When the procedure is run with 20 μL of the standard solution under the above operating conditions, *N*,*N*′-dibenzylethylenediamine and benzylpenicillin are eluted in this order with the resolution between these peaks being not less than 20.

System repeatability: When the test is repeated 6 times with 20 μL of the standard solution under the above operating conditions, the relative standard deviations of the peak areas of *N*,*N*′-dibenzylethylenediamine and benzylpenicillin are not more than 2.0%, respectively.

(2) *N*,*N*′-Dibenzylethylenediamine—Determine the areas, A_T and A_S, of the peak corresponding to *N*,*N*′-dibenzylethylenediamine on the chromatograms obtained in (1) with the sample solution and the standard solution.

Amount (%) of *N*,*N*′-dibenzylethylenediamine ($C_{16}H_{20}N_2$)

$$= \frac{W_S}{W_T} \times \frac{A_T}{A_S} \times 100 \times 0.667$$

W_S: Amount (mg) of *N*,*N*′-dibenzylethylenediamine diacetate

W_T: Amount (mg) of the sample

0.667: Conversion factor for the molecular mass of *N*,*N*′-dibenzylethylenediamine diacetate ($C_{16}H_{20}N_2 \cdot 2CH_3COOH$) to that of *N*,*N*′-dibenzylethylenediamine (benzathine, $C_{16}H_{20}N_2$)

Add the following:

Cefepime Dihydrochloride for Injection

注射用塩酸セフェピム

Cefepime Dihydrochloride for Injection is a preparation for injection, which is dissolved before use.

It contains not less than 95.0% and not more than 110.0% of the labeled amount of cefepime ($C_{19}H_{24}N_6O_5S_2$: 480.56).

Method of preparation Prepare as directed under Injections, with Cefepime Dihydrochloride.

Description Cefepime Dihydrochloride for Injection occurs as a white to pale yellow powder.

Identification (1) Dissolve 40 mg of Cefepime Dihydrochloride in 2 mL of water, add 1 mL of a solution of hydroxylammonium chloride (1 in 10) and 2 mL of sodium hydroxide TS, allow to stand for 5 minutes, then add 3 mL of 1 mol/L hydrochloric acid TS and 3 drops of iron (III) chloride TS: a red-brown color develops.

(2) Determine the absorption spectrum of a solution of Cefepime Dihydrochloride for Injection (1 in 12,500) as directed under the Ultraviolet-visible Spectrophotometry: it exhibits maxima between 233 nm and 237 nm and between 255 nm and 259 nm.

pH The pH of a solution obtained by dissolving an amount of Cefepime Dihydrochloride for Injection, equivalent to 0.5 g (potency) of Cefepime Dihydrochloride according to the labeled amount, in 5 mL of water is between 4.0 and 6.0.

Purity (1) Clarity and color of solution—Dissolve an amount of Cefepime Dihydrochloride for Injection, equivalent to 0.5 g (potency) of Cefepime Dihydrochloride according to the labeled amount, in 5 mL of water: the solution is clear and colorless or light yellow. The color is not darker

than Matching Fluid I.

(2) *N*-Methylpyrrolidine—Weigh accurately an amount of Cefepime Dihydrochloride for Injection, equivalent to about 0.2 g (potency) of Cefepime Dihydrochloride according to the labeled amount, dissolve in diluted nitric acid (2 in 625) to make exactly 20 mL, and use this solution as the sample solution. Separately, transfer 30 mL of water into a 100-mL volumetric flask, weigh accurately the mass of the flask, add about 0.125 g of *N*-methylpyrrolidine, then weigh accurately the mass, and add water to make exactly 100 mL. Pipet 4 mL of this solution, add diluted nitric acid (2 in 3125) to make exactly 100 mL, and use this solution as the standard solution. Perform the test with exactly 100 μL each of the sample solution and the standard solution as directed under the Liquid Chromatography according to the following conditions, and determine the peak areas of *N*-methylpyrrolidine, A_T and A_S, by the automatic integration method within 20 minutes after the sample solution is prepared. Calculate the amount of *N*-methylpyrrolidine per mg (potency) of Cefepime Dihydrochloride for Injection by the following formula: not more than 1.0%.

$$\text{Amount (\%) of } N\text{-methylpyrrolidine} = \frac{W_S \times f}{W_T} \times \frac{A_T}{A_S} \times \frac{1}{125}$$

W_S: Amount (mg) of *N*-methylpyrrolidine
W_T: Amount [mg (potency)] of cefepime in the sample taken
f: Purity (%) of *N*-methylpyrrolidine

Operating conditions—

Proceed as directed in the operating conditions in the Purity (3) under Cefepime Dihydrochloride.

System suitability—

Proceed as directed in the system suitability in the Purity (3) under Cefepime Dihydrochloride.

Water Not more than 4.0% (Weigh accurately about 50 mg of Cefepime Dihydrochloride for Injection, dissolve in exactly 2 mL of methanol for Karl Fischer method, and perform the test with exactly 0.5 mL of this solution. Coulometric titration).

Bacterial endotoxins Less than 0.06 EU/mg (potency).

Mass variation It meets the requirements of the Mass Variation Test.

Foreign insoluble matter Perform the test according to Method 2: it meets the requirements of the Foreign Insoluble Matter Test for Injections.

Insoluble particulate matter Perform the test according to Method 1: it meets the requirements of the Insoluble Particulate Matter Test for Injections.

Sterility Perform the test according to the Membrane filtration method: it meets the requirements of the Sterility Test.

Assay Weigh accurately the mass of the contents of not less than 10 Cefepime Dihydrochloride for Injection. Weigh accurately an amount of the content, equivalent to about 60 mg (potency) of Cefepime Dihydrochloride according to the labeled amount, dissolve in the mobile phase to make exactly 50 mL, and use this solution as the sample solution. Separately, weigh accurately an amount of Cefepime Dihydrochloride Reference Standard, equivalent to about 60 mg (potency), dissolve in the mobile phase to make exactly 50 mL, and use this solution as the standard solution. Proceed as directed in the Assay under Cefepime Dihydrochloride.

$$\text{Amount [}\mu\text{g (potency)] of cefepime } (C_{19}H_{24}N_6O_5S_2) = W_S \times \frac{A_T}{A_S} \times 1000$$

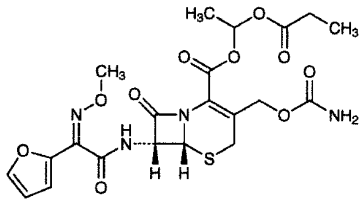
W_S: Amount [mg (potency)] of Cefepime Dihydrochloride Reference Standard

Containers and storage Containers—Hermetic containers. Storage—Light-resistant.

Cefuroxime Axetil

セフロキシムアキセチル

Change the graphic formula to read:



Change the chemical name to read:

(1*RS*)-1-Acetoxyethyl (6*R*,7*R*)-3-carbamoyloxymethyl-7-[(*Z*)-2-furan-2-yl-2-methoxyiminoacetylamino]-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylate

Cefuroxime Sodium

セフロキシムナトリウム

Change the Purity (1) to read:

Purity (1) Clarity and color of solution—Dissolve 1.0 g of Cefuroxime Sodium in 10 mL of water: the solution is clear, and its absorbance at 450 nm is not more than 0.25.

Ciclosporin

シクロスポリン

Change the Purity (3) to read:

Purity

(3) Related substances—Use the sample solution obtained in the Assay as the sample solution. Pipet 2 mL of the sample solution, add a mixture of water and acetonitrile (1:1) to make exactly 200 mL, and use this solution as the standard solution. Perform the test with exactly 20 μL each of the sample solution and the standard soluton as directed under the Liquid Chromatography according to the following conditions. Determine each peak area of both solutions by the automatic integration method: the area of the peak other than the ciclosporin is not more than 0.7 times of the peak area of ciclosporin from the standard solution, and the total area of all peaks other than the ciclosporin is not more than 1.5 times of the peak area of ciclosporin from the standard solution.

Operating conditions—

Detector, column, column temperature, mobile phase, and flow rate: Proceed as directed in the operating conditions in the Assay.

Time span of measurement: About 2 times as long as the retention time of ciclosporin after the solvent peak.

System suitability—

Test for required detection: To exactly 2 mL of the standard solution add a mixture of water and acetonitrile (1:1) to make exactly 20 mL. Confirm that the peak area of ciclosporin obtained from 20 μL of this solution is equivalent to 7 to 13% of that of ciclosporin obtained from 20 μL of the standard solution.

System performance: Proceed as directed in the system suitability in the Assay.

System repeatability: When the test is repeated 6 times with 20 μL of the standard solution under the above operating conditions, the relative standard deviation of the peak area of ciclosporin is not more than 3.0%.

Change the Assay to read:

Assay Weigh accurately about 30 mg each of Ciclosporin and Ciclosporin Reference Standard, previously determined the loss on drying as the same manner as Ciclosporin, and dissolve each in a mixture of water and acetonitrile (1:1) to make exactly 25 mL, and use these solutions as the sample solution and the standard solution. Perform the test with exactly 20 μL each of the sample solution and the standard solution as directed under the Liquid Chromatography according to the following conditions, and determine the peak areas, A_T and A_S, of ciclosporin.

$$\text{Amount (mg) of } C_{62}H_{111}N_{11}O_{12} = W_S \times \frac{A_S}{A_T}$$

W_S: Amount (mg) of Ciclosporin Reference Standard, calculated on the dried basis

Operating conditions—

Detector: An ultraviolet absorption photometer (wavelength: 210 nm).

Column: A stainless steel column 4 mm in inside diameter and 25 cm in length, packed with octadecylsilanized silica gel for liquid chromatography (5 μm in particle diameter). Connect the sample injection port and the column with a stainless steel tube 0.3 mm in inside diameter and 1 m in length.

Column temperature: A constant temperature of about 80°C (including the sample injection port and the connecting tube).

Mobile phase: A mixture of water, acetonitrile, tert-butyl methyl ether and phosphoric acid (520:430:50:1).

Flow rate: Adjust the flow rate so that the retention time of ciclosporin is about 27 minutes.

System suitability—

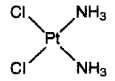
System performance: Dissolve 3 mg of Ciclosporin U in 2.5 mL of a mixture of water and acetonitrile (1:1), and add 2.5 mL of the standard solution. When the procedure is run with 20 μL of this solution under the above operating conditions, ciclosporin U and ciclosporin are eluted in this order with the resolution between these peaks being not less than 1.2.

System repeatability: When the test is repeated 6 times with 20 μL of the standard solution under the above operating conditions, the relative standard deviation of the peak area of ciclosporin is not more than 1.0%.

Add the following:

Cisplatin

シスプラチン



$Cl_2H_6N_2Pt$: 300.05

(*SP*-4-2)-Diamminedichloroplatinum [*15663-27-1*]

Cisplatin, when dried, contains not less than 98.0% and not more than 102.0% of $Cl_2H_6N_2Pt$.

Description Cisplatin occurs as a yellow crystalline powder.

It is sparingly soluble in *N*,*N*-dimethylformamide, slightly soluble in water, and practically insoluble in ethanol (99.5).

Identification **(1)** To 5 mL of a solution of Cisplatin (1 in 2000) add 2 to 3 drops of a solution of tin (II) chloride dihydrate (1 in 100): a brown precipitate is formed.

(2) Determine the absorption spectrum of a solution of Cisplatin in a solution of sodium chloride in 0.01 mol/L hydrochloric acid TS (9 in 1000) (1 in 2000) as directed under the Ultraviolet-visible Spectrophotometry, and compare the spectrum with the Reference Spectrum or the spectrum of a

solution of Cisplatin Reference Standard prepared in the same manner as the sample solution: both spectra exhibit similar intensities of absorption at the same wavelengths.

(3) Determine the infrared absorption spectrum of Cisplatin as directed in the potassium bromide disk method under the Infrared Spectrophotometry, and compare the spectrum with the Reference Spectrum or the spectrum of Cisplatin Reference Standard: both spectra exhibit similar intensities of absorption at the same wave numbers.

(4) A solution of Cisplatin (1 in 2000) responds to the Qualitative Test (1) for chloride.

Purity Ammonium amminetrichloroplatinate—Conduct this procedure using light-resistant vessels. Dissolve 50 mg of Cisplatin in a solution of sodium chloride (9 in 1000) to make exactly 100 mL, and use this solution as the sample solution. Separately, dissolve 10 mg of ammonium amminetrichloroplatinate for liquid chromatography, previously dried at 80°C for 3 hours, in the solution of sodium chloride (9 in 1000) to make exactly 200 mL. Pipet 2 mL of this solution, add the solution of sodium chloride (9 in 1000) to make exactly 20 mL, and use this solution as the standard solution. Perform the test with exactly 40 μL each of the sample solution and the standard solution as directed under the Liquid Chromatography according to the following conditions, and determine the peak area of ammonium amminetrichloroplatinate by the automatic integration method: the peak area from the sample solution is not more than that from the standard solution.

Operating conditions—

Detector: An ultraviolet absorption photometer (wavelength: 209 nm).

Column: A stainless steel column 4.6 mm in inside diameter and 25 cm in length, packed with silica gel for liquid chromatography having quaternary ammonium groups (10 μm in particle diameter).

Column temperature: A constant temperature of about 25°C.

Mobile phase: A solution of ammonium sulfate (1 in 800).

Flow rate: Adjust the flow rate so that the retention time of ammonium amminetrichloroplatinate is about 8 minutes.

System suitability—

System performance: When the procedure is run with 40 μL of the standard solution under the above operating conditions, the number of theoretical plates and the symmetry factor of the peak of ammonium amminetrichloroplatinate are not less than 1500 and not more than 2.0, respectively.

System repeatability: When the test is repeated 6 times with 40 μL of the standard solution under the above operating conditions, the relative standard deviation of the peak area of ammonium amminetrichloroplatinate is not more than 3.0%.

Loss on drying Not more than 0.1% (1 g, 105°C, 4 hours).

Assay Conduct this procedure using light-resistant vessels. Weigh accurately about 25 mg each of Cisplatin and Cisplatin Reference Standard, previously dried, dissolve in *N*,*N*-dimethylformamide to make exactly 25 mL, and use these solutions as the sample solution and the standard solution. Perform the test with exactly 40 μL each of the sample solution and the standard solution as directed under the Liquid Chromatography according to the following conditions, and determine the peak areas, A_T and A_S, of cisplatin by the automatic integration method.

$$\text{Amount (mg) of } Cl_2H_6N_2Pt = W_S \times \frac{A_T}{A_S}$$

W_S: Amount (mg) of Cisplatin Reference Standard

Operating conditions—

Detector: An ultraviolet absorption photometer (wavelength: 310 nm).

Column: A stainless steel column 4.6 mm in inside diameter and 25 cm in length, packed with aminopropylsilanized silica gel for liquid chromatography (5 μm in particle diameter).

Column temperature: A constant temperature of about 25°C.

Mobile phase: A mixture of ethyl acetate, methanol, water and *N*,*N*-dimethylformamide (25:16:5:5).

Flow rate: Adjust the flow rate so that the retention time of cisplatin is about 4 minutes.

System suitability—

System performance: When the procedure is run with 40 μL of the standard solution under the above operating conditions, the number of theoretical plates and the symmetry factor of the peak of cisplatin are not less than 3000 and not more than 2.0, respectively.

System repeatability: When the test is repeated 6 times with 40 μL of the standard solution under the above operating conditions, the relative standard deviation of the peak area of cisplatin is not more than 1.0%.

Containers and storage Containers—Tight containers.

Anhydrous Citric Acid

無水クエン酸

Change the Identification to read:

Identification Determine the infrared absorption spectrum of Anhydrous Citric Acid, previously dried at 105°C for 2 hours, as directed in the potassium bromide disk method under the Infrared Spectrophotometry, and compare the spectrum with the Reference Spectrum: both spectra exhibit similar intensities of absorption at the same wave numbers.

Clarithromycin

クラリスロマイシン

Change the origin/limits of content to read:

Clarithromycin is a derivative of erythromycin.

It contains not less than 950 μg (potency) and not more than 1050 μg (potency) per mg, calculated on the anhydrous basis. The potency of Clarithromycin is expressed as mass (potency) of clarithromycin ($C_{38}H_{69}NO_{13}$).

Change the Melting point to read:

Melting point 220 – 227°C

Change the Purity (2) and (3) to read:

Purity

(2) Arsenic—Prepare the test solution with 1.0 g of Clarithromycin according to Method 3, and perform the test (not more than 2 ppm).

(3) Related substances—Weigh accurately about 0.1 g of Clarithromycin, dissolve in the mobile phase to make exactly 20 mL, and use this solution as the sample solution. Separately, weigh accurately about 10 mg of Clarithromycin Reference Standard, dissolve in the mobile phase to make exactly 20 mL, and use this solution as the standard solution. Perform the test with exactly 10 μL each of the sample solution and the standard solution as directed under the Liquid Chromatography according to the following conditions, and determine the each peak area by the automatic integration method: the amount of each related substance calculated on the anhydrous basis is not more than 2.0%, and the total of them is not more than 5.0%. Exclude any peak with an area of less than 0.05%.

Amount (%) of each related substance calculated on the anhydrous basis

$$= \frac{W_S}{W_T} \times \frac{A_T}{A_S} \times 100$$

Total amount (%) of the related substances calculated on the anhydrous basis

$$= \frac{W_S}{W_T} \times \frac{\Sigma A_T}{A_S} \times 100$$

W_S: Amount (mg) of Clarithromycin Reference Standard
W_T: Amount (mg) of the sample, calculated on the anhydrous basis
A_S: Peak area of clarithromycin obtained with the standard solution
A_T: Peak area of each related substance obtained with the sample solution
ΣA_T: Total area of the peaks other than clarithromycin obtained with the sample solution

Operating conditions—

Detector, column, column temperature, mobile phase, and flow rate: Proceed as directed in the operating conditions in the Assay.

Time span of measurement: About 5 times as long as the retention time of the main peak after 2 minutes of sample injection.

System suitability—

Test for required detection: To exactly 2 mL of the standard solution add the mobile phase to make exactly 10 mL, and use this solution as the solution for system suitability test. Confirm that when the procedure is run with 10 μL of the solution for system suitability test, the peak area of clarithromycin is equivalent to 14 – 26% of that obtained from the standard solution.

System performance: Proceed as directed in the system suitability in the Assay.

System repeatability: When the test is repeated 6 times with 10 μL of the solution for system suitability test under the above operating conditions, the relative standard deviation of the peak area of clarithromycin is not more than 3.0%.

Colchicine

コルヒチン

Change to read except the structural formula and chemical name:

Colchicine contains not less than 97.0% and not more than 102.0% of $C_{22}H_{25}NO_6$, calculated on the anhydrous basis and corrected by the amount of ethyl acetate.

Description Colchicine occurs as a yellowish white powder.

It is very soluble in methanol, freely soluble in *N*,*N*-dimethylformamide, in ethanol (95) and in acetic anhydride, and sparingly soluble in water.

It is colored by light.

Identification **(1)** Determine the absorption spectrum of a solution of Colchicine in ethanol (95) (1 in 100,000) as directed under the Ultraviolet-visible Spectrophotometry, and compare the spectrum with the Reference Spectrum: both spectra exhibit similar intensities of absorption at the same wavelengths.

(2) To 1 g of potassium bromide for infrared absorption spectrum add 0.5 mL of a solution of Colchicine in methanol (1 in 50), grind thoroughly, and dry in vacuum at 80°C for 1 hour. Determine the infrared absorption spectrum of this powder as directed in the potassium bromide disk method under the Infrared Spectrophotometry, and compare the spectrum with the Reference Spectrum: both spectra exhibit similar intensities of absorption at the same wave numbers.

Optical rotation $[\alpha]_D^{20}$: −235 – −250° (0.1 g calculated on the anhydrous basis and corrected by the amount of ethyl

acetate, ethanol (95), 10 mL, 100 mm).

Purity (1) Colchiceine—Dissolve 0.10 g of Colchicine in 10 mL of water, and to 5 mL of this solution add 2 drops of iron (III) chloride TS: no definite green color develops.

(2) Chloroform and ethyl acetate—Weigh accurately about 0.60 g of Colchicine, dissolve in exactly 2 mL of the internal standard solution, add *N*,*N*-dimethylformamide to make 10 mL, and use this solution as the sample solution. Separately, weigh 0.30 g of chloroform using a 100-mL volumetric flask containing about 20 mL of *N*,*N*-dimethylformamide, and add *N*,*N*-dimethylformamide to make exactly 100 mL. Pipet 2 mL of this solution, add *N*,*N*-dimethylformamide to make exactly 200 mL, and use this solution as the standard solution (1). Separately, weigh accurately about 1.8 g of ethyl acetate using a 100-mL volumetric flask containing about 20 mL of *N*,*N*-dimethylformamide, and add *N*,*N*-dimethylformamide to make exactly 100 mL. Pipet 2 mL of this solution, add exactly 2 mL of the internal standard solution and *N*,*N*-dimethylformamide to make 10 mL, and use this solution as the standard solution (2). Perform the test with 2 μL each of the sample solution and the standard solutions (1) and (2) as directed under the Gas Chromatography according to the following conditions: the peak area of chloroform is not more than that from the standard solution (1). Determine the ratios of the peak area of ethyl acetate to that of the internal standard, Q_T and Q_S, of the sample solution and the standard solution (2), and calculate the amount of ethyl acetate by the following formula: the amount of ethyl acetate is not more than 6.0%.

$$\text{Amount (\%) of ethyl acetate } (C_4H_8O_2) = \frac{W_S}{W_T} \times \frac{Q_T}{Q_S} \times 2$$

W_S: Amount (g) of ethyl acetate
W_T: Amount (g) of the sample

Internal standard solution—A solution of 1-propanol in *N*,*N*-dimethylformamide (3 in 200)

Operating conditions—

Detector: A hydrogen flame-ionization detector

Column: A fused silica column 0.53 mm in inside diameter and 30 m in length, coated inside surface with polyethylene glycol 20 M for gas chromatography 1.0 μm in thickness.

Column temperature: 60°C for 7 minutes, then up to 100°C at a rate of 40°C per minute if necessary, and hold at 100°C for 10 minutes.

Injection port temperature: A constant temperature of about 130°C

Detector temperature: A constant temperature of about 200°C

Carrier gas: Helium

Flow rate: Adjust the flow rate so that the retention time of ethyl acetate is about 3 minutes.

Split ratio: 1:20

System suitability—

Test for required detectability: Pipet 2 mL of the standard solution (2), and add *N*,*N*-dimethylformamide to make exactly 25 mL. Pipet 1 mL of this solution, and add *N*,*N*-dimethylformamide to make exactly 50 mL. Confirm that the peak area of ethyl acetate obtained from 2 μL of this solution is equivalent to 0.11 to 0.21% of that obtained from 2 μL of the standard solution (2).

System performance: To 1 mL of chloroform add *N*,*N*-dimethylformamide to make 10 mL. To 1 mL of this solution add 2 mL of ethyl acetate and *N*,*N*-dimethylformamide to make 100 mL. To 2 mL of this solution add 2 mL of the internal standard solution and *N*,*N*-dimethylformamide to make 10 mL. When the procedure is run with 2 μL of this solution under the above operating conditions, ethyl acetate, chloroform and the internal standard are eluted in this order with the resolution between the peaks of chloroform and the internal standard being not less than 2.0.

System repeatability: When the test is repeated 3 times with 2 μL of the standard solution (2) under the above operating conditions, the relative standard deviation of the ratio of the peak area of ethyl acetate to that of the internal standard is not more than 3.0%.

(3) Related substances Dissolve 60 mg of Colchicine in 100 mL of diluted methanol (1 in 2). Pipet 1 mL of this solution, add diluted methanol (1 in 2) to make exactly 100 mL, and use this solution as the sample solution. Perform the test with 20 μL of the sample solution as directed under the Liquid Chromatography according to the following conditions, and determine each peak area by the automatic integration method. Calculate the total amount of the peaks other than colchicine by the area percentage method: not more than 5.0%.

Operating conditions—

Detector: An ultraviolet absorption photometer (wavelength: 254 nm).

Column: A stainless steel column 4.6 mm in inside diameter and 25 cm in length, packed with octylsilanized silica gel for liquid chromatography (5 μm in particle diameter).

Column temperature: A constant temperature of about 25°C.

Mobile phase: To 450 mL of 0.05 mol/L potassium dihydrogen phosphate TS add methanol to make 1000 mL. Adjust the pH to 5.5 with diluted phosphoric acid (7 in 200).

Flow rate: Adjust the flow rate so that the retention time of colchicine is about 7 minutes.

Time span of measurement: About 2 times as long as the retention time of colchicine after the solvent peak.

System suitability—

Test for required detectability: Pipet 1 mL of the sample solution, and add diluted methanol (1 in 2) to make exactly 50 mL. Confirm that the peak area of colchicine obtained from 20 μL of this solution is equivalent to 1.4 to 2.6% of that obtained from 20 μL of the sample solution.

System performance: When the procedure is run with 20 μL of the sample solution under the above operating conditions, the number of theoretical plates and the symmetry factor of the peak of colchicine are not less than 6000 and not more than 1.5, respectively.

System repeatability: When the test is repeated 6 times with 20 μL of the sample solution under the above operating conditions, the relative standard deviation of the peak area

of colchicine is not more than 2.0%.

Water Not more than 2.0% (0.5 g, volumetric titration, back titration).

Assay Weigh accurately about 0.4 g of Colchicine, dissolve in 25 mL of acetic anhydride, and titrate with 0.05 mol/L perchloric acid VS (potentiometric titration). Perform a blank determination in the same manner, and make any necessary correction.

Each mL of 0.05 mol/L perchloric acid VS
= 19.97 mg of $C_{22}H_{25}NO_6$

Containers and storage Containers—Tight containers.
Storage—Light-resistant.

Cytarabine

シタラビン

Change the origin/limits of content to read:

Cytarabine, when dried, contains not less than 98.5% and not more than 101.0% of $C_9H_{13}N_3O_5$.

Change the Description to read:

Description Cytarabine occurs as white crystals or crystalline powder.

It is freely soluble in water, soluble in acetic acid (100), and very slightly soluble in ethanol (99.5).

It dissolves in 0.1 mol/L hydrochloric acid TS.

Melting point: about 214°C (with decomposition).

Change the Identification to read:

Identification **(1)** Determine the absorption spectrum of a solution of Cytarabine in 0.1 mol/L hydrochloric acid TS (1 in 100,000) as directed under the Ultraviolet-visible Spectrophotometry, and compare the spectrum with the Reference Spectrum: both spectra exhibit similar intensities of absorption at the same wavelengths.

(2) Determine the infrared absorption spectrum of Cytarabine as directed in the potassium bromide disk method under the Infrared Spectrophotometry, and compare the spectrum with the Reference Spectrum: both spectra exhibit similar intensities of absorption at the same wave numbers.

Delete the Absorbance:

Change the Purity (4) and (5) to read:

Purity

(4) Related substances—Dissolve 0.10 g of Cytarabine in 10 mL of water, and use this solution as the sample solution. Pipet 1 mL of the sample solution, add water to make exactly 200 mL, and use this solution as the standard solution (1). Pipet 10 mL of the standard solution (1), add water to make exactly 25 mL and use this solution as the standard solution (2). Perform the test with these solutions as directed under the Thin-layer Chromatography. Spot 10 μL each of the sample solution and the standard solutions (1) and (2) on a plate of silica gel with fluorescent indicator for thin-layer chromatography. Develop the plate with 1-butanol saturated with water to a distance of about 12 cm, and air-dry the plate. Examine under ultraviolet light (main wavelength: 254 nm): the spots other than the principal spot from the sample solution are not more intense than the spot from the standard solution (1), and the number of them which are more intense than the spot from the standard solution (2) is not more than two. Spray evenly acidic potassium permanganate TS on the plate: any spot other than the principal spot does not appear.

Deferoxamine Mesilate

メシル酸デフェロキサミン

Change the Purity (6) to read:

Purity

(6) Related substances—Dissolve 50 mg of Deferoxamine Mesilata in 50 mL of the mobile phase, and use this solution as the sample solution. Pipet 3 mL of the sample solution, add the mobile phase to make exactly 50 mL, and use this solution as the standard solution. Perform the test with exactly 20 μL each of the sample solution and the standard solution as directed under the Liquid Chromatography according to the following conditions. Determine each peak area of both solutions by the automatic integration method: the total area of all peaks other than the peak of deferoxamine is not larger than the peak area of deferoxamine from the standard solution.

Operating conditions—

Detector: An ultraviolet absorption photometer (wavelength: 230 nm).

Column: A stainless steel column 4 mm in inside diameter and 20 cm in length, packed with octadecylsilanized silica gel for liquid chromatography (10 μm in particle diameter).

Column temperature: A constant temperature of about 40°C.

Mobile phase: Dissolve 1.32 g of diammonium hydrogen phosphate, 0.37 g of disodium dihydrogen ethylenediamine tetraacetate dihydrate and 1.08 g of sodium 1-heptanesulfonate in 950 mL of water, and adjust the pH of this solution to 2.8 with phosphoric acid. To 800 mL of this solution add 100 mL of 2-propanol.

Flow rate: Adjust the flow rate so that the retention time of deferoxamine is about 15 minutes.

Time span of measurement: About two times as long as the retention time of deferoxamine after the solvent peak.

System suitability—

Test for required detection: To exactly 2 mL of the standard solution add the mobile phase to make exactly 100 mL. Confirm that the peak area of deferoxamine obtained from

20 μL of this solution is equivalent to 1.5 to 2.5% of that of deferoxamine obtained from 20 μL of the standard solution.

System performance: Dissolve 16 mg of Deferoxamine Mesilate and 4 mg of methyl parahydroxybenzoate in 50 mL of the mobile phase. When the procedure is run with 20 μL of this solution under the above operating conditions, deferoxamine and methyl parahydroxybenzoate are eluted in this order with the resolution between these peaks being not less than 4.

System repeatability: When the test is repeated 6 times with 20 μL of the standard solution under the above operating conditions, the relative standard deviation of the peak areas of deferoxamine is not more than 3.0%.

Digoxin

ジゴキシン

Change the Description to read:

Description Digoxin occurs as colorless or white crystals or a white crystalline powder.

It is freely soluble in pyridine, slightly soluble in ethanol (95), very slightly soluble in acetic acid (100), and practically insoluble in water.

Change the Identification (2) to read:

Identification

(2) Determine the infrared absorption spectrum of Digoxin, previously dried, as directed in the potassium bromide disk method under the Infrared Spectrophotometry, and compare the spectrum with the Reference Spectrum: both spectra exhibit similar intensities of absorption at the same wave numbers.

Change the Optical rotation to read:

Optical rotation $[\alpha]_D^{20}$: +10.0 – +13.0° (after drying, 0.20 g, dehydratead pyridine, 10 mL, 100 mm).

Change the Purity (2) to read:

Purity

(2) Related substances—Dissolve exactly 25.0 mg of Digoxin in 50 mL of warm ethanol (95), cool, and add ethanol (95) to make exactly 100 mL. Pipet 10 mL of this solution, add 10 mL of water and dilute ethanol to make 50 mL, and use this solution as the sample solution. Separately, dissolve exactly 5.0 mg of Gitoxin Reference Standard, previously dried under reduced pressure at 105°C for 1 hour, in a mixture of acetonitrile and water (7:3) to make exactly 200 mL. Pipet 2 mL of this solution, add dilute ethanol to make 50 mL, and use this solution as the standard solution. Perform the test with exactly 10 μL each of the sample solution and the standard solution as directed under the Liquid Chromatography according to the following conditions, and determine the peak areas, A_T and A_S, of gitoxin: A_T is not larger than A_S, and the total of the areas of the peaks other than digitoxin and gitoxin, obtained by the area percentage method, is not more than 3%.

Operating conditions—

Detector, column, column temperature, mobile phase, and flow rate: Proceed as directed in the operating conditions in the Assay.

Time span of measurement: About 4 times as long as the retention time of digoxin after the solvent peak.

System suitability—

Test for required detectability: Pipet 1 mL of the sample solution, add the mobile phase to make exactly 100 mL, and use this solution as the solution for system suitability test. Pipet 1 mL of the solution, and add the mobile phase to make exactly 10 mL. Confirm that the peak area of digoxin obtained from 10 μL of this solution is equivalent to 7 to 13% of that from the solution for system suitability test.

System performance: Dissolve 25 mg of digoxin in 50 mL of warm ethanol (95), cool, and add ethanol (95) to make exactly 100 mL. Pipet 10 mL of this solution, add exactly 5 mL of a solution of propyl parahydroxybenzoate in ethanol (95) (1 in 4000), 10 mL of water and dilute ethanol to make 50 mL. When the procedure is run with 10 μL of this solution under the above operating conditions, digoxin and propyl parahydroxybenzoate are eluted in this order with the resolution between these peaks being not less than 5.

System repeatability: When the test is repeated 6 times with 10 μL of the solution for system suitability test under the above operating conditions, the relative standard deviation of the peak area of digoxin is not more than 1.0%.

Change the Assay to read:

Assay Weigh accurately about 25 mg each of Digoxin and Digoxin Reference Standard, previously dried, dissolve in 50 mL of warm ethanol (95), cool, and add ethanol (95) to make exactly 100 mL. Pipet 10 mL of these solutions, add exactly 5 mL of the internal standard solution, 10 mL of water and dilute ethanol to make 50 mL, and use these solutions as the sample solution and the standard solution. Perform the test with 10 μL each of the sample solution and the standard solution as directed under the Liquid Chromatography according to the following conditions, and determine the ratios, Q_T and Q_S, of the peak area of digoxin to that of the internal standard.

$$\text{Amount (mg) of } C_{41}H_{64}O_{14} = W_S \times \frac{Q_T}{Q_S}$$

W_S: Amount (mg) of Digoxin Reference Standard

Internal standard solution—A solution of propyl parahydroxybenzoate in ethanol (95) (1 in 4000).

Operating conditions—

Detector: An ultraviolet absorption photometer (wavelength: 220 nm).

Column: A stainless steel column 4.6 mm in inside diameter and 25 cm in length, packed with octadecylsilanized silica gel for liquid chromatography (5 μm in particle diameter).

Column temperature: A constant temperature of about

30°C.

Mobile phase: A mixture of water and acetonitrile (7:3).

Flow rate: Adjust the flow rate so that the retention time of digoxin is about 10 minutes.

System suitability—

System performance: When the procedure is run with 10 μL of the standard solution under the above operating conditions, digoxin and the internal standard are eluted in this order with the resolution between these peaks being not less than 5.

System repeatability: When the test is repeated 6 times with 10 μL of the standard solution under the above operating conditions, the relative standard deviation of the ratio of the peak area of digoxin to that of the internal standard is not more than 1.0%.

Change to read:

Digoxin Injection

ジゴキシン注射液

Digoxin Injection is an aqueous solution for injection.

It contains not less than 90.0% and not more than 105.0% of the labeled amount of digoxin ($C_{41}H_{64}O_{14}$: 780.94).

Method of preparation Prepare as directed under Injections, with a solution of Digoxin in 5 to 50 vol% ethanol.

Description Digoxin Injection is a clear, colorless liquid.

Identification Dilute Digoxin Injection, if necessary, with methanol so that each mL contains about 0.25 mg of Digoxin according to the labeled amount, and use this solution as the sample solution. In case where ingredients are suspected to affect the test, remove them by means of a solid-phase extraction. Separately, dissolve 0.5 mg of Digoxin Reference Standard in 2 mL of methanol, and use this solution as the standard solution. Perform the test with these solutions as directed under the Thin-layer Chromatography. Spot 10 μL each of the sample solution and the standard solution on a plate of octadecylsilanized silica gel for thin-layer chromatography. Develop the plate with a mixture of methanol and water (7:3) to a distance of about 10 cm, and air-dry the plate. Spray evenly a mixture of a solution of trichloroacetic acid in ethanol (99.5) (1 in 4) and a freshly prepared solution of sodium toluenesulfonchloramide trihydrate (3 in 100) (4:1) on the plate, heat at 110°C for 10 minutes, and examine under ultraviolet light (main wavelength: 366 nm): the *R*f values of the principal spots with the sample solution and the standard solution are not different each other.

Bacterial endotoxins Less than 200 EU/mg.

Actual volume It meets the requirements of Injections.

Foreign insoluble matter Perform the test according to Method 1: it meets the requirements of the Foreign Insoluble Matter Test for Injections.

Insoluble particulate matter Perform the test according to Method 1: it meets the requirement of the Insoluble Particulate Matter Test for Injections.

Sterility Perform the test according to the Membrane filtration method: it meets the requirements of the Sterility Test.

Assay To an exact volume of Digoxin Injection, equivalent to about 2.5 mg of digoxin ($C_{41}H_{64}O_{14}$), add exactly 5 mL of the internal standard solution and dilute ethanol to make 50 mL, and use this solution as the sample solution. Separately, weigh accurately about 25 mg of Digoxin Reference Standard, previously dried under reduced pressure at 105°C for 1 hour, dissolve in 50 mL of warm ethanol (95), cool, and add ethanol (95) to make exactly 100 mL. Pipet 10 mL of this solution, add exactly 5 mL of the internal standard solution, 10 mL of water and dilute ethanol to make 50 mL, and use this solution as the standard solution. Perform the test with 10 μL each of the sample solution and the standard solution as directed under the Liquid Chromatography according to the following conditions, and determine the ratios, Q_T and Q_S, of the peak area of digoxin to that of the internal standard.

$$\text{Amount (mg) of digoxin } (C_{41}H_{64}O_{14}) = W_S \times \frac{Q_T}{Q_S} \times \frac{1}{10}$$

W_S: Amount (mg) of Digoxin Reference Standard

Internal standard solution—A solution of propyl parahydroxybenzoate in ethanol (95) (1 in 4000).

Operating conditions—

Detector: An ultraviolet absorption photometer (wavelength: 220 nm).

Column: A stainless steel column 4.6 mm in inside diameter and 25 cm in length, packed with octadecylsilanized silica gel for liquid chromatography (5 μm in particle diameter).

Column temperature: A constant temperature of about 30°C.

Mobile phase: A mixture of water and acetonitrile (7:3).

Flow rate: Adjust the flow rate so that the retention time of digoxin is about 10 minutes.

System suitability—

System performance: When the procedure is run with 10 μL of the standard solution under the above operating conditions, digoxin and the internal standard are eluted in this order with the resolution between these peaks being not less than 5.

System repeatability: When the test is repeated 6 times with 10 μL of the standard solution under the above operating conditions, the relative standard deviation of the ratio of the peak area of digoxin to that of the internal standard is not more than 1.0%.

Containers and storage Containers—Hermetic containers, and colored containers may be used.

Storage—Light-resistant.

Change to read:

Digoxin Tablets

ジゴキシン錠

Digoxin Tablets contain not less than 90.0% and not more than 105.0% of the labeled amount of digoxin ($C_{41}H_{64}O_{14}$: 780.94).

Method of preparation Prepare as directed under Tablets, with Digoxin.

Identification To an amount of pulverized Digoxin Tablets, equivalent to 0.5 mg of Digoxin according to the labeled amount, add 2 mL of methanol, shake for 10 minutes, filter, and use the filtrate as the sample solution. Separately, dissolve 0.5 mg of Digoxin Reference Standard in 2 mL of methanol, and use this as the standard solution. Perform the test with these solutions as directed under the Thin-layer Chromatography. Spot 10 μL each of the sample solution and the standard solution on a plate of octadecylsilanized silica gel for thin-layer chromatography. Develop the plate with a mixture of methanol and water (7:3) to a distance of about 10 cm, and air-dry the plate. Spray evenly a mixture of a solution of trichloroacetic acid in ethanol (99.5) (1 in 4) and a freshly prepared solution of sodium toluenesulfonchloramide trihydrate (3 in 100) (4:1) on the plate, heat at 110°C for 10 minutes, and examine under ultraviolet light (main wavelength: 366 nm): the *R*f values of the principal spots with the sample solution and the standard solution are not different each other.

Content uniformity Perform the test according to the following method: it meets the requirements of the Content Uniformity Test.

To 1 tablet of Digoxin Tablets add 0.5 mL of water to disintegrate, then add exactly 0.5 mL of the internal standard solution, and add V mL of dilute ethanol so that each mL contains about 21 μg of digoxin ($C_{41}H_{64}O_{14}$). Exposure this solution to ultrasonic waves for 20 minutes, shake for 5 minutes, filter, and use the filtrate as the sample solution. Separately, weigh accurately about 25 mg of Digoxin Reference Standard, previously dried under reduced pressure at 105°C for 1 hour, dissolve in 50 mL of warm ethanol (95), cool, and add ethanol (95) to make exactly 100 mL. Pipet 10 mL of this solution, and add ethanol (95) to make exactly 20 mL. Pipet 1 mL of this solution, add exactly 0.5 mL of the internal standard solution, then add 1.5 mL of water and (V – 2) mL of dilute ethanol, and use this as the standard solution. Proceed with the sample solution and the standard solution as directed in the Assay.

$$\text{Amount (mg) of digoxin } (C_{41}H_{64}O_{14}) = W_S \times \frac{Q_T}{Q_S} \times \frac{1}{200}$$

W_S: Amount (mg) of Digoxin Reference Standard

Internal standard solution—A solution of propyl parahydroxybenzoate in ethanol (95) (1 in 40,000/V).

Dissolution Perform the test with 1 tablet of Digoxin Tablets, using 500 mL of diluted hydrochloric acid (3 in 500) at 100 revolutions per minute according to the Method 1 under the Dissolution Test as the dissolution medium. Withdraw 30 mL or more of the dissolved solution 60 minutes after starting the test, and filter through a membrane filter (less than 0.8 μm in pore size). Discard the first 10 mL of the filtrate, and use the subsequent filtrate as the sample solution. Separately, weigh accurately about 25 mg of Digoxin Reference Standard, previously dried in vacuum at 105°C for 1 hour, dissolve in a small portion of ethanol (95), and add a mixture of ethanol (95) and water (4:1) to make exactly 500 mL. Pipet 5 mL of this solution, add the dissolution medium to make exactly 500 mL, and use this solution as the standard solution. Pipet 2 mL each of the sample solution, the standard solution and the dissolution medium, and transfer to brown glass-stoppered test tubes. Add exactly 10 mL of 0.012 g/dL L-ascorbic acid-hydrochloric acid TS to these tubes, and shake. Immediately add exactly 1 mL of dilute hydrogen peroxide TS, shake well, and allow to stand at a constant temperature between 25°C and 30°C for 45 minutes. Determine the fluorescence intensities, F_T, F_S, and F_B, of these solutions at 360 nm of the excitation wavelength and at 485 nm of the fluorescence wavelength as directed under the Fluorometry, respectively: the dissolution rate in 60 minutes is not less than 65%. No retest requirement is applied to Digoxin Tablets.

Dissolution rate (%) with respect to the labeled amount of digoxin ($C_{41}H_{64}O_{14}$)

$$= W_S \times \frac{F_T - F_B}{F_S - F_B} \times \frac{1}{C}$$

W_S: Amount (mg) of Digoxin Reference Standard

C: The labeled amount (mg) of digoxin ($C_{41}H_{64}O_{14}$) in 1 tablet

Assay Weigh accurately the mass of not less than 20 Digoxin Tablets, and powder. Weigh accurately a portion of the powder, equivalent to about 2.5 mg of digoxin ($C_{41}H_{64}O_{14}$), add 30 mL of dilute ethanol, exposure to ultrasonic waves for 20 minutes, and shake for 5 minutes. After cooling, add exactly 5 mL of the internal standard solution and dilute ethanol to make 50 mL, centrifuge, and use the supernatant liquid as the sample solution. Separately, weigh accurately about 25 mg of Digoxin Reference Standard, previously dried under reduced pressure at 105°C for 1 hour , dissolve in 50 mL of warm ethanol (95), cool, and add ethanol (95) to make exactly 100 mL. Pipet 10 mL of this solution, add exactly 5 mL of the internal standard solution, 10 mL of water and dilute ethanol to make 50 mL, and use this solution as the standard solution. Perform the test with 10 μL each of the sample solution and the standard solution as directed under the Liquid Chromatography according to the following conditions, and determine the ratios, Q_T and Q_S, of the peak area of digoxin to that of the internal standard.

$$\text{Amount (mg) of digoxin } (C_{41}H_{64}O_{14}) = W_S \times \frac{Q_T}{Q_S} \times \frac{1}{10}$$

W_S: Amount (mg) of Digoxin Reference Standard

Internal standard solution—A solution of propyl parahydroxybenzoate in ethanol (95) (1 in 4000).

Operating conditions—

Detector: An ultraviolet absorption photometer (wavelength: 220 nm).

Column: A stainless steel column 4.6 mm in inside diameter and 25 cm in length, packed with octadecylsilanized silica gel for liquid chromatography (5 μm in particle diameter).

Column temperature: A constant temperature of about 30°C.

Mobile phase: A mixture of water and acetonitrile (7:3).

Flow rate: Adjust the flow rate so that the retention time of digoxin is about 10 minutes.

System suitability—

System performance: When the procedure is run with 10 μL of the standard solution under the above operating conditions, digoxin and the internal standard are eluted in this order with the resolution between these peaks being not less than 5.

System repeatability: When the test is repeated 6 times with 10 μL of the standard solution under the above operating conditions, the relative standard deviation of the ratio of the peak area of digoxin to that of the internal standard is not more than 1.0%.

Containers and storage Containers—Tight containers.

Storage—Light-resistant.

Dimorpholamine

ジモルホラミン

Change the origin/limits of the content to read:

Dimorpholamine, when dried, contains not less than 98.0% and not more than 101.0% of $C_{20}H_{38}N_4O_4$.

Change the Description to read:

Description Dimorpholamine is a white to light yellow, crystalline powder, masses or syrupy liquid.

It is very soluble in ethanol (99.5) and in acetic anhydride, and soluble in water.

The pH of a solution prepared by dissolving 1.0 g of Dimorpholamine in 10 mL of water is between 6.0 and 7.0.

It is hygroscopic.

Change the Identification to read:

Identification **(1)** Determine the absorption spectrum of a solution of Dimorpholamine (1 in 50,000) as directed under the Ultraviolet-visible Spectrophotometry, and compare the spectrum with the Reference Spectrum: both spectra exhibit similar intensities of absorption at the same wavelengths.

(2) Determine the infrared absorption spectrum of Dimorpholamine, previously dried, as directed in the potassium bromide disk method under the Infrared Spectrophotometry, and compare the spectrum with the Reference Spectrum: both spectra exhibit similar intensities of absorption at the same wave numbers.

Add the following next to Purity (4):

(5) Related substances—Dissolve 0.20 g of Dimorpholamine in 10 mL of ethanol (99.5), and use this solution as the sample solution. Pipet 1 mL of the sample solution, add ethanol (99.5) to make exactly 100 mL, and use this solution as the standard solution. Perform the test with these solutions as directed under the Thin-layer Chromatography. Spot 10 μL each of the sample solution and the standard solution on a plate of silica gel for thin-layer chromatography. Develop the plate with a mixture of ethanol (99.5) and water (4:1) to a distance of about 10 cm, and air-dry the plate. Allow the plate to stand in iodine vapor for 10 minutes: the spot other than the principal spot from the sample solution is not more intense than the spot from the standard solution.

Change the Assay to read:

Assay Weigh accurately about 0.6 g of Dimorpholamine, previously dried, dissolve in 50 mL of acetic anhydride, and titrate with 0.1 mol/L perchloric acid VS (potentiometric titration). Perform a blank determination, and make any necessary correction.

Each mL of 0.1 mol/L perchloric acid VS
= 39.85 mg of $C_{20}H_{38}N_4O_4$

Dimorpholamine Injection

ジモルホラミン注射液

Change the origin/limits of content to read:

Dimorpholamine Injection is an aqueous solution for injection.

It contains not less than 95.0% and not more than 105.0% of the labeled amount of dimorpholamine ($C_{20}H_{38}N_4O_4$: 398.54).

Change the Description to read:

Description Dimorpholamine Injection is a clear, colorless liquid.

pH: 3.0 – 5.5

Change the Identification (2) to read:

Identification

(2) To a volume of Dimorpholamine Injection, equivalent to 50 mg of Dimorpholamine according to the labeled amount, add 1 mL of dilute hydrochloric acid, and evaporate on a water bath to dryness. Dissolve the residue in

2 mL of hydrochloric acid, boil for 10 minutes under a reflux condenser, and evaporate to dryness on a water bath. Dissolve the residue with 1 mL of water, neurtralize with sodium hydroxide TS, and add 0.2 mL of a solution of acetaldehyde (1 in 20), 0.1 mL of sodium pentacyanonitrosyl ferrate (III) TS and 0.5 mL of sodium carbonate TS: a blue color develops.

Add the following next to Identification:

Bacterial endotoxins Less than 5.0 EU/mg. Perform the test with the sample diluted to 0.15 w/v% with water for bacterial endotoxins test.

Actual volume It meets the requirements of Injections.

Foreign insoluble matter Perform the test according to Method 1: it meets the requirements of the Foreign Insoluble Matter Test for Injections.

Insoluble particulate matter Perform the test according to Method 1: it meets the requirements of the Insoluble Particulate Matter Test for Injections.

Sterility Perform the test according to the Membrane filtration method: it meets the requirements of the Sterility Test.

Change the Assay to read:

Assay Measure exactly a volume of Dimorpholamine Injection, equivalent to about 30 mg of dimorpholamine ($C_{20}H_{38}N_4O_4$), and add water to make exactly 200 mL. Pipet 1 mL of this solution, shake with exactly 4 mL of the inernal standard solution for 5 minutes, and use this solution as the sample solution. Separately, weigh accurately about 0.15 g of dimorpholamine for assay, previously dried in a desiccator (in vacuum, phosphorus (V) oxide) for 8 hours, and dissolve in water to make exactly 1000 mL. Pipet 1 mL of this solutionn, shake with exactly 4 mL of the inernal standard solution for 5 minutes, and use this solution as the standard solution. Perform the test with 10 μL each of the sample solution and the standard solution as directed under the Liquid Chromatography according to the following conditions, and calculate the ratios, Q_T and Q_S, of the peak area of dimorpholamine to that of the inernal standard.

$$\text{Amount (mg) of dimorpholamine } (C_{20}H_{38}N_4O_4) = W_S \times \frac{Q_T}{Q_S} \times \frac{1}{5}$$

W_S: Amount (mg) of dimorpholamine for assay

Internal standard solution—A solution of butyl parahydroxybenzoate in acetonitrile (1 in 25,000).

Operating conditions—

Detector: An ultraviolet absorption photometer (wavelength: 216 nm).

Column: A stainless steel column 4.6 mm in inside diameter and 15 cm in length, packed with octadecylsilanized silica gel for liquid chromatography (5 μm in particle diameter).

Column temperature: A constant temperature of about 40°C.

Mobile phase: A mixture of water and acetonitrile (1:1).

Flow rate: Adjust the flow rate so that the retention time of dimorpholamine is about 4 minutes.

System suitability—

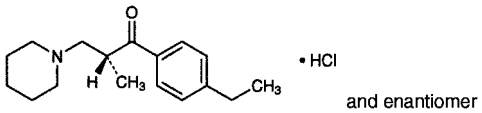
System performance: When the procedure is run with 10 μL of the standard solution under the above operating conditions, dimorpholamine and the internal standard are eluted in this order with the resolution between these peaks being not less than 2.0.

System repeatability: When the test is repeated 6 times with 10 μL of the standard solution under the above operating conditions, the relative standard deviation of the ratio of the peak area of dimorpholamine to that of the internal standard is not more than 1.0%.

Add the following:

Eperisone Hydrochloride

塩酸エペリゾン



$C_{17}H_{25}NO.HCl$: 295.85

(2*RS*)-1-(4-Ethylphenyl)-2-methyl-3-piperidin-1-ylpropan-1-one monohydrochloride [*56839-43-1*]

Eperisone Hydrochloride contains not less than 98.5% and not more than 101.0% of $C_{17}H_{25}NO.HCl$, calculated on the anhydrous basis.

Description Eperisone Hydrochloride occurs as a white crystalline powder.

It is freely soluble in water, in methanol and in acetic acid (100), and soluble in ethanol (99.5).

Melting point: about 167°C (with decomposition).

A solution of Eperisone Hydrochloride in methanol (1 in 100) shows no optical rotation.

Identification **(1)** Determine the absorption spectrum of a solution of Eperisone Hydrochloride in methanol (1 in 100,000) as directed under the Ultraviolet-visible Spectrophotometry, and compare the spectrum with the Reference Spectrum: both spectra exhibit similar intensities of absorption at the same wavelengths.

(2) Determine the infrared absorption spectrum of Eperisone Hydrochloride as directed in the potassium chloride disk method under the Infrared Spectrophotometry, and compare the spectrum with the Reference Spectrum: both spectra exhibit similar intensities of absorption at the same wave numbers.

(3) A solution of Eperisone Hydrochloride (1 in 50) responds to the Qualitative Tests for chloride.

Purity **(1)** Heavy metals—Proceed with 1.0 g of Eperi-

sone Hydrochloride according to Method 1, and perform the test. Prepare the control solution with 2.0 mL of Standard Lead Solution (not more than 20 ppm).

(2) Piperidine hydrochloride—Dissolve 1.0 g of Eperisone Hydrochloride in 20 mL of water, add 2.0 mL of diluted hydrochloric acid (1 in 2), 2.0 mL of a solution of copper (II) sulfate pentahydrate (1 in 20) and 1.5 mL of ammonia solution (28), and use this solution as the sample solution. Separately, to 2.0 mL of a solution of piperidine hydrochloride (1 in 1000) add 18 mL of water, 2.0 mL of diluted hydrochloric acid (1 in 2), 2.0 mL of a solution of copper (II) sulfate pentahydrate (1 in 20) and 1.5 mL of ammonia solution (28), and use this solution as the standard solution. To each of the sample solution and the standard solution add 10 mL of a mixture of isopropylether and carbon disulfide (3:1), shake for 30 seconds, allow them to stand for 2 minutes, and compare the color of the upper layer: the color obtained from the sample solution is not more darker than that from the standard solution.

(3) Related substances—Dissolve 0.1 g of Eperisone Hydrochloride in 100 mL of the mobile phase, and use this solution as the sample solution. Pipet 1 mL of the sample solution, add the mobile phase to make exactly 100 mL, and use this solution as the standard solution. Perform the test with exactly 10 μL each of the sample solution and the standard solution as directed under the Liquid Chromatography according to the following conditions, and determine each peak area by the automatic integration method: the total area of the peaks other than the peak of eperisone is not more than 1/5 of the peak area of eperisone from the standard solution.

Operating conditions—

Detector: An ultraviolet absorption photometer (wavelength: 254 nm).

Column: A stainless steel column 4.6 mm in inside diameter and 15 cm in length, packed with octadecylsilanized silica gel for liquid chromatography (5 μm in particle diameter).

Column temperature: A constant temperature of about 30°C.

Mobile phase: A mixture of methanol, 0.0375 mol/L sodium 1-decanesulfonate TS and perchloric acid (600:400:1).

Flow rate: Adjust the flow rate so that the retention time of eperisone is about 17 minutes.

Time span of measurement: About 2 times as long as the retention time of eperisone.

System suitability—

Test for required detectability: Pipet 1 mL of the standard solution, and add the mobile phase to make exactly 10 mL. Confirm that the peak area of eperisone obtained from 10 μL of this solution is equivalent to 7 to 13% of that obtained from 10 μL of the standard solution.

System performance: When the procedure is run with 10 μL of the standard solution under the above operating conditions, the number of theoretical plates and the symmetry factor of the peak of eperisone are not less than 4000 steps and not more than 2.0, respectively.

System repeatability: When the test is repeated 6 times with 10 μL of the standard solution under the above operating conditions, the relative standard deviation of the peak area of eperisone is not more than 3.0%.

Water Not more than 0.2% (0.1 g, coulometric titration).

Residue on ignition Not more than 0.2% (1 g).

Assay Weigh accurately about 0.6 g of Eperisone Hydrochloride, dissolve in 20 mL of acetic acid (100), add 80 mL of acetic anhydride, and titrate with 0.1 mol/L perchloric acid VS (potentiometric titration). Perform a blank determination in the same manner, and make any necessary correction.

Each mL of 0.1 mol/L perchloric acid VS
= 29.58 mg of $C_{17}H_{25}NO.HCl$

Containers and storage Containers—Well-closed containers.

Ethionamide

エチオナミド

Change to read except the structural formula and chemical name:

Ethionamide, when dried, contains not less than 98.5% and not more than 101.0% of $C_8H_{10}N_2S$.

Description Ethionamide occurs as yellow crystals or crystalline powder, having a characteristic odor.

It is soluble in methanol and in acetic acid (100), sparingly soluble in ethanol (99.5) and in acetone, and practically insoluble in water.

Identification **(1)** Determine the absorption spectrum of a solution of Ethionamide in methanol (3 in 160,000) as directed under the Ultraviolet-visible Spectrophotometry, and compare the spectrum with the Reference Spectrum: both spectra exhibit similar intensities of absorption at the same wavelengths.

(2) Determine the infrared absorption spectrum of Ethionamide as directed in the potassium bromide disk method under the Infrared Spectrophotometry, and compare the spectrum with the Reference Spectrum: both spectra exhibit similar intensities of absorption at the same wave numbers.

Melting point 161 – 165°C

Purity

(1) Acid—Dissolve 3.0 g of Ethionamide in 30 mL of methanol by warming, add 90 mL of water, allow to stand in ice water for 1 hour, and filter. To 80 mL of the filtrate add 0.8 mL of cresol red TS and 0.20 mL of 0.1 mol/L sodium hydroxide VS: a red color develops.

(2) Heavy metals—Proceed with 1.0 g of Ethionamide according to Method 2, and perform the test. Prepare the control solution with 2.0 mL of Standard Lead Solution (not more than 20 ppm).

(3) Arsenic—Prepare the test solution with 1.0 g of Ethionamide according to Method 3. Add 10 mL of a solution of magnesium nitrate hexahydrate in ethanol (95) (1 in 50), then add 1.5 mL of hydrogen peroxide (30), and fire to burn (not more than 2 ppm).

(4) Related substances—Conduct this procedure without exposure to light, using light-resistant vessels. Dissolve 0.20 g of Ethionamide in 10 mL of acetone, and use this solution as the sample solution. Pipet 0.5 mL of the sample solution, add acetone to make exactly 100 mL, and use this solution as the standard solution (1). Separately, pipet exactly 0.2 mL of the sample solution, add acetone to make exactly 100 mL, and use this solution as the standard solution (2). Perform the test with these solutions as directed under the Thin-layer Chromatography. Spot 10 μL each of the sample solution and the standard solutions (1) and (2) on a plate of silica gel with fluorescent indicator for thin-layer chromatography, develop with a mixture of ethyl acetate, hexane and methanol (6:2:1) to a distance of about 15 cm, and air-dry the plate. Examine under ultraviolet light (main wavelength: 254 nm): the spot other than the principal spot obtained with the sample solution is not more intense than the spot with the standard solution (1), and number of the spot other than the principal spot obtained with the sample solution which is more intense than the spot with the standard solution (2) is not more than one.

Loss on drying Not more than 0.5% (1 g, 105°C, 3 hours).

Residue on ignition Not more than 0.10% (1 g).

Assay Weigh accurately about 0.3 g of Ethionamide, previously dried, dissolve in 50 mL of acetic acid (100), and titrate with 0.1 mol/L perchloric acid VS until the color of the solution changes from orange-red to dark orange-brown (indicator: 2 mL of *p*-naphtholbenzein TS). Perform a blank determination in the same manner, and make any necessary correction.

Each mL of 0.1 mol/L perchloric acid VS
= 16.62 mg of $C_8H_{10}N_2S$

Containers and storage Containers—Well-closed containers.

Etilefrine Hydrochloride Tablets

塩酸エチレフリン錠

Change the origin/limits of content to read:

Etilefrine Hydrochloride Tablets contain not less than 93.0% and not more than 107.0% of the labeled amount of etilefrine hydrochloride ($C_{10}H_{15}NO_2$.HCl: 217.69).

Change the Identification to read:

Identification To a quantity of powdered Etilefrine Hydrochloride Tablets, equivalent to 5 mg of Etilefrine Hydrochloride according to the labeled amount, add 60 mL of diluted hydrochloric acid (1 in 1000), shake well, add 40 mL of diluted hydrochloric acid (1 in 1000), and filter. Determine the absorption spectrum of the filtrate as directed under the Ultraviolet-visible Spectrophotometry, using diluted hydrochloric acid (1 in 1000) as the blank: it exhibits a maximum between 271 nm and 275 nm.

Add the following next to Identification:

Content uniformity Perform the test according to the following method: it meets the requirements of the Content Uniformity Test.

To 1 tablet of Etilefrine Hydrochloride Tablets add 60 mL of diluted hydrochloric acid (1 in 1000), and proceed as directed in the Assay.

Amount (mg) of etilefrine hydrochloride ($C_{10}H_{15}NO_2$.HCl)
$$= W_S \times \frac{A_T}{A_S} \times \frac{1}{10}$$

W_S: Amount (mg) of etilefrine hydrochloride for assay

Change the Assay to read:

Assay Weigh accurately the mass of not less than 20 Etilefrine Hydrochloride Tablets, and powder. Weigh accurately a portion of the powder, equivalent to about 5 mg of etilefrine hydrochloride ($C_{10}H_{15}NO_2$.HCl), add 60 mL of diluted hydrochloric acid (1 in 1000), shake for 10 minutes, add diluted hydrochloric acid (1 in 1000) to make exactly 100 mL, and filter. Discard the first 20 mL of the filtrate, and use the subsequent filtrate as the sample solution. Separately, weigh accurately about 50 mg of etilefrine hydrochloride for assay, previously dried at 105°C for 4 hours, and dissolve in diluted hydrochloric acid (1 in 1000) to make exactly 100 mL. Pipet 10 mL of this solution, add diluted hydrochloric acid (1 in 1000) to make exactly 100 mL, and use this solution as the standard solution. Perform the test with exactly 20 μL each of the sample solution and the standard solution as direct under the Liquid Chromatography according to the following conditions, and determine the peak areas, A_T and A_S, of etilefrine.

Amount (mg) of etilefrine hydrochloride ($C_{10}H_{15}NO_2$.HCl)
$$= W_S \times \frac{A_T}{A_S} \times \frac{1}{10}$$

W_S: Amount (mg) of etilefrine hydrochloride for assay

Operating conditions—

Detector: An ultraviolet absorption photometer (wavelength: 220 nm).

Column: A stainless steel column 4.6 mm in inside diameter and 25 cm in length, packed with octylsilanized silica gel for liquid chromatography (5 μm in particle diameter).

Column temperature: A constant temperature of about 40°C.

Mobile phase: Dissolve 5 g of sodium lauryl sulfate in 940 mL of water and 500 mL of acetonitrile, and adjust the pH to 2.3 with phosphoric acid.

Flow rate: Adjust the flow rate so that the retention time of etilefrine is about 6 minutes.

System suitability—

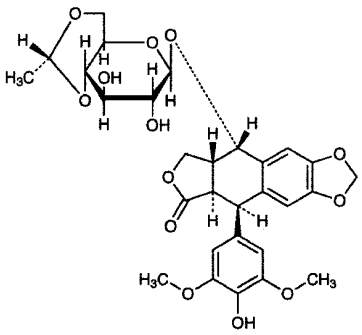
System performance: Dissolve 4 mg of bamethan sulfate and 4 mg of etilefrine hydrochloride in the mobile phase to make 50 mL. When the procedure is run with 20 μL of this solution under the above operating conditions, etilefrine and bamethan are eluted in this order with the resolution between these peaks being not less than 5.

System repeatability: When the test is repeated 6 times with 20 μL of the standard solution under the above operating conditions, the relative standard deviation of the peak area of etilefrine is not more than 1.0%.

Add the following:

Etoposide

エトポシド



$C_{29}H_{32}O_{13}$: 588.56

(5*R*,5a*R*,8a*R*,9*S*)-9-{[4,6-*O*-(1*R*)-Ethylidene-*β*-D-glucopyranosyl]oxy}-5,8,8a,9-tetrahydro-5-(4-hydroxy-3,5-dimethoxyphenyl)furo[3′,4′:6,7]naphtha[2,3-*d*]-1,3-dioxol-6(5a*H*)-one [*33419-42-0*]

Etoposide contains not less than 98.0% and not more than 102.0% of $C_{29}H_{32}O_{13}$, calculated on the anhydrous basis.

Description Etoposide occurs as white crystals or crystalline powder.

It is sparingly soluble in methanol, slightly soluble in ethanol (99.5), and very slightly soluble in water.

Melting point: about 260°C (with decomposition).

Identification **(1)** Determine the absorption spectrum of a solution of Etoposide in methanol (1 in 10,000) as directed under the Ultraviolet-visible Spectrophotometry, and compare the spectrum with the Reference Spectrum or the spectrum of a solution of Etoposide Reference Standard prepared in the same manner as the sample solution: both spectra exhibit similar intensities of absorption at the same wavelengths.

(2) Determine the infrared absorption spectrum of Etoposide as directed in the potassium bromide disk method under the Infrared Spectrophotometry, and compare the spectrum with the Reference Spectrum or the spectrum of Etoposide Reference Standard: both spectra exhibit similar intensities of absorption at the same wave numbers.

Optical rotation $[\alpha]_D^{20}$: -100 – $-105°$ (0.1 g calculated on the anhydrous basis, methanol, 20 mL, 100 mm).

Purity **(1)** Heavy metals—Proceed with 2.0 g of Etoposide according to Method 2, and perform the test. Prepare the control solution with 2.0 mL of Standard Lead Solution (not more than 10 ppm).

(2) Related substances—Dissolve 50 mg of Etoposide in 10 mL of methanol, add the mobile phase to make 50 mL, and use this solution as the sample solution. Pipet 2 mL of the sample solution, add the mobile phase to make exactly 200 mL, and use this solution as the standard solution. Perform the test with exactly 50 μL each of the sample solution and the standard solution as directed under the Liquid Chromatography according to the following conditions, and determine each peak area by the automatic integration method: the area of the peak other than etoposide is not larger than 1/5 times the peak area of etoposide with the standard solution, and the total area of the peaks other than the peak of etoposide with the sample solution is not larger than 1/2 times the peak area of etoposide with the standard solution.

Operating conditions—

Detector, column, column temperature, mobile phase, and flow rate: Proceed as directed in the operating conditions in the Assay.

Time span of measurement: About 3 times as long as the retention time of etoposide after the solvent peak.

System suitability—

Test for required detectability: Measure exactly 1 mL of the standard solution, and add the mobile phase to make exactly 10 mL. Confirm that the peak area of etoposide obtained with 50 μL of this solution is equivalent to 7 to 13% of that with 50 μL of the standard solution.

System performance: Proceed as directed in the system suitability in the Assay.

System repeatability: When the test is repeated 6 times with 50 μL of the standard solution under the above operating conditions, the relative standard deviation of the peak area of etoposide is not more than 2.0%.

Water Not more than 4.0% (0.5 g, volumetric titration, direct titration).

Residue on ignition Not more than 0.1% (1 g).

Assay Weigh accurately about 25 mg each of Etoposide and Etoposide Reference Standard, separately determined the water content, and dissolve separately in methanol to make exactly 25 mL. Pipet 10 mL each of these solutions, add exactly 5 mL of the internal standard solution and the mobile phase to make 50 mL, and use these solutions as the sample solution and the standard solution. Perform the test with 50 μL each of the sample solution and the standard

solution as directed under the Liquid Chromatography according to the following conditions, and determine the ratios, Q_T and Q_S, of the peak area of etoposide to that of the internal standard.

$$\text{Amount (mg) of } C_{29}H_{32}O_{13} = W_S \times \frac{Q_T}{Q_S}$$

W_S: Amount (mg) of Etoposide Reference Standard, calculated on the anhydrous basis

Internal standard solution—A solution of 2,6-dichlorophenol in methanol (3 in 2500).

Operating conditions—

Detector: An ultraviolet absorption photometer (wavelength: 290 nm).

Column: A stainless steel column 3.9 mm in inside diameter and 30 cm in length, packed with phenylsilanized silica gel for liquid chromatography (10 μm in particle diameter).

Column temperature: A constant temperature of about 35°C.

Mobile phase: Dissolve 6.44 g of sodium sulfate decahydrate in diluted acetic acid (100) (1 in 100) to make 1000 mL, and add 250 mL of acetonitrile.

Flow rate: Adjust the flow rate so that the retention time of etoposide is about 20 minutes.

System suitability—

System performance: Dissolve 10 mg of Etoposide in 2 mL of methanol, add 8 mL of the mobile phase, and mix well. Add 0.1 mL of diluted acetic acid (100) (1 in 25) and 0.1 mL of phenolphthalein TS, and add sodium hydroxide TS until the color of the solution changes to faintly red. After allowing to stand for 15 minutes, add 0.1 mL of diluted acetic acid (100) (1 in 25). When the procedure is run with 10 μL of this solution under the above operating conditions, the resolution between the peak of etoposide and the peak having the relative retention time of about 1.3 with respect to etoposide is not less than 3.

System repeatability: When the test is repeated 6 times with 50 μL of the standard solution under the above operating conditions, the relative standard deviation of the ratio of the peak area of etoposide to that of the internal standard is not more than 1.0%.

Containers and storage Containers—Tight containers.

Storage—Light-resistant.

Flavin Adenine Dinucleotide Sodium

フラビンアデニンジヌクレオチドナトリウム

Change the Purity (5) to read:

Purity

(5) Related substances—Dissolve 0.10 g of Flavin Adenine Dinucleotide Sodium in 200 mL of the mobile phase, and use this solution as the sample solution. Perform the test with 20 μL of the sample solution as directed under the Liquid Chromatography according to the following conditions. Determine the peak area, A, of flavin adenine dinucleotide and the total area, S, of peaks other than the peak of flavin adenine dinucleotide by the automatic integration method: $S/(A+S)$ is not more than 0.10.

Operating conditions—

Column, column temperature, mobile phase, flow rate, and time span of measurement: Proceed as directed in the operating conditions in the Procedure (ii) under Assay (1).

Detector: An ultraviolet absorption photometer (wavelength: 260 nm).

System suitability—

Test for required detection: To exactly 2 mL of the sample solution add the mobile phase to make exactly 20 mL, and use this solution as the solution for system suitability test. Confirm that the peak area of flavin adenine dinucleotide obtained from 20 μL of this solution is equivalent to 8 to 12% of that of flavin adenine dinucleotide obtained from 20 μL of the sample solution.

System performance: Proceed as directed in the system suitability in the Procedure (ii) under Assay (1).

System repeatability: When the test is repeated 6 times with 20 μL of the solution for system suitability test under the above operating conditions, the relative standard deviation of the peak area of flavin adenine dinucleotide is not more than 1.0%.

Change the Assay (1) to read:

Assay (1) Procedure (i) Total flavin content—Conduct this procedure without exposure to daylight, using light-resistant vessels. Weigh accurately about 0.1 g of Flavin Adenine Dinucleotide Sodium, and dissolve in water to make exactly 200 mL. Pipet 5 mL of this solution, add 5 mL of zinc chloride TS, and heat in a water bath for 30 minutes. After cooling, add water to make exactly 100 mL, and use this solution as the sample solution. Separately, weigh accurately about 50 mg of Riboflavin Reference Standard, previously dried at 105°C for 2 hours, dissolve in 200 mL of diluted acetic acid (100) (1 in 100) by warming, cool, add water to make exactly 500 mL. Pipet 10 mL of this solution, add water to make exactly 100 mL, and use this solution as the standard solution. Determine the absorbances, A_T and A_S, of the sample solution and the standard solution at 450 nm as directed under the Ultraviolet-visible Spectrophotometry, using water as the blank.

$$\text{Total amout (mg) of flavin} = W_S \times \frac{A_T}{A_S} \times \frac{4}{5}$$

W_S: Amount (mg) of Riboflavin Reference Standard

(ii) Peak area ratio of flavin adenine dinucleotide—Dissolve 0.1 g of Flavin Adenine Dinucleotide Sodium in 200 mL of water, and use this solution as the sample solution. Perform the test with 5 μL of this solution as directed under the Liquid Chromatography according to the following conditions. Determine the peak area, A of flavin adenine

dinucleotide, and the total area, S, of the peaks other than flavin adenine dinucleotide by the automatic integration method.

$$\text{Peak area ratio of flavin adenine dinucleotide} = \frac{1.08 \times A}{1.08 \times A + S}$$

Operating conditions—

Detector: A visible spectrophotometer (wavelength: 450 nm).

Column: A stainless steel column 4 mm in inside diameter and 15 cm in length, packed with octadecylsilanized silica gel for liquid chromatography (5 μm in particle diameter).

Column temperature: A constant temperature of about 35°C.

Mobile phase: A mixture of a solution of potassium dihydrogen phosphate (1 in 500) and methanol (4:1).

Flow rate: Adjust the flow rate so that the retention time of flavin adenine dinucleotide is about 10 minutes.

Time span of measurement: About 4.5 times as long as the retention time of flavin adenine dinucleotide.

System suitability—

Test for required detection: To exactly 2 mL of the sample solution add water to make exactly 20 mL, and use this solution as the solution for system suitability test. Pipet 2 mL of the solution, and add water to make exactly 20 mL. Confirm that the peak area of flavin adenine dinucleotide obtained from 5 μL of this solution is equivalent to 8 to 12% of that of flavin adenine dinucleotide obtained from 5 μL of the solution for system suitability test.

System performance: Dissolve 20 mg each of Flavin Adenine Dinucleotide Sodium and riboflavin sodium phosphate in 100 mL of water. When the procedure is run with 5 μL of this solution under the above operating conditions, flavin adenine dinucleotide and riboflavin phosphate are eluted in this order with the resolution between these peaks being not less than 2.0.

System repeatability: When the test is repeated 6 times with 5 μL of the solution for system suitability test under the above operating conditions, the relative standard deviation of the peak area of flavin adenine dinucleotide is not more than 1.0%.

(2) Calculation

$$\text{Amount (mg) of } C_{27}H_{31}N_9Na_2O_{15}P_2 = f_T \times f_R \times 2.2040$$

f_T: Total amount (mg) of flavin in Flavin Adenine Dinucleotide Sodium obtained from the procedure (i).

f_R: Peak area ratio of flavin adenine dinucleotide in Flavin Adenine Dinucleotide Sodium obtained from the procedure (ii).

Add the following:

Flomoxef Sodium for Injection

注射用フロモキセフナトリウム

Flomoxef Sodium for Injection is a preparation for injection which is dissolved before use.

It contains not less than 90.0% and not more than 110.0% of the labeled amount of flomoxef ($C_{15}H_{18}F_2N_6O_7S_2$: 496.47).

Method of preparation Prepare as directed under Injections, with Flomoxef Sodium.

Description Flomoxef Sodium for Injection occurs as white to light yellowish white, friable masses or powder.

Identification Proceed as directed in the Identification (3) under Flomoxef Sodium.

pH The pH of a solution obtained by dissolving an amount of Flomoxef Sodium for Injection, equivalent to 0.5 g (potency) of Flomoxef Sodium according to the labeled amount, in 5 mL of water is between 4.0 and 5.5.

Purity **(1)** Clarity and color of solution—Dissolve an amount of Flomoxef Sodium for Injection, equivalent to 1.0 g (potency) of Flomoxef Sodium according to the labeled amount, in 10 mL of water: the solution is clear and colorless or pale yellow.

(2) 1-(2-Hydroxyethyl)-1*H*-tetrazol-5-thiol—Use the sample solution obtained in the Assay as the sample solution. Weigh accurately about 20 mg of 1-(2-hydroxyethyl)-1*H*-tetrazol-5-thiol, and dissolve in water to make exactly 100 mL. Pipet 5 mL of this solution, add exactly 25 mL of the internal standard solution and water to make 50 mL, and use this solution as the standard solution. Perform the test with 5 μL each of the sample solution and the standard solution as directed under the Liquid Chromatography according to the following conditions, and determine the ratios, Q_T and Q_S, of the peak area of 1-(2-hydroxyethyl)-1*H*-tetrazol-5-thiol to that of the internal standard. Calculate the amount of 1-(2-hydroxyethyl)-1*H*-tetrazol-5-thiol per 1 g (potency) of Flomoxef Sodium for Injection by the following formula: not more than 10 mg.

$$\text{Amount (mg) of 1-(2-hydroxyethyl)-1}H\text{-tetrazol-5-thiol } (C_3H_6N_4OS) = W_S \times \frac{Q_T}{Q_S} \times \frac{1}{10}$$

W_S: Amount (mg) of 1-(2-hydroxyethyl)-1*H*-tetrazol-5-thiol

Operating conditions—

Proceed as directed in the Assay.

System suitability—

Test for required detectability: Pipet 1 mL of the standard solution, and add water to make exactly 20 mL. Confirm that the peak area of 1-(2-hydroxyethyl)-1*H*-tetrazol-5-thiol obtained from 5 μL of this solution is equivalent to 3.5 – 6.5% of that obtained from 5 μL of the standard solution.

System performance: When the procedure is run with 5 μL of the standard solution under the above operating conditions, 1-(2-hydroxyethyl)-1*H*-tetrazol-5-thiol and the internal standard are eluted in this order with the resolution between these peaks being not less than 20.

System repeatability: When the test is repeated 3 times with 5 μL of the standard solution under the above operating conditions, the relative standard deviation of the ratio of the peak area of 1-(2-hydroxyethyl)-1*H*-tetrazol-5-thiol to that of the internal standard is not more than 1.0%.

Water Not more than 1.5% (0.5 g, volumetric titration, back titration).

Bacterial endotoxins Less than 0.025 EU/mg (potency).

Mass variation It meets the requirements of the Mass Variation Test.

Foreign insoluble matter Perform the test according to Method 2: it meets the requirements of the Foreign Insoluble Matter Test for Injections.

Insoluble particulate matter Perform the test according to Method 1: it meets the requirements of the Insoluble Particulate Matter Test for Injections.

Sterility Perform the test according to the Membrane filtration method: it meets the requirements of the Sterility Test.

Assay Weigh accurately the mass of the contents of not less than 10 Flomoxef Sodium for Injection, and calculate the average mass of the content. Spread out thinly about 1 g of the content in a petri dish, allow the dish to stand in a desiccator containing a saturated solution of magnesium bromide without light exposure to equilibrate the sample to constant water content. Determine the water content, separately, with about 0.1 g of the sample according to the method described in Water. Weigh accurately an amount of the sample, equivalent to about 50 mg (potency) of Flomoxef Sodium according to the labeled amount, add exactly 50 mL of the internal standard solution to dissolve, add water to make 100 mL, and use this solution as the sample solution. Separately weigh accurately about 50 mg (potency) of Flomoxef Triethylammonium Reference Standard, add exactly 50 mL of the internal standard solution to dissolve, add water to make 100 mL, and use this solution as the standard solution. Proceed as directed in the Assay under Flomoxef Sodium.

$$\text{Amount [}\mu\text{g (potency)] of flomoxef } (C_{15}H_{18}F_2N_6O_7S_2) = W_S \times \frac{Q_T}{Q_S} \times 1000$$

W_S: Amount [mg (potency)] of Flomoxef Triethylammonium Reference Standard

Internal standard solution—A solution of *m*-cresol (3 in 1000).

Containers and storage Containers—Hermetic containers. Polyethylene or polypropylene containers for aqueous injection may be used.

Add the following:

Flopropione Capsules

フロプロピオンカプセル

Flopropione Capsules contain not less than 93.0% and not more than 107.0% of the labeled amount of flopropione ($C_9H_{10}O_4$: 182.17).

Method of preparation Prepare as directed under the Capsules, with Flopropione.

Identification **(1)** Powder the contents of Flopropione Capsules. To a portion of the powder, equivalent to 60 mg of Flopropione according to the labeled amount, add 40 mL of water, shake well, and filter. To 5 mL of the filtrate add 1 mL of iron (III) nitrate TS: a red-purple color appears.

(2) Powder the contents of Flopropione Capsules. To a portion of the powder, equivalent to 90 mg of Flopropione according to the labeled amount, add 100 mL of ethanol (99.5), shake well, and filter. To 5 mL of the filtrate add ethanol (99.5) to make 50 mL. To 5 mL of this solution add ethanol (99.5) to make 100 mL, and use this solution as the sample solution. Determine the absorption spectrum of the sample solution as directed under the Ultraviolet-visible Spectrophotometry: it exhibits a maximum between 283 nm and 287 nm.

Content uniformity Perform the test as directed in the Assay, and determine the content: it meets the requirements of the Content Uniformity Test.

Dissolution Being specified separately.

Assay To 1 capsule of Flopropione Capsules add 43 mL of a mixture of water and phosphoric acid (86:1), and disintegrate the capsule at 50°C in a water bath. After cooling, add acetonitrile to make exactly 100 mL, stir for 10 minutes, and centrifuge a part of this solution for 5 minutes at 3000 rpm. Use the supernatant liquid as the sample solution. Separately, weigh accurately about 40 mg of flopropione for assay, separately determine the water content in the same manner as Flopropione, add 70 mL of the mobile phase, and dissolve by exposure for 10 minutes to ultrasonic vibration. Add the mobile phase to make exactly 100 mL, and use this solution as the standard solution. Perform the test with exactly 5 μL each of the sample solution and the standard solution as directed under the Liquid chromatography according to the following conditions, and determine the peak areas, A_T and A_S, of flopropione. Repeat the procedure above with totally not less than 10 capsules, and calculate the mean.

$$\text{Amount (mg) of flopropione } (C_9H_{10}O_4) = W_S \times \frac{A_T}{A_S}$$

W_S: Amount (mg) of flopropione for assay, calculated on

the anhydrous basis

Operating conditions—

Detector: An ultraviolet absorption photometer (wavelength: 267 nm).

Column: A stainless steel column 4.6 mm in inside diameter and 15 cm in length, packed with octadecylsilanized silica gel for liquid chromatography (5 μm in particle diameter).

Column temperature: A constant temperature of about 35°C.

Mobile phase: A mixture of acetonitrile, water and phosphoric acid (114:86:1)

Flow rate: Adjust the flow rate so that the retention time of flopropione is about 3 minutes.

System suitability—

System performance: Dissolve 50 mg of flopropione in 50 mL of the mobile phase. To 20 mL of the solution add 25 mL of a solution prepared by dissolving 25 mg of ethyl parahydroxybenzoate in 30 mL of acetonitrile and add water to make 50 mL, and then add the mobile phase to make 50 mL. When the procedure is run with 5 μL of this solution under the above operating conditions, flopropione and ethyl parahydroxybenzoate are eluted in this order with the resolution between these peaks being not less than 2.0.

System repeatability: When the test is repeated 6 times with 5 μL of the standard solution under the above operating conditions, the relative standard deviation of the peak area of flopropione is not more than 1.0%.

Containers and storage Containers—Tight containers.

Fosfomycin Calcium

ホスホマイシンカルシウム

Change the origin/limits of content to read:

Fosfomycin Calcium is the calcium salt of a substance having antibacterial actively produced by the growth of *Streptomyces fradiae* or by the chemical synthesis.

It contains not less than 725 μg (potency) and not more than 805 μg (potency) per mg, calculated on the anhydrous basis. The potency of Fosfomycin Calcium is expressed as mass (potency) of fosfomycin ($C_3H_7O_4P$: 138.06).

Change the Description to read:

Description Fosfomycin Calcium occurs as a white crystalline powder.

It is slightly soluble in water, and practically insoluble in methanol and in ethanol (99.5).

Change the Identification (2) to read:

Identification

(2) Determine the spectrum of a solution of Fosfomycin Calcium in heavy water for nuclear magnetic resonance spectroscopy (1 in 300), using sodium 3-trimethylsilylpropanesulfonate for nuclear magnetic resonance spectroscopy as an internal reference compound, as directed under the Nuclear Magnetic Resonance Spectroscopy: it exhibits a double signal at around δ 1.5 ppm, a duple double signal at around δ 2.9 ppm, a multiple signal at around δ 3.3 ppm, and no signal at around δ 1.4 ppm.

Change the Purity (1) to read:

Purity (1) Heavy metals—To 1.0 g of Fosfomycin Calcium add 40 mL of 0.25 mol/L acetic acid TS and water to make 50 mL. Proceed with this solution according to Method 1, and perform the test. Prepare the control solution with 2.0 mL of Standard Lead Solution (not more than 20 ppm).

Fosfomycin Sodium

ホスホマイシンナトリウム

Change the origin/limits of content to read:

Fosfomycin Sodium is the sodium salt of a substance having antibacterial activity produced by the growth of *Streptomyces fradiae* or by the chemical synthesis.

It contains not less than 725 μg (potency) and not more than 770 μg (potency) per mg, calculated on the anhydrous basis. The potency of Fosfomycin Sodium is expressed as mass (potency) of fosfomycin ($C_3H_7O_4P$: 138.06).

Change the Description to read:

Description Fosfomycin Sodium occurs as a white crystalline powder.

It is very soluble in water, sparingly soluble in methanol, and practically insoluble in ethanol (99.5).

Change the Identification (2) to read:

Identification

(2) Determine the spectrum of a solution of Fosfomycin Sodium in heavy water for nuclear magnetic resonance spectroscopy (1 in 300), using sodium 3-trimethylsilylpropanesulfonate for nuclear magnetic resonance spectroscopy as an internal reference compound, as directed under the Nuclear Magnetic Resonance Spectroscopy: it exhibits a double signal at around δ 1.5 ppm, a duple double signal at around δ 2.8 ppm, a multiple signal at around δ 3.3 ppm, and no signal at around δ 1.3 ppm.

Furosemide

フロセミド

Change the origin/limits of content to read:

Furosemide, when dried, contains not less than 98.0% and not more than 101.0% of $C_{12}H_{11}ClN_2O_5S$.

Change the Description to read:

Description Furosemide occurs as white, crystals or crystalline powder.

It is freely soluble in *N*,*N*-dimethylformamide, soluble in methanol, sparingly soluble in ethanol (99.5), slightly soluble in acetonitrile and in acetic acid (100), and practically insoluble in water.

It dissolves in dilute sodium hydroxide TS.

It is gradually colored by light.

Melting point: about 205°C (with decomposition).

Change the Identification to read:

Identification **(1)** Dissolve 25 mg of Furosemide in 10 mL of methanol. To 1 mL of this solution add 10 mL of 2 mol/L hydrochloric acid TS. Heat the solution under a reflux condenser on a water bath for 15 minutes, cool, and add 18 mL of sodium hydroxide TS to make weakly acidic: the solution responds to the Qualitative Tests for primary aromatic amines, producing a red to red-purple color.

(2) Determine the absorption spectrum of a solution of Furosemide in dilute sodium hydroxide TS (1 in 125,000) as directed under the Ultraviolet-visible Spectrophotometry, and compare the spectrum with the Reference Spectrum or the spectrum of a solution of Furosemide Reference Standard prepared in the same manner as the sample solution: both spectra exhibit similar intensities of absorption at the same wavelengths.

(3) Determine the infrared absorption spectrum of Furosemide as directed in the potassium bromide disk method under the Infrared Spectrophotometry, and compare the spectrum with the Reference Spectrum or the spectrum of Furosemide Reference Standard: both spectra exhibit similar intensities of absorption at the same wave numbers.

Change the Purity to read:

Purity **(1)** Clarity and color of solution—Dissolve 0.5 g of Furosemide in 10 mL of a solution of sodium hydroxide (1 in 50): the solution is clear and colorless.

(2) Chloride—Dissolve 2.6 g of Furosemide in 90 mL of dilute sodium hydroxide TS, add 2 mL of nitric acid, and filter. To 25 mL of the filtrate add 6 mL of dilute nitric acid and water to make 50 mL, and perform the test using this solution as the test solution. Prepare the control solution as follows: To 0.40 mL of 0.01 mol/L hydrochloric acid VS add 6 mL of dilute nitric acid and water to make 50 mL (not more than 0.020%).

(3) Sulfate—To 20 mL of the filtrate obtained in (2) add 1 mL of dilute hydrochloric acid and water to make 50 mL, and perform the test using this solution as the test solution. Prepare the control solution as follows: To 0.35 mL of 0.005 mol/L sulfuric acid VS add 1 mL of dilute hydrochloric acid and water to make 50 mL (not more than 0.030%).

(4) Heavy metals—Proceed with 2.0 g of Furosemide according to Method 2, and perform the test. Prepare the control solution with 2.0 mL of Standard Lead Solution (not more than 10 ppm).

(5) Related substances—Dissolve 25 mg of Furosemide in 25 mL of the dissolving solution, and use this solution as the sample solution. Pipet 1 mL of the sample solution, add the dissolving solution to make exactly 200 mL, and use this solution as the standard solution. Perform the test with 20 μL each of the sample solution and the standard solution as directed under the Liquid Chromatography according to the following conditions, and determine each peak area by the automatic integration method: the area of each peak appeared ahead of the peak of furosemide is not more than 2/5 times the peak area of furosemide from the standard solution, the area of each peak appeared behind the peak of furosemide is not more than 1/4 times the peak area of furosemide from the standard solution, and the total area of these peaks is not more than 2 times the peak area of furosemide from the standard solution.

Dissolving solution—To 22 mL of acetic acid (100) add a mixture of water and acetonitrile (1:1) to make 1000 mL.

Operating conditions—

Detector: An ultraviolet absorption photometer (wavelength: 272 nm).

Column: A stainless steel column 4.6 mm in inside diameter and 25 cm in length, packed with octadecylsilanized silica gel for liquid chromatography (5 μm in particle diameter).

Column temperature: A constant temperature of about 25°C.

Mobile phase: A mixture of water, tetrahydrofuran and acetic acid (100) (70:30:1).

Flow rate: Adjust the flow rate so that the retention time of furosemide is about 18 minutes.

Time span of measurement: About 2.5 times as long as the retention time of furosemide after the solvent peak.

System suitability—

Test for required detectability: Measure exactly 2 mL of the standard solution, and add the dissolving solution to make exactly 50 mL. Confirm that the peak area of furosemide obtained from 20 μL of this solution is equivalent to 3.2 to 4.8% of that obtained from 20 μL of the standard solution.

System performance: When the procedure is run with 20 μL of the standard solution under the above operating conditions, the number of theoretical plates and the symmetry factor of the peak of furosemide is not less than 7000 and not more than 1.5, respectively.

System repeatability: When the test is repeated 6 times with 20 μL of the standard solution under the above operat-

ing conditions, the relative standard deviation of the peak area of furosemide is not more than 2.0%.

Change the Assay to read:

Assay Weigh accurately about 0.5 g of Furosemide, previously dried, dissolve in 50 mL of *N*,*N*-dimethylformamide, and titrate with 0.1 mol/L sodium hydroxide VS until the color of the solution changes from yellow to blue (indicator: 3 drops of bromothymol blue TS). Perform a blank determination with a mixture of 50 mL of *N*,*N*-dimethylformamide and 15 mL of water, and make any necessary correction

Each mL of 0.1 mol/L sodium hydroxide VS
= 33.07 mg of $C_{12}H_{11}ClN_2O_5S$

Add the following:

Furosemide Tablets

フロセミド錠

Furosemide Tablets contain not less than 95.0% and not more than 105.0% of the labeled amount of furosemide ($C_{12}H_{11}ClN_2O_5S$: 330.74).

Method of preparation Prepare as directed under Tablets, with Furosemide.

Identification **(1)** Shake well a quantity of powdered Furosemide Tablets, equivalent to 0.2 g of Furosemide according to the labeled amount, with 40 mL of acetone, and filter. To 0.5 mL of the filtrate add 10 mL of 2 mol/L hydrochloric acid TS, and heat under a reflux condenser on a water bath for 15 minutes. After cooling, add 18 mL of sodium hydroxide TS to make the solution slightly acetic: the solution responds to the Qualitative Tests for primary aromatic amines, producing a red to red-purple color.

(2) Determine the absorption spectrum of the sample solution obtained in the Assay as directed under the Ultraviolet-visible Spectrophotometry: it exhibits maxima between 227 nm and 231 nm, between 269 nm and 273 nm, and between 330 nm and 336 nm.

Purity To a quantity of powdered Furosemide Tablets, equivalent to 40 mg of Furosemide according to the labeled amount, add about 30 mL of acetone, shake well, and add acetone to make exactly 50 mL. Centrifuge the solution, add 3.0 mL of water to 1.0 mL of the supernatant liquid, cool in ice, add 3.0 mL of dilute hydrochloric acid and 0.15 mL of sodium nitrite TS, shake, and allow to stand for 1 minute. Add 1.0 mL of ammonium amidosulfate TS, shake well, allow to stand for 3 minutes, add 1.0 mL of *N*,*N*-diethyl-*N*′-1-naphthylethylenediamine oxalate TS, shake well, and allow to stand for 5 minutes. Perform the test with this solution as directed under the Ultraviolet-visible Spectrophotometry, using a solution prepared in the same manner with 1.0 mL of acetone as the blank: the absorbance at 530 nm is not more than 0.10.

Content uniformity Perform the test according to the following method: it meets the requirements of the Content Uniformity Test.

To 1 tablet of Furosemide Tablets add a suitable amount of 0.05 mol/L sodium hydroxide TS, shake to disintegrate, then add 0.05 mol/L sodium hydroxide TS to make exactly V mL so that each mL contains about 0.4 mg of furosemide ($C_{12}H_{11}ClN_2O_5S$). Filter the solution, discard the first 10 mL of the filtrate, pipet the subsequent 2 mL of the filtrate, add 0.05 mol/L sodium hydroxide TS to make exactly 100 mL, and use this solution as the sample solution. Proceed as directed in the Assay.

$$\text{Amount (mg) of furosemide } (C_{12}H_{11}ClN_2O_5S) = W_S \times \frac{A_T}{A_S} \times \frac{V}{100}$$

W_S: Amount (mg) of Furosemide Reference Standard

Dissolution Perform the test with 1 tablet of Furosemide Tablets at 50 revolutions per minute according to Method 2 under the Dissolution Test, using 900 mL of diluted phosphate buffer solution, pH 6.8 (1 in 2) as the dissolution medium. Withdraw 20 mL or more of the dissolution medium 15 minutes after starting the test for a 20-mg tablet or 30 minutes after for a 40-mg tablet, and filter through a membrane filter with pore size of not more than 0.45 μm. Discard the first 10 mL of the filtrate, pipet V mL of the subsequent filtrate, add diluted phosphate buffer solution, pH 6.8 (1 in 2) to make exactly V' mL so that each mL contains about 10 μg of furosemide ($C_{12}H_{11}ClN_2O_5S$) according to the labeled amount, and use this solution as the sample solution. Separately, weigh accurately about 20 mg of Furosemide Reference Standard, previously dried at 105°C for 4 hours, and dissolve in 5 mL of methanol, and add diluted phosphate buffer solution, pH 6.8 (1 in 2) to make exactly 100 mL. Pipet 5 mL of this solution, add diluted phosphate buffer solution, pH 6.8 (1 in 2) to make exactly 100 mL, and use this solution as the standard solution. Determine the absorbances, A_T and A_S, of the sample solution and the standard solution at 277 nm as directed under the Ultraviolet-visible Spectrophotometry: the dissolution rates for a 20-mg tablet in 15 minutes and for a 40-mg tablet in 30 minutes are not less than 80%, respectively.

Dissolution rate (%) with respect to the labeled amount of furosemide ($C_{12}H_{11}ClN_2O_5S$)

$$= W_S \times \frac{A_T}{A_S} \times \frac{V'}{V} \times \frac{1}{C} \times 45$$

W_S: Amount (mg) of Furosemide Reference Standard
C: Labeled amount (mg) of furosemide ($C_{12}H_{11}ClN_2O_5S$) in 1 tablet

Assay Weigh accurately the mass of not less than 20 Furosemide Tablets, and powder. Weigh accurately a portion of the powder, equivalent to about 40 mg of furosemide ($C_{12}H_{11}ClN_2O_5S$), add about 70 mL of 0.05 mol/L sodium hydroxide TS, shake well, and add 0.05 mol/L sodium hydroxide TS to make exactly 100 mL. Filter, discard the first 10 mL of the filtrate, pipet 2 mL of the subsequent

filtrate, add 0.05 mol/L sodium hydroxide TS to make exactly 100 mL, and use this solution as the sample solution. Separately, weigh accurately about 20 mg of Furosemide Reference Standard, previously dried at 105°C for 4 hours, and dissolve in 0.05 mol/L sodium hydroxide TS to make exactly 50 mL. Pipet 2 mL of this solution, add 0.05 mol/L sodium hydroxide TS to make exactly 100 mL, and use this solution as the standard solution. Determine the absorbances, A_T and A_S, of the sample solution and the standard solution at 271 nm as directed under the Ultraviolet-visible Spectrophotometry.

$$\text{Amount (mg) of furosemide } (C_{12}H_{11}ClN_2O_5S) = W_S \times \frac{A_T}{A_S}$$

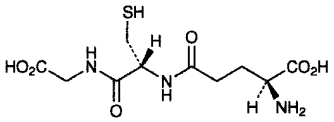
W_S: Amount (mg) of Furosemide Reference Standard

Containers and storage Containers—Tight containers.
Storage—Light-resistant.

Add the following:

Glutathione

グルタチオン



$C_{10}H_{17}N_3O_6S$: 307.32
(2*S*)-2-Amino-4-[1-(carboxymethyl)carbamoyl-(2*R*)-2-sulfanylethylcarbamoyl]butanoic acid [*70-18-8*]

Glutathione, when dried, contains not less than 98.0% and not more than 101.0% of $C_{10}H_{17}N_3O_6S$.

Description Glutathione occurs as a white crystalline powder.

It is freely soluble in water, and practically insoluble in ethanol (99.5).

Melting point: about 185°C (with decomposition).

Identification Determine the infrared absorption spectrum of Glutathione, previously dried, as directed in the potassium bromide disk method under the Infrared Spectrophotometry, and compare the spectrum with the Reference Spectrum: both spectra exhibit similar intensities of absorption at the same wave numbers.

Optical rotation $[\alpha]_D^{20}$: -15.5 – $-17.5°$ (after drying, 2 g, water, 50 mL, 100 mm).

Purity **(1)** Clarity and color of solution—Dissolve 1.0 g of Glutathione in 10 mL of water: the solution is clear and colorless.

(2) Heavy metals—Proceed with 2.0 g of Glutathione according to Method 2, and perform the test. Prepare the control solution with 2.0 mL of Standard Lead Solution (not more than 10 ppm).

(3) Arsenic—Prepare the test solution with 1.0 g of Glutathione according to Method 1, and perform the test (not more than 2 ppm).

(4) Related substances—Dissolve 50 mg of Glutathione in 100 mL of the mobile phase, and use this solution as the sample solution. Pipet 2 mL of the sample solution, add the mobile phase to make exactly 100 mL, and use this solution as the standard solution. Perform the test with exactly 10 μL each of the sample solution and the standard solution as directed under the Liquid Chromatography according to the following conditions, and determine each peak area by the automatic integration method: the area of the peak having the relative retention time of about 4 with respect to glutathione is not more than 3/4 times the peak area of glutathione from the standard solution, and the total area of the peaks other than the peak of glutathione is not more than the peak area of glutathione from the standard solution.

Operating conditions—

Detector: An ultraviolet absorption photometer (wavelength: 210 nm).

Column: A stainless steel column 4.6 mm in inside diameter and 15 cm in length, packed with octadecylsilanized silica gel for liquid chromatography (5 μm in particle diameter).

Column temperature: A constant temperature of about 30°C.

Mobile phase: Dissolve 6.8 g of potassium dihydrogen phosphate and 2.02 g of sodium 1-heptane sulfonate in 1000 mL of water, and adjust the pH to 3.0 with phosphoric acid. To 970 mL of this solution add 30 mL of methanol.

Flow rate: Adjust the flow rate so that the retention time of glutathione is about 5 minutes.

Time span of measurement: About 6 times as long as the retention time of glutathione after the solvent peak.

System suitability—

Test for required detectability: Pipet 10 mL of the standard solution, and add the mobile phase to make exactly 100 mL. Confirm that the peak area of glutathione obtained from 10 μL of this solution is equivalent to 8 to 12% of that obtained from 10 μL of the standard solution.

System performance: Dissolve 50 mg of glutathione, 10 mg of D-phenylglycine and 50 mg of ascorbic acid in 100 mL of water. When the procedure is run with 10 μL of this solution under the above operating conditions, ascorbic acid, glutathione and D-phenylglycine are eluted in this order, and the resolutions between the peaks of ascorbic acid and glutathione and between the peaks of glutathione and D-phenylglycine are not less than 5, respectively.

System repeatability: When the test is repeated 6 times with 10 μL of the standard solution under the above operating conditions, the relative standard deviation of the peak area of glutathione is not more than 1.5%.

Loss on drying Not more than 0.5% (1 g, 105°C, 3 hours).

Residue on ignition Not more than 0.1% (1 g).

Assay Weigh accurately about 0.5 g of Glutathione, previously dried, dissolve in 50 mL of a solution of

metaphosphoric acid (1 in 50), and titrate with 0.05 mol/L iodine VS (indicator: 1 mL of starch TS). Perform a blank determination in the same manner, and make any necessary correction.

Each mL of 0.05 mol/L iodine VS
= 30.73 mg of $C_{10}H_{17}N_3O_6S$

Containers and storage Containers—Tight containers.

Heparin Sodium

ヘパリンナトリウム

Change the Assay to read:

Assay (i) Substrate solution: Dissolve 15 mg of *N*-benzoyl-L-isoleucyl-L-glutamyl(γ-OR)-glycyl-L-arginyl-*p*-nitroanilide hydrochloride in 20 mL of water.

(ii) Antithrombin III solution: Dissolve human antithrombin III in water to make a solution containing 1 Unit per mL.

(iii) Activated blood coagulation factor X solution: Dissolve bovine activated blood coagulation factor X in water to make a solution containing 0.426 Units per mL.

(iv) Human normal plasma: Dissolve an amount of dried human normal plasma powder, equivalent to 1 mL of human normal plasma, in 1 mL of water. Store at 2 – 10°C and use within a week.

(v) Buffer solution: Dissolve 6.06 g of 2-amino-2-hydroxymethyl-1,3-propanediol in 750 mL of water, adjust the pH to 8.4 with 1 mol/L hydrochloric acid TS, and add water to make 1000 mL.

(vi) Reaction stop solution: To 20 mL of acetic acid (100) add water to make 40 mL.

(vii) Heparin standard solution: Dissolve Heparin Sodium Reference Standard in isotonic sodium chloride solution to make a solution containing 10 Units per mL, and use as the standard stock solution. To 100 μL of the standard stock solution add the buffer solution to make exactly 5 mL, and use this solution as the standard solution. Prepare the heparin standard solutions (1), (2), (3), (4) and (5) by addition of antithrombin III solution, human normal plasma and the buffer solution to the standard solution as directed in the following table.

Heparin standard solution		Buffer solution (μL)	Antithrombin III solution (μL)	Human normal plasma (μL)	Standard solution (μL)
No.	Heparin concentration (Unit/mL)				
(1)	0	800	100	100	0
(2)	0.02	700	100	100	100
(3)	0.04	600	100	100	200
(4)	0.06	500	100	100	300
(5)	0.08	400	100	100	400

(viii) Sample solution: Weigh accurately an adequate amount of Heparin Sodium, dissolve in isotonic sodium chloride solution so that each mL contains about 0.5 Units according to the labeled amount. To 100 μL of this solution add 100 μL of antithrombin III solution, 100 μL of human normal plasma and 700 μL of the buffer solution, and use this solution as the sample solution.

(ix) Procedure: Transfer 400 μL of the sample solution to a test tube, and warm at 37°C for 4 minutes. Add 200 μL of the activated blood coagulation factor X solution, mix well, warm at 37°C for exactly 30 seconds, add 400 μL of the substrate solution, previously warmed at 37°C, and mix well. Allow the tube to stand at 37°C for exactly 3 minutes, add 600 μL of the reaction stop solution, mix immediately, and determine the absorbance at 405 nm, using the blank solution prepared by addition of 600 μL of the reaction stop solution and 600 μL of water to 400 μL of the sample solution. Proceed the same way with the heparin standard solution (1), the heparin standard solution (2), the heparin standard solution (3), the heparin standard solution (4) and the heparin standard solution (5), and determine their absorbances.

(x) Calculation: Plot the absorbances of the standard solutions on the vertical axis and their heparin concentrations on the horizontal axis to prepare a calibration curve. Determine the heparin concentration, *C*, of the sample solution from its absorbance by using the curve, and calculate heparin Units per mg of Heparin Sodium from the following formula.

$$\text{Units per mg of Heparin Sodium} = C \times 10 \times \frac{b}{a}$$

a: Amount of sample (mg)
b: Total volume (mL) of isotonic sodium chloride solution used to dissolve the sample to make the solution containing about 0.5 Units per mL

Heparin Sodium Injection

ヘパリンナトリウム注射液

Add the following next to Bacterial endotoxins:

Actual volume It meets the requirements of Injections.

Foreign insoluble matter Perform the test according to Method 1: it meets the requirements of the Foreign Insoluble Matter Test for Injections.

Insoluble particulate matter Perform the test according to Method 1: it meets the requirements of the Insoluble Particulate Matter Test for Injections.

Sterility Perform the test according to the Membrane filtration method: it meets the requirements of the Sterility Test.

Change the Assay to read:

Assay Proceed as directed in the Assay under Heparin Sodium, replacing the sample solution indicated in (viii) and the calculation in (x) with the following.

Sample solution: Measure exactly an adequate portion of Heparin Sodium Injection according to the labeled Units, dilute it with isotonic sodium chloride solution so that each mL contains about 0.5 Units. To 100 μL of this solution add 100 μL of antithrombin III solution, 100 μL of human normal plasma and 700 μL of the buffer solution, and use this solution as the sample solution.

Calculation: Plot the absorbances of the standard solutions on the vertical axis and their heparin concentrations on the horizontal axis to prepare a calibration curve. Determine the heparin concentration, C, of the sample solution from its absorbance by using the curve, and calculate heparin Units per mL of Heparin Sodium Injection from the following formula.

$$\text{Units per mL of Heparin Sodium Injection} = C \times 10 \times \frac{b}{a}$$

a: Amount of sample (mL)
b: Total volume (mL) of isotonic sodium chloride solution used to dilute the sample to make the solution containing about 0.5 Units per mL

Kallidinogenase

カリジノゲナーゼ

Change the Assay to read:

Assay Weigh accurately an appropriate amount of Kallidinogenase according to the labeled Units, dissolve in 0.05 mol/L phosphate buffer solution, pH 7.0 to prepare a solution containing about 10 Kallidinogenase Units per mL, and use this solution as the sample stock solution. Pipet 4 mL of the sample stock solution, add exactly 1 mL of trypsin inhibitor TS and 0.05 mol/L phosphate buffer solution, pH 7.0 to make exactly 10 mL, and use this solution as the sample solution. Pipet 2.5 mL of substrate TS for kallidinogenase assay (1), previously warmed at 30±0.5°C for 5 minutes, place in a 1-cm cell, add exactly 0.5 mL of the sample solution, warmed at 30±0.5°C for 5 minutes, and start simultaneously a chronograph. Perform the test at 30±0.5°C as directed under the Ultraviolet-visible Spectrophotometry using water as the blank, and determine the absorbances at 405 nm, A_{T2} and A_{T6}, of this solution after allowing to stand for exactly 2 and 6 minutes. Separately, dissolve Kallidinogenase Reference Standard in 0.05 mol/L phosphate buffer solution, pH 7.0 to make a solutin so that each mL contains exactly 10 Units, and use this solution as the standard stock solution. Pipet 4 mL of the stock solution, add exactly 1 mL of trypsin inhibitor TS and 0.05 mol/L phosphate buffer solution, pH 7.0 to make exactly 10 mL, and use this solution as the standard solution. Take exactly 0.5 mL of the standard solution, perform the test in the same manner as described for the sample solution, and determine the absorbances, A_{S2} and A_{S6}, of the solution after allowing to stand for exactly 2 and 6 minutes. Separately, take exactly 1 mL of the trypsin inhibitor TS, and add 0.05 mol/L phosphate buffer solution, pH 7.0 to make exactly 10 mL. Pipet 0.5 mL of this solution, perform the test in the same manner as described for the sample solution, and determine the absorbances, A_{O2} and A_{O6}, of the solution after allowing to stand for exactly 2 and 6 minutes.

Units per 1 mg of Kallidinogenase

$$= \frac{(A_{T6} - A_{T2}) - (A_{O6} - A_{O2})}{(A_{S6} - A_{S2}) - (A_{O6} - A_{O2})} \times \frac{W_S}{a} \times \frac{1}{b}$$

W_S: Amount (Units) of Kallidinogenase Reference Standard
a: Volume (mL) of the standard stock solution
b: Amount (mg) of Kallidinogenase in 1 mL of the sample stock solution

D-Mannitol

D-マンニトール

Change the Identification (2) to read:

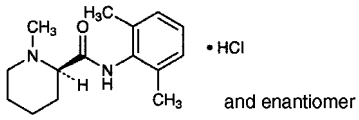
Identification

(2) Determine the infrared absorption spectrum of D-Mannitol as directed in the potassium bromide disk method under the Infrared Spectrophotometry, and compare the spectrum with the Reference Spectrum: both spectra exhibit similar intensities of absorption at the same wave numbers. If any difference appears between the spectra, dissolve 1 g of D-Mannitol in 3 mL of warm water, then allow to stand at 5°C for 24 hours or until crystals appear, and filter. Wash the crystals so obtained with a few amount of cold water, dry at 105°C for 4 hours, and perform the test with the crystals.

Mepivacaine Hydrochloride

塩酸メピバカイン

Change the graphic formula to read:



Change the chemical name to read:

(2*RS*)-*N*-(2,6-Dimethylphenyl)-1-methylpiperidine-2-carboxamide monohydrochloride

Meropenem Trihydrate

メロペネム 三水和物

Change the water to read:

Water Not less than 11.4% and not more than 13.4% (0.35 g, volumetric titration, direct titration).

dl-Methylephedrine Hydrochloride

dl-塩酸メチルエフェドリン

Change the origin/limits of the content to read:

dl-Methylephedrine Hydrochloride, when dried, contains not less than 99.0% and not more than 101.0% of $C_{11}H_{17}NO.HCl$.

Change the Description to read:

Description *dl*-Methylephedrine Hydrochloride occurs as colorless crystals or a white, crystalline powder.

It is freely soluble in water, sparingly soluble in ethanol (99.5), slightly soluble in acetic acid (100), and practically insoluble in acetic anhydride.

A solution of *dl*-Methylephedrine Hydrochloride (1 in 20) shows no optical rotation.

Change the Identification to read:

Identification **(1)** Determine the absorption spectrum of a solution of *dl*-Methylephedrine Hydrochloride (1 in 2000) as directed under the Ultraviolet-visible Spectrophotometry, and compare the spectrum with the Reference Spectrum: both spectra exhibit similar intensities of absorption at the same wavelengths.

(2) Determine the infrared absorption spectrum of *dl*-Methylephedrine Hydrochloride, previously dried, as directed in the potassium chloride disk method under the Infrared Spectrophotometry, and compare the spectrum with the Reference Spectrum: both spectra exhibit similar intensities of absorption at the same wave numbers.

(3) A solution of *dl*-Methylephedrine Hydrochloride (1 in 10) responds to the Qualitative Tests for chloride.

Add the following next to Identification:

pH The pH of a solution prepared by dissolving 1.0 g of *dl*-Methylephedrine Hydrochloride in 20 mL of water is between 4.5 and 6.0.

Change the Purity to read:

Purity **(1)** Clarity and color of solution—Dissolve 1.0 g of *dl*-Methylephedrine Hydrochloride in 10 mL of water: the solution is clear and colorless.

(2) Heavy metals—Proceed with 1.0 g of *dl*-Methylephedrine Hydrochloride according to Method 4, and perform the test. Prepare the control solution with 1.0 mL of Standard Lead Solution (not more than 10 ppm).

(3) Related substances—Dissolve 50 mg of *dl*-Methylephedrine Hydrochloride in 20 mL of water, and use this solution as the sample solution. Pipet 1 mL of the sample solution, add water to make exactly 100 mL, and use this solution as the standard solution. Perform the test with exactly 20 μL each of the sample solution and the standard solution as directed under the Liquid Chromatography according to the following conditions, and determine each peak area by the automatic integration method: the total area of the peaks other than the peak of methylephedrine is not more than the peak area of methylephedrine from the standard solution.

Operating conditions—

Detector: An ultraviolet absorption photometer (wavelength: 257 nm).

Column: A stainless steel column 4.6 mm in inside diameter and 15 cm in length, packed with octadecylsilanized silica gel for liquid chromatography (5 μm in particle diameter).

Column temperature: A constant temperature of about 40°C.

Mobile phase: Dissolve 13.6 g of potassium dihydrogen phosphate and 3 g of sodium 1-heptane sulfonate in 1000 mL of water, and adjust the pH to 2.5 with phosphoric acid. To 900 mL of this solution add 200 mL of acetonitrile.

Flow rate: Adjust the flow rate so that the retention time of methylephedrine is about 10 minutes.

Time span of measurement: About 2 times as long as the retention time of methylephedrine after the solvent peak.

System suitability—

Test for required detectability: To exactly 2 mL of the standard solution add water to make exactly 20 mL. Confirm that the peak area of methylephedrine obtained from 20 μL of this solution is equivalent to 7 to 13% of that of methylephedrine obtained from 20 μL of the standard solution.

System performance: Dissolve 50 mg of *dl*-Methylephedrine Hydrochloride and 0.4 mg of methyl parahydroxybenzoate in 50 mL of water. When the procedure is run with

20 μL of this solution under the above operating conditions, methylephedrine and methyl parahydroxybenzoate are eluted in this order with the resolution between these peaks being not less than 3.

System repeatability: When the test is repeated 6 times with 20 μL of the standard solution under the above operating conditions, the relative standard deviation of the peak area of methylephedrine is not more than 2.0%.

10% *dl*-Methylephedrine Hydrochloride Powder

dl-塩酸メチルエフェドリン散10%

Delete the Description:

Change the Identification to read:

Identification Determine the absorption spectrum of a solution of 10% *dl*-Methylephedrine Hydrochloride Powder (1 in 200) as directed under the Ultraviolet-visible Spectrophotometry: it exhibits maxima between 250 nm and 253 nm, between 255 nm and 259 nm, and between 261 nm and 264 nm.

Change the Assay to read:

Assay Weigh accurately about 0.5 g of 10% *dl*-Methylephedrine Hydrochloride Powder, add exactly 4 mL of the internal standard solution and 25 mL of water, shake vigorously for 20 minutes to dissolve, add water to make 50 mL, filter through a membrane filter with pore size of 0.45 μm, if necessary, discard the first 10 mL of the filtrate, and use the subsequent filtrate as the sample solution. Separately, weigh accurately about 50 mg of *dl*-methylephedrine hydrochloride for assay, previously dried at 105°C for 3 hours, add exactly 4 mL of the internal standard solution and water to make 50 mL, and use this solution as the standard solution. Perform the test with 20 μL each of the sample solution and the standard solution as directed under the Liquid Chromatography according to the following conditions, and determine the ratios of the peak area, Q_T and Q_S, of methylephedrine to that of the internal standard.

$$\text{Amount (mg) of } dl\text{-methylephedrine hydrochloride } (C_{11}H_{17}NO.HCl) = W_S \times \frac{Q_T}{Q_S}$$

W_S: Amount (mg) of *dl*-methylephedrine hydrochloride for assay

Internal standard solution—A solution of methyl parahydroxybenzoate in acetonitrile (1 in 10,000).

Operating conditions—

Detector: An ultraviolet absorption photometer (wavelength: 257 nm).

Column: A stainless steel column 4.6 mm in inside diameter and 15 cm in length, packed with octadecylsilanized silica gel for liquid chromatography (5 μm in particle diameter).

Column temperature: A constant temperature of about 40°C.

Mobile phase: Dissolve 13.6 g of potassium dihydrogen phosphate and 3 g of sodium 1-heptane sulfonate in 1000 mL of water, and adjust the pH to 2.5 with phosphoric acid. To 900 mL of this solution add 200 mL of acetonitrile.

Flow rate: Adjust the flow rate so that the retention time of methylephedrine is about 10 minutes.

System suitability—

System performance: When the procedure is run with 20 μL of the standard solution under the above operating conditions, methylephedrine and the internal standard are eluted in this order with the resolution between these peaks being not less than 3.

System repeatability: When the test is repeated 6 times with 20 μL of the standard solution under the above operating conditions, the relative standard deviation of the ratio of the peak area of methylephedrine to that of the internal standard is not more than 1.0%.

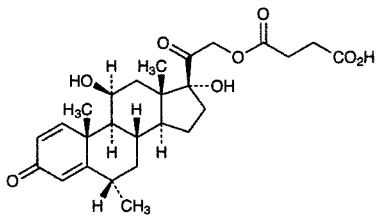
Add the following:

Methylprednisolone Succinate

コハク酸メチルプレドニゾロン

$C_{26}H_{34}O_8$: 474.54

11β,17,21-Trihydroxy-6α-methylpregna-1,4-diene-3,20-dione 21-(hydrogen succinate) [*2921-57-5*]



Methylprednisolone Succinate, when dried, contains not less than 97.0% and not more than 103.0% of $C_{26}H_{34}O_8$.

Description Methylprednisolone Succinate occurs as a white, crystals or crystalline powder.

It is soluble in methanol, sparingly soluble in ethanol (95), and practically insoluble in water.

Melting point: about 235°C (with decomposition).

Identification **(1)** Determine the absorption spectrum of a solution of Methylprednisolone Succinate in methanol (1 in 50,000) as directed under the Ultraviolet-visible Spectrophotometry, and compare the spectrum with the Reference Spectrum or the spectrum of a solution of Methylprednisolone Succinate Reference Standard prepared in the same manner as the sample solution: both spectra exhibit similar intensities of absorption at the same wavelengths.

(2) Determine the infrared absorption spectrum of

Methylprednisolone Succinate, previously dried, as directed in the potassium bromide disk method under the Infrared Spectrophotometry, and compare the spectrum with the Reference Spectrum or the spectrum of previously dried Methylprednisolone Succinate Reference Standard: both spectra exhibit similar intensities of absorption at the same wave numbers. In case when some differences are found between the spectra, repeat the test with residues obtained by dissolving these substances in ethanol (95), evaporating to dryness, and drying.

Optical rotation $[\alpha]_D^{25}$: +99 – +103° (after drying, 0.2 g, ethanol (95), 20 mL, 100 mm).

Purity **(1)** Heavy metals—Proceed with 1.0 g of Methylprednisolone Succinate according to Method 4, and perform the test. Prepare the control solution with 1.0 mL of Standard Lead Solution (not more than 10 ppm).

(2) Arsenic—Prepare the test solution with 2.0 g of Methylprednisolone Succinate according to Method 3, and perform the test (not more than 1 ppm).

(3) Related substances—Dissolve 15 mg of Methylprednisolone Succinate in 5 mL of methanol, add a mixture of 0.05 mol/L phosphate buffer solution, pH 3.5 and acetonitrile (1:1) to make 50 mL, and use this solution as the sample solution. Pipet 1 mL of the sample solution, add the mixture of 0.05 mol/L phosphate buffer solution, pH 3.5 and acetonitrile (1:1) to make exactly 100 mL, and use this solution as the standard solution. Perform the test with exactly 5 μL each of the sample solution and the standard solution as directed under the Liquid Chromatography according to the following conditions, and determine each peak area by the automatic integration method: the area of the peaks other than the peak of methylprednisolone succinate is not more than 1/2 of the peak area of methylprednisolone succinate from the standard solution, and the total area of the peaks other than the peak of methylprednisolone succinate is not more than the peak area of methylprednisolone succinate from the standard solution.

Operating conditions—

Detector, column, column temperature, mobile phase, and flow rate: Proceed as directed in the operating conditions in the Assay.

Time span of measurement: About 3 times as long as the retention time of methylprednisolone succinate.

System suitability—

Test for required detectability: Pipet 1 mL of the standard solution, and add the mixture of 0.05 mol/L phosphate buffer solution, pH 3.5 and acetonitrile (1:1) to make exactly 10 mL. Confirm that the peak area of methylprednisolone succinate obtained from 5 μL of this solution is equivalent to 7 to 13% of that obtained from 5 μL of the standard solution.

System performance: Proceed as directed in the System suitability in the Assay.

System repeatability: When the test is repeated 6 times with 5 μL of the standard solution under the above operating conditions, the relative standard deviation of the peak area of methylprednisolone succinate is not more than 1.0%.

Loss on drying Not more than 1.0% (1 g, 105°C, 3 hours).

Residue on ignition Not more than 0.2% (0.5 g).

Assay Weigh accurately about 15 mg each of Methylprednisolone Succinate and Methylprednisolone Succinate Reference Standard, previously dried, dissolve separately in 5 mL of methanol, and add the mixture of 0.05 mol/L phosphate buffer solution, pH 3.5 and acetonitrile (1:1) to make exactly 50 mL. Pipet 5 mL each of these solutions, add exactly 5 mL of the internal standard solution, and use these solutions as the sample solution and the standard solution, respectively. Perform the test with 5 μL each of the sample solution and the standard solution as directed under the Liquid Chromatography according to the following conditions, and determine the ratios, Q_T and Q_S, of the peak area of methylprednisolone succinate to that of the internal standard.

$$\text{Amount (mg) of } C_{26}H_{34}O_8 = W_S \times \frac{Q_T}{Q_S}$$

W_S: Amount (mg) of Methylprednisolone Succinate Reference Standard

Internal standard solution—A solution of ethyl parahydroxybenzoate in a mixture of 0.05 mol/L phosphate buffer solution, pH 3.5 and acetonitrile (1:1) (3 in 20,000).

Operating conditions—

Detector: An ultraviolet absorption photometer (wavelength: 254 nm).

Column: A stainless steel column 4.6 mm in inside diameter and 25 cm in length, packed with octadecylsilanized silica gel for liquid chromatography (5 μm in particle diameter).

Column temperature: A constant temperature of about 25°C.

Mobile phase: To 1000 mL of 0.05 mol/L potassium dihydrogen phosphate TS add a suitable amount of 0.05 mol/L disodium hydrogen phosphate TS to make a solution having pH 5.5. To 640 mL of this solution add 360 mL of acetonitrile.

Flow rate: Adjust the flow rate so that the retention time of methylprednisolone succinate is about 6 minutes.

System suitability—

System performance: When the procedure is run with 5 μL of the standard solution under the above operating conditions, methylprednisolone succinate and the internal standard are eluted in this order with the resolution between these peaks being not less than 6.

System repeatability: When the test is repeated 6 times with 5 μL of the standard solution under the above operating conditions, the relative standard deviation of the ratio of the peak area of methylprednisolone succinate to that of the internal standard is not more than 1.0%.

Containers and storage Containers—Tight containers.

Methyltestosterone

メチルテストステロン

Change the origin/limits of content to read:

Methyltestosterone, when dried, contains not less than 98.0% and not more than 102.0% of $C_{20}H_{30}O_2$.

Change the Description to read:

Description Methyltestosterone occurs as white to pale yellow, crystals or crystalline powder.

It is freely soluble in methanol and in ethanol (95), and practically insoluble in water.

Change the Identification to read:

Identification

(1) Determine the absorption spectrum of a solution of Methyltestosterone in ethanol (95) (1 in 100,000) as directed under the Ultraviolet-visible Spectrophotometry, and compare the spectrum with the Reference Spectrum or the spectrum of a solution of Methyltestosterone Reference Standard prepared in the same manner as the sample solution: both spectra exhibit similar intensities of absorption at the same wavelengths.

(2) Determine the infrared absorption spectrum of Methyltestosterone, previously dried, as directed in the potassium bromide disk method under the Infrared Spectrophotometry, and compare the spectrum with the Reference Spectrum or the spectrum of dried Methyltestosterone Reference Standard: both spectra exhibit similar intensities of absorption at the same wave numbers.

Change the Assay to read:

Assay Weigh accurately about 20 mg each of Methyltestosterone and Methyltestosterone Reference Standard, previously dried in a desiccator (in vacuum, phosphorus (V) oxide) for 10 hours, dissolve each in methanol to make exactly 200 mL. Pipet 5 mL each of these solutions, add exactly 5 mL of the internal standard solution, add methanol to make 50 mL, and use these solutions as the sample solution and the standard solution. Perform the test with 10 μL each of the sample solution and the standard solution as directed under the Liquid Chromatography according to the following conditions, and determine the ratios, Q_T and Q_S, of the peak area of methyltestosterone to that of the internal standard.

$$\text{Amount (mg) of } C_{20}H_{30}O_2 = W_S \times \frac{Q_T}{Q_S}$$

W_S: Amount (mg) of Methyltestosterone Reference Standard

Internal standard solution—A solution of propyl parahydroxybenzoate in methanol (1 in 10,000).

Operating conditions—

Detector: An ultraviolet absorption photometer (wavelength: 241 nm).

Column: A stainless steel column 6 mm in inside diameter and 15 cm in length, packed with octadecylsilanized silica gel for liquid chromatography (5 μm in particle diameter).

Column temperature: A constant temperature of about 35°C.

Mobile phase: A mixture of acetonitrile and water (11:9).

Flow rate: Adjust the flow rate so that the retention time of methyltestosterone is about 10 minutes.

System suitability—

System performance: When the procedure is run with 10 μL of the standard solution under the above operating conditions, the internal standard and methyltestosterone are eluted in this order with the resolution between these peaks being not less than 9.

System repeatability: When the test is repeated 6 times with 10 μL of the standard solution under the above operating conditions, the relative standard deviation of the ratios of the peak area of methyltestosterone to that of the internal standard is not more than 1.0%.

Methyltestosterone Tablets

メチルテストステロン錠

Change the origin/limits of content to read:

Methyltestosterone Tablets contain not less than 90.0% and not more than 110.0% of the labeled amount of methyltestosterone ($C_{20}H_{30}O_2$: 302.45).

Change the Identification to read:

Identification To a portion of powdered Methyltestosterone Tablets, equivalent to 0.01 g of Methyltestosterone according to the labeled amount, add 50 mL of acetone, shake for 30 minutes, and filter. Evaporate the filtrate to dryness, dissolve the residue in 10 mL of acetone, and use this solution as the sample solution. Separately, dissolve 0.01 g of Methyltestosterone Reference Standard in 10 mL of acetone, and use this solution as the standard solution. Perform the test with these solutions as directed under the Thin-layer Chromatography. Spot 10 μL each of the sample solution and the standard solution on a plate of silica gel for thin-layer chromatography. Develop the plate with a mixture of chloroform and ethanol (95) (9:1) to a distance of about 12 cm, and air-dry the plate. Spray evenly dilute sulfuric acid on the plate, and heat at 110°C for 10 minutes: the spot from the sample solution and the standard solution show the same *R*f value.

Change the Assay to read:

Assay Weigh accurately the mass of not less than 20 Methyltestosterone Tablets, and powder. Weigh accurately a portion of the powder, equivalent to about 25 mg of

methyltestosterone ($C_{20}H_{30}O_2$), add about 70 mL of methanol, shake for 30 minutes, and add methanol to make exactly 100 mL. Pipet 2 mL of this solution, add exactly 5 mL of the internal standard solution and methanol to make 50 mL, filter through a membrane filter (not exceeding 0.45 μm in pore size), and use the filtrate as the sample solution. Separately, weigh accurately about 20 mg of Methyltestosterone Reference Standard, previously dried in a desiccator (in vacuum, phosphorus (V) oxide) for 10 hours, dissolve in methanol to make exactly 200 mL. Pipet 5 mL of this solution, add exactly 5 mL of the internal standard solution, add methanol to make 50 mL, and use this solution as the standard solution. Perform the test with 10 μL each of the sample solution and the standard solution as directed under the Liquid Chromatography according to the following conditions, and determine the ratios, Q_T and Q_S, of the peak area of methyltestosterone to that of the internal standard.

$$\text{Amount (mg) of methyltestosterone } (C_{20}H_{30}O_2) = W_S \times \frac{Q_T}{Q_S} \times \frac{5}{2}$$

W_S: Amount (mg) of Methyltestosterone Reference Standard

Internal standard solution—A solution of propyl parahydroxybenzoate in methanol (1 in 10,000).

Operating conditions—

Detector: An ultraviolet absorption photometer (wavelength: 241 nm).

Column: A stainless steel column 6 mm in inside diameter and 15 cm in length, packed with octadecylsilanized silica gel for liquid chromatography (5 μm in particle diameter).

Column temperature: A constant temperature of about 35°C.

Mobile phase: A mixture of acetonitrile and water (11:9).

Flow rate: Adjust the flow rate so that the retention time of methyltestosterone is about 10 minutes.

System suitability—

System performance: When the procedure is run with 10 μL of the standard solution under the above operating conditions, the internal standard and methyltestosterone are eluted in this order with the resolution between these peaks being not less than 9.

System repeatability: When the test is repeated 6 times with 10 μL of the standard solution under the above operating conditions, the relative standard deviation of the ratio of the peak area of methyltestosterone to that of the internal standard is not more than 1.0%.

Add the following:

Metoclopramide Tablets

メトクロプラミド錠

Metoclopramide Tablets contain not less than 95.0% and not more than 105.0% of the labeled amount of metoclopramide ($C_{14}H_{22}ClN_3O_2$: 299.80).

Method of preparation Prepare as directed under Tablets, with Metoclopramide.

Identification (1) To a quantity of powdered Metoclopramide Tablets, equivalent to 50 mg of Metoclopramide according to the labeled amount, add 15 mL of 0.5 mol/L hydrochloric acid TS, and heat in a water bath at 70°C for 15 minutes while frequent shaking. After cooling, centrifuge for 10 minutes, and to 5 mL of the supernatant liquid add 1 mL of 4-dimethylaminobenzaldehyde-hydrochloric acid TS: a yellow color develops.

(2) Determine the absorption spectrum of the sample solution obtained in the Assay as directed under the Ultraviolet-visible Spectrophotometry: it exhibits maxima between 270 nm and 274 nm, and between 306 nm and 310 nm.

Content uniformity Perform the test according to the following method: it meets the requirements of the Content Uniformity Test.

To 1 tablet of Metoclopramide Tablets add 10 mL of 0.1 mol/L hydrochloric acid TS, disperse the particles with the aid of ultrasonic waves, then add 0.1 mol/L hydrochloric acid TS to make exactly 25 mL, and centrifuge for 10 minutes. Pipet 4 mL of the supernatant liquid, add 0.1 mol/L hydrochloric acid TS to make exactly V mL of a solution so that each mL contains about 12 μg of metoclopramide ($C_{14}H_{22}ClN_3O_2$), and use this solution as the sample solution. Separately, weigh accurately about 80 mg of metoclopramide for assay, previously dried at 105°C for 3 hours, and dissolve in 0.1 mol/L hydrochloric acid TS to make exactly 500 mL. Pipet 4 mL of this solution, add 0.1 mol/L hydrochloric acid TS to make exactly 50 mL, and use this solution as the standard solution. Determine the absorbances, A_T and A_S, of the sample solution and the standard solution at 308 nm as directed under the Ultraviolet-visible Spectrophotometry.

$$\text{Amount (mg) of metoclopramide } (C_{14}H_{22}ClN_3O_2) = W_S \times \frac{A_T}{A_S} \times \frac{V}{1000}$$

W_S: Amount (mg) of metoclopramide for assay

Dissolution Perform the test with 1 tablet of Metoclopramide Tablets at 50 revolutions per minute according to Method 2 under the Dissolution Test, using 900 mL of diluted phosphate buffer solution, pH 6.8 (1 in 2) as the dissolution medium. Withdraw 20 mL or more of the dissolution medium 45 minutes after starting the test for a 3.84-mg tablet or 15 minutes after starting the test for a

7.67-mg tablet, and filter through a membrane filter with pore size of not more than 0.5 μm. Discard the first 10 mL of the filtrate, pipet V mL of the subsequent filtrate, add diluted phosphate buffer solution, pH 6.8 (1 in 2) to make exactly V' mL so that each mL contains about 4 μg of metoclopramide ($C_{14}H_{22}ClN_3O_2$) according to the labeled amount, and use this solution as the sample solution. Separately, weigh accurately about 20 mg of metoclopramide for assay, previously dried at 105°C for 3 hours, and dissolve in diluted phosphate buffer solution, pH 6.8 (1 in 2) to make exactly 100 mL. Pipet 2 mL of this solution, add diluted phosphate buffer solution, pH 6.8 (1 in 2) to make exactly 100 mL, and use this solution as the standard solution. Perform the test with exactly 50 μL each of the sample solution and the standard solution as directed under the Liquid Chromatography according to the following conditions, and determine the peak areas, A_T and A_S, of metoclopramide. The dissolution rates for a 3.84-mg tablet in 45 minutes and for a 7.67-mg tablet in 15 minutes are not less than 80% and not less than 85%, respectively.

Dissolution rate (%) with respect to the labeled amount of metoclopramide ($C_{14}H_{22}ClN_3O_2$)

$$= W_S \times \frac{A_T}{A_S} \times \frac{V'}{V} \times \frac{1}{C} \times 18$$

W_S: Amount (mg) of metoclopramide for assay
C: Labeled amount (mg) of metoclopramide ($C_{14}H_{22}ClN_3O_2$) in 1 tablet

Operating conditions—

Detector: An ultraviolet absorption photometer (wavelength: 275 nm).

Column: A stainless steel column 4 mm in inside diameter and 15 cm in length, packed with octadecylsilanized silica gel for liquid chromatography (5 μm in particle diameter).

Column temperature: A constant temperature of about 25°C.

Mobile phase: Dissolve 0.79 g of sodium lauryl sulfate in 550 mL of water, and add 450 mL of acetonitrile and 0.3 mL of acetic acid (100).

Flow rate: Adjust the flow rate so that the retention time of metoclopramide is about 5 minutes.

System suitability—

System performance: When the procedure is run with 50 μL of the standard solution under the above operating conditions, the number of theoretical plates and the symmetry factor of the peak of metoclopramide are not less than 3000 and not more than 1.5, respectively.

System repeatability: When the test is repeated 6 times with 50 μL of the standard solution under the above operating conditions, the relative standard deviation of the peak area of metoclopramide is not more than 1.0%.

Assay Weigh accurately not less than 20 Metoclopramide Tablets, and powder. Weigh accurately a portion of the powder, equivalent to about 75 mg of metoclopramide ($C_{14}H_{22}ClN_3O_2$), add 300 mL of 0.1 mol/L hydrochloric acid TS, shake for 1 hour, and add 0.1 mol/L hydrochloric acid TS to make exactly 500 mL. Centrifuge for 10 minutes, pipet 4 mL of the supernatant liquid, add 0.1 mol/hydrochloric acid TS to make exactly 50 mL, and use this solution as the sample solution. Separately, weigh accurately about 80 mg of metoclopramide for assay, previously dried at 105°C for 3 hours, and dissolve in 0.1 mol/L hydrochloric acid TS to make exactly 500 mL. Pipet 4 mL of this solution, add 0.1 mol/hydrochloric acid TS to make exactly 50 mL, and use this solution as the standard solution. Determine the absorbances, A_T and A_S, of the sample solution and the standard solution at 308 nm as directed under the Ultraviolet-visible Spectrophotometry.

Amount (mg) of metoclopramide ($C_{14}H_{22}ClN_3O_2$)

$$= W_S \times \frac{A_T}{A_S}$$

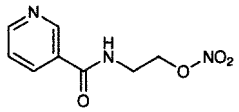
W_S: Amount (mg) of metoclopramide for assay

Containers and storage Containers—Tight containers.

Add the following:

Nicorandil

ニコランジル



$C_8H_9N_3O_4$: 211.17
N-[2-(Nitrooxy)ethyl]pyridine-3-carboxamide [*65141-46-0*]

Nicorandil contains not less than 98.5% and not more than 101.0% of $C_8H_9N_3O_4$, calculated on the anhydrous basis.

Description Nicorandil occurs as white crystals.

It is freely soluble in methanol, in ethanol (99.5) and in acetic acid (100), soluble in acetic anhydride, and sparingly soluble in water.

Melting point: about 92°C (with decomposition).

Identification Determine the infrared absorption spectrum of Nicorandil as directed in the potassium bromide disk method under the Infrared Spectrophotometry, and compare the spectrum with the Reference Spectrum: both spectra exhibit similar intensities of absorption at the same wave numbers.

Purity **(1)** Sulfate—Dissolve 2.0 g of Nicorandil in 20 mL of dilute ethanol, add 1 mL of dilute hydrochloric acid and water to make 50 mL, and perform the test using this solution as the test solution. Prepare the control solution with 0.40 mL of 0.005 mol/L sulfuric acid VS, 20 mL of dilute ethanol and 1 mL of dilute hydrochloric acid, and dilute with water to make 50 mL (not more than 0.010%).

(2) Heavy metals—Proceed with 2.0 g of Nicorandil according to Method 2, and perform the test. Prepare the control solution with 2.0 mL of Standard Lead Solution (not more than 10 ppm).

(3) Related substances—Dissolve 20 mg of Nicorandil in 10 mL of the mobile phase, and use this solution as the sample solution. Perform the test with 10 μL of the sample solution as directed under the Liquid Chromatography according to the following conditions, and determine each peak area by the automatic integration method: the peak area of *N*-(2-hydroxyethyl)isonicotinamide nitric ester, having the relative retention time of about 0.86 with respect to nicorandil, is not more than 0.5% of the peak area of nicorandil, the area of all other peaks is less than 0.1%, and the sum area of the peaks other than nicorandil and *N*-(2-hydroxyethyl)isonicotinamide nitric ester is not more than 0.25% of the total peak area.

Operating conditions—

Detector: An ultraviolet absorption photometer (wavelength: 254 nm).

Column: A stainless steel column 4 mm in inside diameter and 25 cm in length, packed with octadecylsilanized silica gel for liquid chromatography (5 μm in particle diameter).

Column temperature: A constant temperature of about 25°C.

Mobile phase: A mixture of water, tetrahydrofuran, triethylamine and trifluoroacetic acid (982:10:5:3).

Flow rate: Adjust the flow rate so that the retention time of nicorandil is about 18 minutes.

Time span of measurement: About 3 times as long as the retention time of nicorandil after the solvent peak.

System suitability—

Test for required detectability: Measure exactly 1 mL of the sample solution, add the mobile phase to make exactly 500 mL, and use this solution as the solution for system suitability test. Pipet 1 mL of the solution for system suitability test, and add the mobile phase to make exactly 20 mL. Confirm that the peak area of nicorandil obtained with 10 μL of this solution is equivalent to 2 to 8% of that with 10 μL of the solution for system suitability test.

System performance: Dissolve 10 mg of *N*-(2-hydroxyethyl)isonicotinamide nitric ester in the mobile phase to make 100 mL. To 1 mL of this solution add 10 mL of the sample solution. When the procedure is run with this solution under the above operating conditions, *N*-(2-hydroxyethyl)isonicotinamide nitric ester and nicorandil are eluted in this order with the resolution between these peaks being not less than 3.0.

System repeatability: When the test is repeated 6 times with 10 μL of the solution for system suitability test under the above operating conditions, the relative standard deviation of the peak area of nicorandil is not more than 1.5%.

Water Not more than 0.1% (2 g, volumetric titration, direct titration).

Residue on ignition Not more than 0.1% (1 g).

Assay Weigh accurately about 0.3 g of Nicorandil, dissolve in 30 mL of a mixture of acetic anhydride and acetic acid (100) (7:3), and titrate with 0.1 mol/L perchloric acid VS (potentiometric titration). Perform a blank determination in the same manner, and make any necessary correction.

Each mL of 0.1 mol/L perchloric acid VS
= 21.12 mg of $C_8H_9N_3O_4$

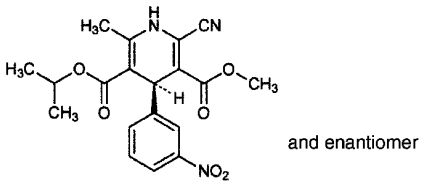
Containers and storage Containers—Tight containers.

Storage—At a temperature between 2°C and 8°C.

Add the following:

Nilvadipine

ニルバジピン



and enantiomer

$C_{19}H_{19}N_3O_6$: 385.37

5-Isopropyl 3-methyl (4*RS*)-2-cyano-1,4-dihydro-6-methyl-4-(3-nitrophenyl)pyridine-3,5-dicarboxylate [*75530-68-6*]

Nilvadipine contains not less than 98.0% and not more than 102.0% of $C_{19}H_{19}N_3O_6$.

Description Nilvadipine occurs as a yellow crystalline powder.

It is freely soluble in acetonitrile, soluble in methanol, sparingly soluble in ethanol (99.5), and practically insoluble in water.

A solution of Nilvadipine in acetonitrile (1 in 20) shows no optical rotation.

Identification (1) Determine the absorption spectrum of a solution of Nilvadipine in ethanol (99.5) (1 in 100,000) as directed under the Ultraviolet-visible Spectrophotometry, and compare the spectrum with the Reference Spectrum or the spectrum of a solution of Nilvadipine Reference Standard prepared in the same manner as the sample solution: both spectra exhibit similar intensities of absorption at the same wavelengths.

(2) Determine the infrared absorption spectrum of Nilvadipine as directed in the potassium bromide disk method under the Infrared Spectrophotometry, and compare the spectrum with the Reference Spectrum or the spectrum of Nilvadipine Reference Standard: both spectra exhibit similar intensities of absorption at the same wave numbers.

Melting point 167 – 171°C

Purity (1) Heavy metals—Proceed with 2.0 g of Nilvadipine according to Method 2, and perform the test. Prepare the control solution with 2.0 mL of Standard Lead Solution (not more than 10 ppm).

(2) Related substances—Dissolve 20 mg of Nilvadipine in 20 mL of acetonitrile, and use this solution as the sample

solution. Perform the test with 5 μL of the sample solution as directed under the Liquid Chromatography according to the following conditions. Determine each peak area by the automatic integration method, and calculate the amount of them by the area percentage method: the amount of each related substance is not more than 0.3%, and the total of them is not more than 0.5%.

Operating conditions—

Detector: An ultraviolet absorption photometer (wavelength: 240 nm).

Column: A stainless steel column 4.6 mm in inside diameter and 15 cm in length, packed with octadecylsilanized silica gel for liquid chromatography (5 μm in particle diameter).

Column temperature: A constant temperature of about 25°C.

Mobile phase: A mixture of phosphate buffer solution, pH 7.4, methanol and acetonitrile (32:27:18).

Flow rate: Adjust the flow rate so that the retention time of nilvadipine is about 12 minutes.

Time span of measurement: About 2.5 times as long as the retention time of nilvadipine after the solvent peak.

System suitability—

Test for required detectability: Pipet 1 mL of the sample solution, add acetonitrile to make exactly 100 mL, and use this solution as the solution for system suitability test. Pipet 1 mL of the solution for system suitability test, and add acetonitrile to make exactly 10 mL. Confirm that the peak area of nilvadipine obtained from 5 μL of this solution is equivalent to 7 to 13% of that obtained from 5 μL of the solution for system suitability test.

System performance: When the procedure is run with 5 μL of the solution for system suitability test under the above operating conditions, the number of theoretical plates and the symmetry factor of the peak of nilvadipine is not less than 3300 and not more than 1.3, respectively.

System repeatability: Pipet 1 mL of the solution for system suitability test, and add acetonitrile to make exactly 10 mL. When the test is repeated 6 times with 5 μL of this solution under the above operating conditions, the relative standard deviation of the peak area of nilvadipine is not more than 1.5%.

Loss on drying Not more than 0.1% (1 g, 105°C, 2 hours).

Residue on ignition Not more than 0.1% (1 g).

Assay Weigh accurately about 25 mg each of Nilvadipine and Nilvadipine Reference Standard, dissolve in methanol to make exactly 25 mL. Pipet 10 mL each of these solutions, add exactly 20 mL of the internal standard solution, 20 mL of water and methanol to make 100 mL, and use these solutions as the sample solution and the standard solution, respectively. Perform the test with 5 μL each of the sample solution and the standard solution as directed under the Liquid Chromatography according to the following conditions, and determine the ratios, Q_T and Q_S, of the peak area of nilvadipine to that of the internal standard.

$$\text{Amount (mg) of } C_{19}H_{19}N_3O_6 = W_S \times \frac{Q_T}{Q_S}$$

W_S: Amount (mg) of Nilvadipine Reference Standard

Internal standard solution—A solution of acenaphthene in methanol (1 in 200).

Operating conditions—

Detector: An ultraviolet absorption photometer (wavelength: 254 nm).

Column: A stainless steel column 4 mm in inside diameter and 15 cm in length, packed with octadecylsilanized silica gel for liquid chromatography (5 μm in particle diameter).

Column temperature: A constant temperature of about 25°C.

Mobile phase: Dissolve 2.5 g of diammonium hydrogen phosphate in 1000 mL of water, add 10 mL of tetrabutylammonium hydoxide TS, adjust the pH to 7.0 with diluted phosphoric acid (1 in 10), and add 900 mL of acetonitrile.

Flow rate: Adjust the flow rate so that the retention time of nilvadipine is about 12 minutes.

System suitability—

System performance: When the procedure is run with 5 μL of the standard solution under the above operating conditions, nilvadipine and the internal standard are eluted in this order with the resolution between these peaks being not less than 8.

System repeatability: When the test is repeated 6 times with 5 μL of the standard solution under the above operating conditions, the relative standard deviation of the ratio of the peak area of nilvadipine to that of the internal standard is not more than 1.0%.

Containers and storage Containers—Well-closed containers.

Add the following:

Nilvadipine Tablets

ニルバジピン錠

Nilvadipine Tablets contain not less than 93.0% and not more than 107.0% of the labeled amount of nilvadipine ($C_{19}H_{19}N_3O_6$: 385.37).

Method of preparation Prepare as directed under Tablets, with Nilvadipine.

Identification To a quantity of powdered Nilvadipine Tablets, equivalent to 1 mg of Nilvadipine according to the labeled amount, add 100 mL of ethanol (99.5), shake for 10 minutes, centrifuge, and use the supernatant liquid as the sample solution. Determine the absorption spectrum of the sample solution as directed under the Ultraviolet-visible Spectrophotometry: it exhibits a maximum between 239 nm and 243 nm and a maximum having a broad-ranging absorption between 371 nm and 381 nm.

Content uniformity Perform the test according to the following method: it meets the requirements of the Content Uniformity Test.

To 1 tablet of Nilvadipine Tablets add V mL of a mixture of acetonitrile and water (7:3) so that each mL of the solution contains about 200 μg of nilvadipine ($C_{19}H_{19}N_3O_6$) according to the labeled amount, add exactly V mL of the internal standard solution obtained in the Assay, and disperse the particles with the aid of ultrasonic waves. Centrifuge for 10 minutes, and use the supernatant liquid as the sample solution. Separately, weigh accurately about 20 mg of Nilvadipine Reference Standard, dissolve in the mixture of acetonitrile and water (7:3) to make exactly 20 mL. Pipet 5 mL of this solution, add exactly 25 mL of the internal standard solution obtained in the Assay and the mixture of acetonitrile and water (7:3) to make 50 mL, and use this solution as the standard solution. Proceed as directed in the Assay.

$$\text{Amount (mg) of nilvadipine } (C_{19}H_{19}N_3O_6) = W_S \times \frac{Q_T}{Q_S} \times \frac{V}{100}$$

W_S: Amount (mg) of Nilvadipine Reference Standard

Dissolution Perform the test with 1 tablet of Nilvadipine Tablets at 50 revolutions per minute according to Method 2 under the Dissolution Test, using 900 mL of water as the dissolution medium. Withdraw 20 mL or more of the dissolution medium 30 minutes after starting the test, and filter through a membrane filter with pore size of not more than 0.5 μm. Discard the first 10 mL of the filtrate, pipet 10 mL of the subsequent filtrate, add exactly 1 mL of methanol, and use this solution as the sample solution. Separately, weigh accurately an amount of Nilvadipine Reference Standard, equivalent to 10 times the labeled amount of Nilvadipine Tablets, and dissolve in methanol to make exactly 50 mL. Pipet 5 mL of this solution, and add methanol to make exactly 100 mL. Pipet 1 mL of this solution, add exactly 10 mL of water, and use this solution as the standard solution. Perform the test with exactly 20 μL each of the sample solution and the standard solution as directed under the Liquid Chromatography according to the following conditions, and determine the peak areas, A_T and A_S, of nilvadipine: the dissolution rate in 30 minutes is not less than 85%.

$$\text{Dissolution rate (\%) with respect to the labeled amount of nilvadipine } (C_{19}H_{19}N_3O_6) = W_S \times \frac{A_T}{A_S} \times \frac{1}{C} \times 9$$

W_S: Amount (mg) of Nilvadipine Reference Standard
C: Labeled amount (mg) of nilvadipine ($C_{19}H_{19}N_3O_6$) in 1 tablet

Operating conditions—

Detector: An ultraviolet absorption photometer (wavelength: 242 nm).

Column: A stainless steel column 4 mm in inside diameter and 15 cm in length, packed with octadecylsilanized silica gel for liquid chromatography (5 μm in particle diameter).

Column temperature: A constant temperature of about 25°C.

Mobile phase: A mixture of phosphate buffer solution, pH 7.4, methanol and acetonitrile (7:7:6)

Flow rate: Adjust the flow rate so that the retention time of nilvadipineis about 5 minutes.

System suitability—

System performance: When the procedure is run with 20 μL of the standard solution under the above operating conditions, the number of theoretical plates and the symmetry factor of the peak of nilvadipine are not less than 2000 and not more than 1.5, respectively.

System repeatability: When the test is repeated 6 times with 20 μL of the standard solution under the above operating conditions, the relative standard deviation of the peak area of nilvadipine is not more than 1.5%.

Assay Weigh accurately not less than 20 Nilvadipine Tablets, and powder. Weigh accurately an amount of the powder, equivalent to about 5 mg of nilvadipine ($C_{19}H_{19}N_3O_6$), add 10 mL of a mixture of acetonitrile and water (7:3) and exactly 25 mL of the internal standard solution, shake for 15 minutes, and add the mixture of acetonitrile and water (7:3) to make 50 mL. Centrifuge, and use the supernatant liquid as the sample solution. Separately, weigh accurately about 20 mg of Nilvadipine Reference Standard, dissolve in the mixture of acetonitrile and water (7:3) to make exactly 20 mL. Pipet 5 mL of this solution, add exactly 25 mL of the internal standard solution and the mixture of acetonitrile and water (7:3) to make 50 mL, and use this solution as the standard solution. Perform the test with 5 μL each of the sample solution and the standard solution as directed under the Liquid Chromatography according to the following conditions, and determine the ratios, Q_T and Q_S, of the peak area of nilvadipine to that of the internal standard.

$$\text{Amount (mg) of nilvadipine } (C_{19}H_{19}N_3O_6) = W_S \times \frac{Q_T}{Q_S} \times \frac{1}{4}$$

W_S: Amount (mg) of Nilvadipine Reference Standard

Internal standard solution—A solution of acenaphthene in acetonitrile (1 in 500).

Operating conditions—

Detector: An ultraviolet absorption photometer (wavelength: 254 nm).

Column: A stainless steel column 4 mm in inside diameter and 15 cm in length, packed with octadecylsilanized silica gel for liquid chromatography (5 μm in particle diameter).

Column temperature: A constant temperature of about 25°C.

Mobile phase: Dissolve 2.5 g of diammonium hydrogen phosphate in 1000 mL of water, add 10 mL of tetrabutylammonium hydoxide TS, adjust the pH to 7.0 with diluted phosphoric acid (1 in 10), and add 900 mL of acetonitrile.

Flow rate: Adjust the flow rate so that the retention time of nilvadipine is about 12 minutes.

System suitability—

System performance: When the procedure is run with 5 μL

of the standard solution under the above operating conditions, nilvadipine and the internal standard are eluted in this order with the resolution between these peaks being not less than 8.

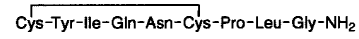
System repeatability: When the test is repeated 6 times with 5 μL of the standard solution under the above operating conditions, the relative standard deviation of the ratio of the peak area of nilvadipine to that of the internal standard is not more than 1.0%.

Containers and storage Containers—Well-closed containers.

Add the following:

Oxytocin

オキシトシン



$C_{43}H_{66}N_{12}O_{12}S_2$: 1007.19
[*50-56-6*]

Oxytocin is a synthetic peptide having the property of causing the contraction of uterine smooth muscle.

It contains not less than 540 oxytocin Units and not more than 600 oxytocin Units per mg, calculated on the dehydrated and de-acetic acid basis.

Description Oxytocin occurs as a white powder.

It is very soluble in water, and freely soluble in ethanol (99.5).

It dissolves in hydrochloric acid TS.

The pH of a solution prepared by dissolving 0.10 g of Oxytocin in 10 mL of freshly boiled and cooled water is between 4.0 and 6.0.

It is hygroscopic.

Identification Determine the absorption spectrum of a solution of Oxytocin (1 in 2000) as directed under the Ultraviolet-visible Spectrophotometry, and compare the spectrum with the Reference Spectrum: both spectra exhibit similar intensities of absorption at the same wavelengths.

Constituent amino acids Put about 1 mg of Oxytocin in a test tube for hydrolysis, add 6 mol/L hydrochloric acid TS to dissolve, replace the air in the tube with Nitrogen, seal the tube under reduced pressure, and heat at 110 to 115°C for 16 hours. After cooling, open the tube, evaporate the hydrolyzate to dryness under reduced pressure, add 2 mL of 0.02 mol/L hydrochloric acid TS to dissolve the residue, and use this solution as the sample solution. Separately, weigh accurately about 27 mg of L-aspartic acid, about 24 mg of L-threonine, about 21 mg of L-serine, about 29 mg of L-glutamic acid, about 23 mg of L-proline, about 15 mg of glycine, about 18 mg of L-alanine, about 23 mg of L-valine, about 48 mg of L-cystine, about 30 mg of methionine, about 26 mg of L-isoleucine, about 26 mg of L-leucine, about 36 mg of L-tyrosine, about 33 mg of phenylalanine, about 37 mg of L-lysine hydrochloride, about 42 mg of L-histidine hydrochloride monohydrate and about 42 mg of L-arginine hydrochloride, dissolve them in 10 mL of 1 mol/L hydrochloric acid TS, and add water to make exactly 100 mL. Pipet 5 mL of this solution, add water to make exactly 20 mL, and use this solution as the standard solution. Perform the test with exactly 20 μL each of the sample solution and the standard solution as directed under the Liquid Chromatography according to the following conditions, and calculate the respective molar ratios with respect to leucine: 0.95 – 1.05 for aspartic acid, 0.95 – 1.05 for glutamic acid, 0.95 – 1.05 for proline, 0.95 – 1.05 for glycine, 0.80 – 1.10 for isoleucine, 0.80 – 1.05 for tyrosine and 0.80 – 1.05 for cystine, and not more than 0.01 each for others.

Operating conditions—

Detector: A visible spectrophotometer (wavelength: 440 nm and 570 nm).

Column: A stainless steel column 4.6 mm in inside diameter and 8 cm in length, packed with strongly acidic ion-exchange resin for liquid chromatography (sodium type) composed with a sulfonated polystyrene copolymer (3 μm in particle diameter).

Column temperature: A constant temperature of about 57°C.

Chemical reaction bath temperature: A constant temperature of about 130°C.

Color developing time: About 1 minute.

Mobile phase: Prepare mobile phases A, B and C according to the following table.

Mobile phase	A	B	C
Citric acid monohydrate	19.80 g	22.00 g	6.10 g
Trisodium citrate dihydrate	6.19 g	7.74 g	26.67 g
Sodium chloride	5.66 g	7.07 g	54.35 g
Ethanol (99.5)	260.0 mL	20.0 mL	—
Benzyl alcohol	—	—	5.0 mL
Thiodiglycol	5.0 mL	5.0 mL	—
Lauromacrogol solution (1 in 4)	4.0 mL	4.0 mL	4.0 mL
Capryric acid	0.1 mL	0.1 mL	0.1 mL
Water	a sufficient amount	a sufficient amount	a sufficient amount
Total amount	2000 mL	1000 mL	1000 mL
pH	3.3	3.2	4.9

Flowing of the mobile phase: Control the gradient by mixing the mobile phases A, B and C as directed in the following table.

Time after injection of sample (min)	Mobile phase A (%)	Mobile phase B (%)	Mobile phase C (%)
0 – 9	100	0	0
9 – 25	0	100	0
25 – 61	0	100 → 0	0 → 100
61 – 80	0	0	100

Reaction reagent: Mix 407 g of lithium acetate dihydrate, 245 mL of acetic acid (100) and 801 mL of 1-methoxy-2-propanol, add water to make 2000 mL, stir for more than 10 minutes while passing Nitrogen, and use this solution as Solution A. Separately, to 1957 mL of 1-methoxy-2-propanol add 77 g of ninhydrin and 0.134 g of sodium borohydride, stir for more than 30 minutes while passing Nitrogen, and use this solution as Solution B. Mix Solution A and Solution B before use.

Flow rate of mobile phase: About 0.26 mL per minute.

Flow rate of reaction reagent: About 0.3 mL per minute.

System suitability—

System performance: When the procedure is run with 20 μL of the standard solution under the above operating conditions, aspartic acid, threonine, serine, glutamic acid, proline, glycine, alanine, valine, cystine, methionine, isoleucine, leucine, tyrosine, phenylalanine, lysine, histidine and arginine are eluted in this order with the resolutions between the peaks of threonine and serine, glycine and alanine, and isoleucine and leucine being not less than 1.5, 1.4 and 1.2, respectively.

System repeatability: When the test is repeated 3 times with 20 μL of the standard solution under the above operating conditions, the relative standard deviations of the peak area of aspartic acid, proline, valine and arginine are not more than 2.0%, respectively.

Purity (1) Acetic acid—Weigh accurately about 15 mg of Oxytocin, dissolve in the internal standard solution to make exactly 10 mL, and use this solution as the sample solution. Separately, weigh accurately about 1 g of acetic acid (100), add the internal standard solution to make exactly 100 mL. Pipet 2 mL of this solution, add the internal standard solution to make exactly 200 mL, and use this solution as the standard solution. Perform the test with 10 μL each of the sample solution and the standard solution as directed under the Liquid Chromatography according to the following conditions, and calculate the ratios, Q_T and Q_S, of the peak area of acetic acid to that of the internal standard: the amount of acetic acid is not less than 6.0% and not more than 10.0%.

$$\text{Amount (\%) of acetic acid } (C_2H_4O_2) = \frac{W_S}{W_T} \times \frac{Q_T}{Q_S} \times \frac{1}{10}$$

W_S: Amount (mg) of acetic acid (100)

W_T: Amount (mg) of the sample

Internal standard solution—A solution of propionic acid in the mobile phase (1 in 10,000).

Operating conditions—

Detector: An ultraviolet absorption photometer (wavelength: 210 nm).

Column: A stainless steel column 4.6 mm in inside diameter and 15 cm in length, packed with octadecylsilanized silica gel for liquid chromatography (5 μm in particle diameter).

Column temperature: A constant temperature of about 40°C.

Mobile phase: To 0.7 mL of phosphoric acid add 900 mL of water, adjust the pH to 3.0 with 8 mol/L sodium hydroxide TS, and add water to make 1000 mL. To 950 mL of this solution add 50 mL of methanol.

Flow rate: Adjust the flow rate so that the retention time of acetic acid is about 3 minutes.

System suitability—

System performance: When the procedure is run with 10 μL of the standard solution under the above operating conditions, acetic acid and propionic acid are eluted in this order with the resolution between these peaks being not less than 14.

System repeatability: When the test is repeated 6 times with 10 μL of the standard solution under the above operating conditions, the relative standard deviation of the ratio of the peak area of acetic acid to that of the internal standard is not more than 2.0%.

(2) Related substances—Dissolve 25 mg of Oxytocin in 100 mL of the mobile phase A, and use this solution as the sample solution. Perform the test with 50 μL of the sample solution as directed under the Liquid Chromatography according to the following conditions, determine each peak area by the automatic integration method, and calculate the amount of them by the area percentage method: the amount of each peak other than Oxytocin is not more than 1.5%, and the total of them is not more than 5.0%.

Operating conditions—

Detector, column, column temperature, mobile phase, flowing of mobile phase, and flow rate: Proceed as directed in the operating conditions in the Assay.

Time span of measurement: About 2.5 times as long as the retention time of oxytocin.

System suitability—

Test for required detectability: Measure exactly 1 mL of the sample solution, add the mobile phase A to make exactly 100 mL, and use this solution as the solution for system suitability test. Pipet 1 mL of the solution for system suitability test, and add the mobile phase A to make exactly 10 mL. Confirm that the peak area of oxytocin obtained from 50 μL of this solution is equivalent to 5 to 15% of that from 50 μL of the solution for system suitability test.

System performance: Dissolve an adequate amount of oxytocin and vasopressin in the mobile phase A, so that each mL contains about 0.1 mg each of them. When the procedure is run with 50 μL of this solution under the above operating conditions, vasopressin and oxytocin are eluted in this order with the resolution between these peaks being not less than 14, and the symmetry factor of the peak of oxytocin is not more than 1.5.

System repeatability: When the test is repeated 6 times with 50 μL of the solution for system suitability test under the above operating conditions, the relative standard deviation of the peak area of oxytocin is not more than 2.0%.

Water Not more than 5.0% (50 mg, coulometric titration).

Assay Weigh accurately an amount of Oxytocin, equivalent to about 13,000 Units, dissolve in the mobile phase A to make exactly 100 mL, and use this solution as the sample solution. Separately, dissolve oxytocin in 1 bottle of the Posterior Pituitary Reference Standard in the mobile phase A to make a known concentration solution containing each mL contains about 130 Units, and use this solution as the standard solution. Perform the test with exactly 25 μL each of the sample solution and the standard solution as directed under the Liquid Chromatography according to the following conditions, and determine the peak areas, A_T and A_S, of oxytocin.

Units per mg of Oxytocin, calculated on the dehydrated and de-acetic acid basis

$$= \frac{W_S}{W_T} \times \frac{A_T}{A_S} \times 100$$

W_S: Units per mL of the standard solution
W_T: Amount (mg) of the sample, calculated on the dehydrated and de-acetic acid basis

Operating conditions—

Detector: An ultraviolet absorption photometer (wavelength: 220 nm).

Column: A stainless steel column 4.6 mm in inside diameter and 15 cm in length, packed with octadecylsilanized silica gel for liquid chromatography (5 μm in particle diameter).

Column temperature: A constant temperature of about 25°C.

Mobile phase A: Dissolve 15.6 g of sodium dihydrogen phosphate dihydrate in 1000 mL of water.

Mobile phase B: A mixture of water and acetonitrile (1:1).

Flowing of the mobile phase: Control the gradient by mixing the mobile phases A and B as directed in the following table.

Time after injection of sample (min)	Mobile phase A (%)	Mobile phase B (%)
0 – 30	70 → 40	30 → 60
30 – 30.1	40 → 70	60 → 30
30.1 – 45	70	30

Flow rate: About 1.0 mL per minute.

System suitability—

System performance: Dissolve an adequate amount of oxytocin and vasopressin in the mobile phase A, so that each mL contains about 0.1 mg each of them. When the procedure is run with 25 μL of this solution under the above operating conditions, vasopressin and oxytocin are eluted in this order with the resolution between these peaks being not less than 14, and the symmetry factor of the peak of oxytocin is not more than 1.5.

System repeatability: When the test is repeated 6 times with 25 μL of the standard solution under the above operating conditions, the relative standard deviation of the peak area of oxytocin is not more than 1.0%.

Containers and storage Containers—Tight containers.

Storage—At 2 to 8°C.

Change to read:

Oxytocin Injection

オキシトシン注射液

Oxytocin Injection is an aqueous solution for injection.

It contains not less than 90.0% and not more than 110.0% of the labeled oxytocin Units.

Method of preparation Prepare as directed under Injections, with Oxytocin.

Description Oxytocin Injection is a colorless, clear liquid.

pH 2.5 – 4.5

Bacterial endotoxins Less than 10 EU/oxytocin Unit.

Actual volume It meets the requirements of the Injections.

Foreign insoluble matter Perform the test according to Method 1: it meets the requirements of the Foreign Insoluble Matter Test for Injections.

Insoluble particulate matter Perform the test according to Method 1: it meets the requirements of the Insoluble Particulate Matter Test for Injections.

Sterility Perform the test according to the Membrane filtration method: it meets the requirements of the Sterility Test.

Assay Measure exactly a portion of Oxytocin Injection according to the labeled Units, dilute with the diluent so that each mL contains about 1 Unit, and use this solution as the sample solution. Separately, dissolve oxytocin in 1 bottle of Posterior Pituitary Reference Standard in the mobile phase A to make exactly 20 mL. Pipet a suitable volume of this solution, dilute with the diluent to make a known concentration solution so that each mL contains about 1 Unit, and use this solution as the standard solution. Perform the test with exactly 100 μL each of the sample solution and the standard solution as directed under the Liquid Chromatography according to the following conditions, and determine the peak areas, A_T and A_S, of oxytocin.

$$\text{Units per mL of Oxytocin Injection} = W_S \times \frac{A_T}{A_S} \times \frac{b}{a}$$

W_S: Units per mL of the standard solution
a: Volume (mL) of the sample
b: Total volume of the sample solution prepared by dilut-

ing with the diluent

Diluent: Dissolve 5 g of chlorobutanol, 1.1 g of sodium acetate trihydrate, 5 g of acetic acid (100) and 6 mL of ethanol (99.5) in water to make 1000 mL.

Operating conditions—

Detector: An ultraviolet absorption photometer (wavelength: 220 nm).

Column: A stainless steel column 4.6 mm in inside diameter and 15 cm in length, packed with octadecylsilanized silica gel for liquid chromatography (5 μm in particle diameter).

Column temperature: A constant temperature of about 25°C.

Mobile phase A: Dissolve 15.6 g of sodium dihydrogen phosphate dihydrate in 1000 mL of water.

Mobile phase B: A mixture of water and acetonitrile (1:1).

Flowing of the mobile phase: Control the gradient by mixing the mobile phases A and B as directed in the following table.

Time after injection of sample (min)	Mobile phase A (%)	Mobile phase B (%)
0 – 30	70 → 40	30 → 60
30 – 30.1	40 → 70	60 → 30
30.1 – 45	70	30

Flow rate: About 1.0 mL per minute.

System suitability—

System performance: Dissolve an adequate amount of oxytocin and vasopressin in the mobile phase A, so that each mL contains about 0.02 mg each of them. When the procedure is run with 100 μL of this solution under the above operating conditions, vasopressin and oxytocin are eluted in this order with the resolution between these peaks being not less than 14, and the symmetry factor of the peak of oxytocin is not more than 1.5.

System repeatability: When the test is repeated 6 times with 100 μL of the standard solution under the above operating conditions, the relative standard deviation of the peak area of oxytocin is not more than 2.0%.

Containers and storage Containers—Hermetic containers.

Storage—In a cold place, and avoid freezing.

Pirenoxine

ピレノキシン

Change the Purity (2) to read:

Purity

(2) Related substances—Dissolve 10 mg of Pirenoxine in 50 mL of the mobile phase, and use this solution as the sample solution. Pipet 3 mL of the sample solution, add the mobile phase to make exactly 200 mL, and use this solution as the standard solution. Perform the test with exactly 5 μL each of the sample solution and the standard solution as directed under the Liquid Chromatography according to the following conditions. Determine each peak area of both solutions by the automatic integration method: the total area of the peaks other than pirenoxine is not larger than the peak area of pirenoxine from the standard solution.

Operating conditions—

Detector: An ultraviolet absorption photometer (wavelength: 230 nm).

Column: A stainless steel column 4 mm in inside diameter and 15 cm in length, packed with octadecylsilanized silica gel for liquid chromatography (5 μm in particle diameter).

Column temperature: A constant temperature of about 35°C.

Mobile phase: Dissolve 1.39 g of tetra *n*-butylammonium chloride and 4.5 g of disodium hydrogen phosphate 12-water in 1000 mL of water, and adjust the pH to 6.5 with phosphoric acid. To 700 mL of this solution add 200 mL of acetonitrile and 30 mL of tetrahydrofuran, and mix.

Flow rate: Adjust the flow rate so that the retention time of pirenoxine is about 10 minutes.

Time span of measurement: About 3 times as long as the retention time of pirenoxine.

System suitability—

Test for required detectability: To exactly 2 mL of the standard solution add the mobile phase to make exactly 30 mL. Confirm that the peak area of pirenoxine obtained from 5 μL of this solution is equivalent to 5 to 8% of that of pirenoxine obtained from 5 μL of the standard solution.

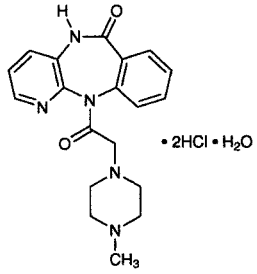
System performance: Dissolve 3 mg of Pirenoxine and 16 mg of methyl parahydroxybenzoate in 100 mL of the mobile phase. When the procedure is run with 5 μL of this solution under the above operating conditions, pirenoxine and methyl parahydroxybenzoate are eluted in this order with the resolution between these peaks being not less than 2.0.

System repeatability: When the test is repeated 6 times with 5 μL of the standard solution under the above operating conditions, the relative standard deviation of the peak area of pirenoxine is not more than 1.0%.

Add the following:

Pirenzepine Hydrochloride Hydrate

塩酸ピレンゼピン水和物



$C_{19}H_{21}N_5O_2.2HCl.H_2O$: 442.34

5,11-Dihydro-11-[(4-methylpiperazin-1-yl)acetyl]-6*H*-pyrido[2,3-*b*][1,4]benzodiazepin-6-one dihydrochloride monohydrate [*29868-97-1*, anhydride]

Pirenzepine Hydrochloride Hydrate contains not less than 98.5% and not more than 101.0% of pirenzepine hydrochloride ($C_{19}H_{21}N_5O_2.2HCl$: 424.32), calculated on the anhydrous basis.

Description Pirenzepine Hydrochloride Hydrate occurs as a white to pale yellow crystalline powder.

It is freely soluble in water and in formic acid, slightly soluble in methanol, and very slightly soluble in ethanol (99.5).

The pH of a solution by dissolving 1 g of Pirenzepine Hydrochloride Hydrate in 10 mL of water is between 1.0 and 2.0.

Melting point: about 245°C (with decomposition).

It is gradually colored by light.

Identification **(1)** Determine the absorption spectrum of a solution of Pirenzepine Hydrochloride Hydrate (1 in 40,000) as directed under the Ultraviolet-visible Spectrophotometry, and compare the spectrum with the Reference Spectrum: both spectra exhibit similar intensities of absorption at the same wavelengths.

(2) Determine the infrared absorption spectrum of Pirenzepine Hydrochloride Hydrate as directed in the potassium chloride disk method under the Infrared Spectrophotometry, and compare the spectrum with the Reference Spectrum: both spectra exhibit similar intensities of absorption at the same wave numbers.

(3) A solution of Pirenzepine Hydrochloride Hydrate (1 in 50) responds to the Qualitative Tests for chloride.

Purity **(1)** Clarity and color of solution—A solution obtained by dissolving 1.0 g of Pirenzepine Hydrochloride Hydrate in 10 mL of water is clear and not more color than that of the following control solution.

Control solution: To 1.2 mL of Matching fluid for color F add 8.8 mL of diluted hydrochloric acid (1 in 40).

(2) Heavy metals—Proceed with 2.0 g of Pirenzepine Hydrochloride Hydrate according to Method 2, and perform the test. Prepare the control solution with 2.0 mL of Standard Lead Solution (not more than 10 ppm).

(3) Related substances—Dissolve 0.3 g of Pirenzepine Hydrochloride Hydrate in 10 mL of water. To 1 mL of this solution add 5 mL of methanol and the mobile phase A to make 10 mL, and use this solution as the sample solution. Pipet 1 mL of the sample solution, and add 5 mL of methanol and the mobile phase A to make exactly 10 mL. Pipet 1 mL of this solution, add 5 mL of methanol and the mobile phase A to make exactly 10 mL, and use this solution as the standard solution. Perform the test with exactly 10 μL each of the sample solution and the standard solution as directed under the Liquid Chromatography according to the following conditions, and determine each peak area by the automatic integration method: the area of the peak other than pirenzepine is not more than 3/10 times the peak area of pirenzepine from the standard solution, and the total area of the peaks other than pirenzepine is not more than 3/5 times the peak area of pirenzepine from the standard solution.

Operating conditions—

Detector: An ultraviolet absorption photometer (wavelength: 283 nm).

Column: A stainless steel column 4.6 mm in inside diameter and 15 cm in length, packed with octadecylsilanized silica gel for liquid chromatography (5 μm in particle diameter).

Column temperature: A constant temperature of about 40°C.

Mobile phase A: Dissolve 2 g of sodium lauryl sulfate in 900 mL of water, adjust the pH to 3.2 with acetic acid (100), and add water to make 1000 mL.

Mobile phase B: Methanol

Mobile phase C: Acetonitrile

Flowing of the mobile phase: Control the gradient by mixing the mobile phases A, B and C as directed in the following table.

Time after injection of sample (min)	Mobile phase A (%)	Mobile phase B (%)	Mobile phase C (%)
0 – 15	55 → 25	30	15 → 45
15 –	25	30	45

Flow rate: Adjust the flow rate so that the retention time of pirenzepine is about 8 minutes.

Time span of measurement: About 2 times as long as the retention time of pirenzepine after the solvent peak.

System suitability—

Test for required detectability: Pipet 1 mL of the standard solution, and add 5 mL of methanol and the mobile phase A to make exactly 10 mL. Confirm that the peak area of pirenzepine obtained from 10 μL of this solution is equivalent to 7 to 13% of that from 10 μL of the standard solution.

System performance: Dissolve 0.1 g of phenylpiperazine hydrochloride in 10 mL of methanol. Mix 1 mL of this solution and 1 mL of the sample solution, and add 5 mL of methanol and the mobile phase A to make 10 mL. When the procedure is run with 10 μL of this solution under the above

operating conditions, pirenzepine and phenylpiperazine are eluted in this order with the resolution between these peaks being not less than 5.

System repeatability: When the test is repeated 6 times with 10 μL of the standard solution under the above operating conditions, the relative standard deviation of the peak area of pirenzepine is not more than 2.0%.

Water Not less than 3.5% and not more than 5.0% (0.3 g, volumetric titration, direct titration).

Residue on ignition Not more than 0.1% (1 g).

Assay Weigh accurately about 0.2 g of Pirenzepine Hydrochloride Hydrate, dissolve in 2 mL of formic acid, add 60 mL of acetic anhydride, and titrate with 0.1 mol/L perchloric acid VS (potentiometric titration). Perform a blank determination in the same manner, and make any necessary correction.

Each mL of 0.1 mol/L perchloric acid VS
= 14.14 mg of $C_{19}H_{21}N_5O_2.2HCl$

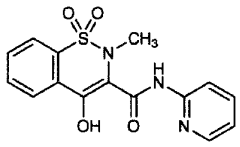
Containers and storage Containers—Well-closed containers.

Storage—Light-resistant.

Add the following:

Piroxicam

ピロキシカム



$C_{15}H_{13}N_3O_4S$: 331.35

4-Hydroxy-2-methyl-*N*-(pyridin-2-yl)-2*H*-1,2-benzothiazine-3-carboxamide 1,1-dioxide [*36322-90-4*]

Piroxicam contains not less than 98.5% and not more than 101.0% of $C_{15}H_{13}N_3O_4S$, calculated on the dried basis.

Description Piroxicam occurs as a white to pale yellow crystalline powder.

It is sparingly soluble in acetic anhydride, slightly soluble in acetonitrile, in methanol and in ethanol (99.5), very slightly soluble in acetic acid (100), and practically insoluble in water.

Melting point: about 200°C (with decomposition).

Identification (1) Dissolve 0.1 g of Piroxicam in a mixture of methanol and 0.5 mol/L hydrochloric acid TS (490:1) to make 200 mL. To 1 mL of this solution add the mixture of methanol and 0.5 mol/L hydrochloric acid TS (490:1) to make 100 mL. Determine the absorption spectrum of this solution as directed under the Ultraviolet-visible Spectrophotometry, and compare the spectrum with the Reference Spectrum: both spectra exhibit similar intensities of absorption at the same wavelengths.

(2) Determine the infrared absorption spectrum of Piroxicam as directed in the potassium bromide disk method under the Infrared Spectrophotometry, and compare the spectrum with the Reference Spectrum: both spectra exhibit similar intensities of absorption at the same wave numbers. If any difference appears between the spectra, dissolve the sample with dichloromethane, evaporate the solvent, dry the residue on a water bath, and perform the test.

Purity (1) Heavy metals—Proceed with 1.0 g of Piroxicam according to Method 2, and perform the test. Prepare the control solution with 2.0 mL of Standard Lead Solution (not more than 20 ppm).

(2) Related substances—Dissolve 75 mg of Piroxicam in 50 mL of acetonitrile for liquid chromatography, and use this solution as the sample solution. Pipet 1 mL of the sample solution, add acetonitrile for liquid chromatography to make exactly 10 mL. Pipet 1 mL of this solution, add acetonitrile for liquid chromatography to make exactly 50 mL, and use this solution as the standard solution. Perform the test with exactly 20 μL each of the sample solution and the standard solution as directed under the Liquid Chromatography according to the following conditions, and determine each peak area by the automatic integration method: the area of the peak other than piroxicam is not larger than the peak area of piroxicam with the standard solution, and the total area of the peaks other than piroxicam is not larger than 2 times the peak area of piroxicam with the standard solution.

Operating conditions—

Detector: An ultraviolet absorption photometer (wavelength: 230 nm).

Column: A stainless steel column 4.6 mm in inside diameter and 25 cm in length, packed with octadecylsilanized silica gel for liquid chromatography (5 μm in particle diameter).

Column temperature: A constant temperature of about 40°C.

Mobile phase: A mixture of 0.05 mol/L potassium dihydrogen phosphate TS, pH 3.0 and acetonitrile for liquid chromatography (3:2).

Flow rate: Adjust the flow rate so that the retention time of piroxicam is about 10 minutes.

Time span of measurement: About 5 times as long as the retention time of piroxicam after the solvent peak.

System suitability—

Test for required detectability: To exactly 5 mL of the standard solution add acetonitrile for liquid chromatography to make exactly 20 mL. Confirm that the peak area of piroxicam obtained with 20 μL of this solution is equivalent to 17.5 to 32.5% of that with 20 μL of the standard solution.

System performance: When the procedure is run with 20 μL of the standard solution under the above operating conditions, the number of theoretical plates and the symmetry factor of the peak of piroxicam are not less than 6000 and

not more than 1.5, respectively.

System repeatability: When the test is repeated 6 times with 20 μL of the standard solution under the above operating conditions, the relative standard deviation of the peak area of piroxicam is not more than 2.0%.

Loss on drying Not more than 0.5% (1 g, 105°C, 3 hours).

Residue on ignition Not more than 0.2% (1 g).

Assay Weigh accurately about 0.25 g of Piroxicam, dissolve in 60 mL of a mixture of acetic anhydride and acetic acid (100) (1:1), and titrate with 0.1 mol/L perchloric acid VS (potentiometric titration). Perform a blank determination in the same manner, and make any necessary correction.

Each mL of 0.1 mol/L perchloric acid VS
= 33.14 mg of $C_{15}H_{13}N_3O_4S$

Containers and storage Containers—Tight containers.

Pyrazinamide

ピラジナミド

Change the Description to read:

Description Pyrazinamide occurs as white crystals or crystalline powder.

It is sparingly soluble in water and in methanol, and slightly soluble in ethanol (99.5) and in acetic anhydride.

Change the Purity to read:

Purity

(1) Heavy metals—Proceed with 1.0 g of Pyrazinamide according to Method 2, and perform the test. Prepare the control solution with 2.0 mL of Standard Lead Solution (not more than 20 ppm).

(2) Related substances—Dissolve 0.10 g of Pyrazinamide in 10 mL of methanol, and use this solution as the sample solution. Pipet 1 mL of the sample solution, add methanol to make exactly 200 mL, and use this solution as the standard solution. Perform the test with these solutions as directed under the Thin-layer Chromatography. Spot 20 μL each of the sample solution and the standard solution on a plate of silica gel with fluorescent indicator for thin-layer chromatography. Develop the plate with a mixture of 1-butanol, water and acetic acid (100) (3:1:1) to a distance of about 10 cm, and air-dry the plate. Examine under ultraviolet light (main wavelength: 254 nm): the spot other than the principal spot obtained from the sample solution is not more intense than the spot from the standard solution.

Change the Assay to read:

Assay Weigh accurately about 0.1 g of Pyrazinamide, previously dried, dissolve in 50 mL of acetic anhydride, and titrate with 0.1 mol/L perchloric acid VS (potentiometric titration). Perform a blank determination in the same manner, and make any necessary correction.

Each mL of 0.1 mol/L perchloric acid VS
= 12.31 mg of $C_5H_5N_3O$

Pyridoxine Hydrochloride

塩酸ピリドキシン

Change the origin/limits of content to read:

Pyridoxine Hydrochloride, when dried, contains not less than 98.0% and not more than 101.0% of $C_8H_{11}NO_3.HCl$.

Change the Description to read:

Description Pyridoxine Hydrochloride occurs as a white to pale yellow, crystalline powder.

It is freely soluble in water, slightly soluble in ethanol (99.5), and practically insoluble in acetic anhydride and in acetic acid (100).

It is gradually affected by light.

Melting point: about 206°C (with decomposition).

Change the Identification to read:

Identification

(1) Determine the absorption spectrum of a solution of Pyridoxine Hydrochloride in 0.1 mol/L hydrochloric acid TS (1 in 100,000) as directed under the Ultraviolet-visible Spectrophotometry, and compare the spectrum with the Reference Spectrum or the spectrum of a solution of Pyridoxine Hydrochloride Reference Standard prepared in the same manner as the sample solution: both spectra exhibit similar intensities of absorption at the same wavelengths.

(2) Determine the infrared absorption spectrum of Pyridoxine Hydrochloride as directed in the potassium chloride disk method under the Infrared Spectrophotometry, and compare the spectrum with the Reference Spectrum or the spectrum of Pyridoxine Hydrochloride Reference Standard: both spectra exhibit similar intensities of absorption at the same wave numbers.

(3) A solution of Pyridoxine Hydrochloride (1 in 10) responds to the Qualitative Tests for chloride.

Add the following next to Identification:

pH The pH of a solution prepared by dissolving 1.0 g of Pyridoxine Hydrochloride in 50 mL of water is between 2.5 and 3.5.

Add the following next to Purity (2):

(3) Related substances—Dissolve 1.0 g of Pyridoxine Hydrochloride in 10 mL of water, and use this solution as the sample solution. Pipet 2.5 mL of the sample solution, and add water to make exactly 100 mL. Pipet 1 mL of this solution, add water to make exactly 10 mL, and use this

solution as the standard solution. Perform the test with these solutions as directed under the Thin-layer Chromatography. Spot 2 μL each of the sample solution and the standard solution on a plate of silica gel for thin-layer chromatography, and air-dry the plate. Develop the plate with a mixture of acetone, tetrahydrofuran, hexane and ammonia solution (28) (65:13:13:9) to a distance of about 10 cm, and air-dry the plate. Spray evenly a solution of sodium carbonate in diluted ethanol (99.5) (3 in 10) (1 in 20) on the plate. After air-drying, spray evenly a solution of 2,6-dibromo-*N*-chloro-1,4-benzoquinone monoimine in ethanol (99.5) (1 in 1000) on the plate, and air-dry: the spot other than the principal spot obtained from the sample solution is not more intense than the spot from the standard solution.

Pyridoxine Hydrochloride Injection

塩酸ピリドキシン注射液

Change the origin/limits of content to read:

Pyridoxine Hydrochloride Injection is an aqueous solution for injection.

It contains not less than 95.0% and not more than 105.0% of the labeled amount of pyridoxine hydrochloride ($C_8H_{11}NO_3$.HCl: 205.64).

Change the Identification to read:

Identification

(1) To a volume of Pyridoxine Hydrochloride Injection, equivalent to 50 mg of Pyridoxine Hydrochloride according to the labeled amount, add 0.1 mol/L hydrochloric acid TS to make 100 mL. To 2 mL of this solution add 0.1 mol/L hydrochloric acid TS to make 100 mL. Determine the absorption spectrum of this solution as directed under the Ultraviolet-visible spectrophotometry: it exhibits a maximum between 288 nm and 292 nm.

(2) To a volume of Pyridoxine Hydrochloride Injection, equivalent to 10 mg of Pyridoxine Hydrochloride according to the labeled amount, add water to make 10 mL, and use this solution as the sample solution. Separately, dissolve 10 mg of Pyridoxine Hydrochloride Reference Standard in 10 mL of water, and use this solution as the standard solution. Perform the test with these solutions as directed under the Thin-layer Chromatography. Spot 2 μL each of the sample solution and the standard solution on a plate of silica gel for thin-layer chromatography, and air-dry the plate. Develop the plate with a mixture of acetone, tetrahydrofuran, hexane and ammonia solution (28) (65:13:13:9) to a distance of about 10 cm, and air-dry the plate. Spray evenly a solution of sodium carbonate in diluted ethanol (99.5) (3 in 10) (1 in 20) on the plate. After air-drying, spray evenly a solution of 2,6-dibromo-*N*-chloro-1,4-benzoquinone monoimine in ethanol (99.5) (1 in 1000) on the plate: the spots obtained from the sample solution and the standard solution are blue in color and have the same *R*f value.

Add the following next to Identification:

Endotoxins Less than 3.0 EU/mg.

Delete the following Monograph:

Santonin Tablets

Add the following:

Serrapeptase

セラペプターゼ

[*95077-02-4*]

Serrapeptase is the enzyme preparation having proteolytic activity, produced by the growth of *Serratia* species. Usually, it is diluted with Lactose.

It contains not less than 2000 serrapeptase Units and not more than 2600 serrapeptase Units per mg.

It is hygroscopic.

Description Serrapeptase occurs as a grayish white to light brown powder, having a slight characteristic odor.

Identification Dissolve 0.4 g of Serrapeptase in 100 mL of acetic acid-sodium acetate buffer solution, pH 5.0, transfer exactly 1 mL each of this solution into three tubes, and refer to them as A, B and C. To tube A add exactly 1 mL of water, to tubes B and C add exactly 1 mL of 0.04 mol/L disodium dihydrogen ethylenediamine tetraacetate TS, mix gently, and allow them to stand in a water bath at 4 ± 1°C for about 1 hour. Then, to the tube B add exactly 2 mL of 0.04 mol/L zinc chloride TS, to the tubes A and C add exactly 2 mL of water, mix gently, and allow them to stand in a water bath at 4 ± 1°C for about 1 hour. Pipet 1 mL each of these solutions, add borate-hydrochloric acid buffer solution, pH 9.0 to the solutions A and B to make exactly 200 mL, to the solution C to make exactly 50 mL, and use these solutions as the sample solutions. Proceed with these sample solutions as directed in the Assay: the activities of the solutions A and B are almost the same, and the activity of the solution C is not more than 5% of that of the solution A.

$$\text{Activity of solutions A, B or C} = \frac{A_T}{A_S} \times \frac{1}{20} \times D \times 176$$

A_S: Absorbance of the standard solution

A_T: Absorbance of the sample solution

20: Reaction time (minute)

D: Dilution rate (200 for solution A and B, 50 for solution C)

176: Conversion factor (Total volume of enzyme reaction solution/volume of filtrate taken × amount of tyrosine in 2 mL of tyrosine standard solution)

Purity **(1)** Heavy metals—Put 1.0 g of Serrapeptase in a

porcelain crucible, add 2 drops each of sulfuric acid and nitric acid, and incinerate by ignition. After cooling, to the residue add 2 mL of hydrochloric acid, evaporate to dryness on a water bath, add 10 mL of a solution of hydroxylamine hydrochloride (3 in 100) and 2 mL of dilute acetic acid, and heat on a water bath for 5 minutes. After cooling, filter if necessary, wash the filter paper with 10 mL of water, put the filtrate and washing in a Nessler tube, add water to make 50 mL, and use this solution as the test solution. Prepare the control solution as follows: Evaporate to dryness 2 drops each of sulfuric acid and nitric acid on a sand bath, add 2 mL of hydrochloric acid to the residue, evaporate to dryness on a water bath, add 2.0 mL of Standard Lead Solution, 10 mL of a solution of hydroxylamine hydrochloride (3 in 100) and 2 mL of dilute acetic acid, and heat on a water bath for 5 minutes. Proceed in the same manner as directed for the preparation of the test solution, and add water to make 50 mL (not more than 20 ppm).

(2) Arsenic—Prepare the test solution with 0.40 g of Serrapeptase according to Method 3, excepting addition of 5 mL of a solution of magnesium nitrate hexahydrate in ethanol (95) (3 in 10) instead of a solution of magnesium nitrate hexahydrate in ethanol (95) (1 in 50), evaporating to dryness on a water bath, then incinerating with a small flame, and perform the test (not more than 5 ppm).

Loss on drying Not more than 7.0% (1 g, 105°C, 4 hours).

Residue on ignition Not more than 1.5% (1 g).

Assay (i) Sample solution: Dissolve exactly 0.100 g of Serrapeptase in a solution of ammonium sulfate (1 in 20) to make exactly 100 mL. Pipet 1 mL of this solution, add borate-hydrochloric acid buffer solution, pH 9.0 to make exactly 200 mL, and use this solution as the sample solution.

(ii) Tyrosine standard solution: Dissolve exactly 0.160 g of Tyrosine Reference Standard, previously dried at 105°C for 3 hours, in 0.2 mol/L hydrochloric acid TS to make exactly 1000 mL. Pipet 10 mL of this solution, and add 0.2 mol/L hydrochloric acid TS to make exactly 100 mL. Prepare before use.

(iii) Substrate solution: Previously determine the loss on drying (60°C, reduced pressure not exceeding 0.67 kPa, 3 hours) of milk casein, previously dried. To exactly 1.20 g of the milk casein, calculated based on the loss on drying, add 160 mL of a solution of sodium borate (19 in 1000), and heat in a water bath to dissolve. After cooling, adjust the pH to exactly 9.0 with 1 mol/L hydrochloric acid TS, and add borate-hydrochloric acid buffer solution, pH 9.0 to make exactly 200 mL. Use after warming to 37 ± 0.5°C. Prepare before use.

(iv) Precipitation reagent: Trichloroacetic acid TS for serrapeptase. Use after warming to 37 ± 0.5°C.

(v) Procedure: Pipet 1 mL of the sample solution, put in a glass-stoppered tube (15 × 130 mm), allow to stand at 37 ± 0.5°C for 5 minutes, add exactly 5 mL of the substrate solution, and mix well immediately. Allow to stand at 37 ± 0.5°C for exactly 20 minutes, add exactly 5 mL of trichloroacetic acid TS for serrapeptase, mix, allow to stand at 37 ± 0.5°C for 30 minutes, and filter through a dried filter paper. Pipet 2 mL of the filtrate, add exactly 5 mL of a solution of anhydrous sodium carbonate (3 in 50), mix, add exactly 1 mL of diluted Folin's TS (1 in 3), mix well, and allow to stand at 37 ± 0.5°C for 30 minutes. Determine the absorbance of this solution at 660 nm, A_1, as directed under the Ultraviolet-visible Spectrophotometry using water as the blank. Separately, pipet 1 mL of the sample solution, add exactly 5 mL of trichloroacetic acid TS for serrapeptase, mix, add exactly 5 mL of the substrate solution, allow to stand at 37 ± 0.5°C for 30 minutes, and proceed in the same manner as directed above to determine the absorbance A_2. Separately, pipet 2 mL of the tyrosine standard solution, add exactly 5 mL of a solution of anhydrous sodium carbonate (3 in 50), mix, add exactly 1 mL of diluted Folin's TS (1 in 3), mix well, and proceed in the same manner as directed above to determine the absorbance A_3. Separately, pipet 2 mL of 0.2 mol/L hydrochloric acid TS, and proceed in the same manner as directed above to determine the absorbance A_4.

$$\text{Serrapeptase Unit per mg of Serrapeptase} = \frac{A_1 - A_2}{A_3 - A_4} \times \frac{1}{20} \times 200 \times 176$$

20: Reaction time (minute)
200: Dilution rate
176: Conversion factor (Total volume of enzyme reaction solution/volume of filtrate taken × amount of tyrosine in 2 mL of tyrosine standard solution)

One serrapeptase Unit corresponds to the amount of serrapeptase which produces 5 μg of tyrosine per minute from 5 mL of the substrate solution under the above conditions.

Containers and storage Containers—Tight containers.

Sodium Aurothiomalate

金チオリンゴ酸ナトリウム

Change the origin/limits of content to read:

Sodium Aurothiomalate contains not less than 49.0% and not more than 52.5% of gold (Au: 196.97), calculated on the anhydrous basis and corrected by the amount of ethanol.

Change the Description to read:

Description Sodium Aurothiomalate occurs as white to light yellow, powder or granules.

It is very soluble in water, and practically insoluble in ethanol (99.5).

It is hygroscopic.

It changes in color by light to greenish pale yellow.

Change the Identification (3) to read:

Identification

(3) Place 2 mL of a solution of Sodium Aurothiomalate (1 in 10) in a porcelain crucible, add 1 mL of ammonia TS and 1 mL of hydrogen peroxide (30), evaporate to dryness, and ignite. Add 20 mL of water to the residue, and filter: the residue on the filter paper occurs as a yellow or dark yellow, powder or granules.

Add the following next to Identification (3):

Identification

(4) The filtrate obtained in (3) responds to the Qualitative Tests for sodium salt.

(5) The filtrate obtained in (3) responds to the Qualitative Tests for sulfate.

Add the following next to Purity (3):

Purity

(4) Ethanol—Weigh accurately about 0.2 g of Sodium Aurothiomalate, add exactly 3 mL of the internal standard solution and 2 mL of water to dissolve, and use this solution as the sample solution. Separately, pipet 3 mL of ethanol (99.5), and add water to make exactly 1000 mL. Pipet 2 mL of this solution, add exactly 3 mL of the internal standard solution, and use this solution as the standard solution. Perform the test with 2 μL each of the sample solution and the standard solution as directed under the Gas Chromatography according to the following conditions, and determine the ratios of the peak area of ethanol to that of the internal standard, Q_T and Q_S: the amount of ethanol is not more than 3.0%.

$$\text{Amount (mg) of ethanol} = \frac{Q_T}{Q_S} \times 6 \times 0.793$$

0.793: Density (g/mL) of ethanol (99.5) at 20°C

Internal standard solution—A solution of 2-propanol (1 in 500).

Operating conditions—

Detector: Hydrogen flame-ionization detector.

Column: A column 3 mm in inside diameter and 3 m in length, packed with porous styrene-divinylbenzene copolymer for gas chromatography (particle diameter: 150 – 180 μm) (average pore size: 0.0085 μm; 300 – 400 m^2/g).

Column temperature: A constant temperature of about 180°C.

Carrier gas: Nitrogen

Flow rate: Adjust the flow rate so that the retention time of the internal standard is about 7 minutes.

System suitability—

System performance: When the procedure is run with 2 μL of the standard solution under the above operating conditions, ethanol and the internal standard are eluted in this order with the resolution between these peaks being not less than 4.

System repeatability: When the test is repeated 6 times with 2 μL of the standard solution under the above operating conditions, the relative standard deviation of the ratio of the peak area of ethanol to that of the internal standard is not more than 2.0%.

Add the following next to Purity:

Water Not more than 5.0% (0.1 g, coulometric titration). Use a water vaporizer (heating temperature: 105°C; heating time: 30 minutes).

Delete the Loss on drying:

Change the Assay to read:

Assay Weigh accurately about 25 mg of Sodium Aurothiomalate, and dissolve in 2 mL of aqua regia by heating. After cooling, add water to make exactly 100 mL. Pipet 2 mL of the solution, add water to make exactly 25 mL, and use this solution as the sample solution. Separately, pipet 5 mL, 10 mL and 15 mL of Standard Gold Solution for atomic absorption spectrophotometry, add water to make exactly 25 mL, and use these solutions as the standard solutions (1), (2) and (3), respectively. Perform the test with the sample solution and the standard solutions (1), (2) and (3) as directed under the Atomic Absorption Spectrophotometry under the following conditions. Determine the amount of gold in the sample solution using the calibration curve obtained from the absorbances of the standard solutions.

Gas: Combustible gas—Acetylene
Supporting gas—Air

Lamp: Gold hollow-cathode lamp

Wavelength: 242.8 nm

Sodium Polystyrene Sulfonate

ポリスチレンスルホン酸ナトリウム

Change the Purity (4) to read:

(4) Styrene—To 10.0 g of Sodium Polystyrene Sulfonate add 10 mL of acetone, shake for 30 minutes, centrifuge, and use the supernatant liquid as the sample solution. Separately, dissolve 10 mg of styrene in acetone to make exactly 100 mL. Pipet 1 mL of this solution, add acetone to make exactly 100 mL, and use this solution as the standard solution. Perform the test with 20 μL each of the sample solution and the standard solution as directed under the Liquid Chromatography according to the following conditions, and determine peak areas, A_T and A_S, of styrene in each solution: A_T is not larger than A_S.

Operating conditions—

Detector: An ultraviolet absorption photometer (wavelength: 254 nm).

Column: A stainless steel column 4 mm in inside diameter and 15 cm in length, packed with octadecylsilanized silica gel for liquid chromatography (5 μm in particle diameter).

Column temperature: A constant temperature of about 25°C.

Mobile phase: A mixture of water and acetonitrile (1:1).

Flow rate: Adjust the flow rate so that the retention time

of styrene is about 8 minutes.

System suitability—

System performance: Dissolve 20 mg each of styrene and butyl parahydroxybenzoate in 100 mL of acetone. To 5 mL of this solution add acetone to make 100 mL. When the procedure is run with 20 μL of this solution under the above operating conditions, butyl parahydroxybenzoate and styrene are eluted in this order with the resolution between these peaks being not less than 5.

System repeatability: When the test is repeated 6 times with 20 μL of the standard solution under the above operating conditions, the relative standard deviation of the peak area of styrene is not more than 2.0%.

Sodium Thiosulfate

チオ硫酸ナトリウム

Change the origin/limits of content to read:

Sodium Thiosulfate, when dried, contains not less than 99.0% and not more than 101.0% of sodium thiosulfate ($Na_2S_2O_3$: 158.11).

Change the Description to read:

Description Sodium Thiosulfate occurs as colorless, crystals or crystalline powder. It is odorless.

It is very soluble in water, and practically insoluble in ethanol (99.5).

It effloresces in dry air, and is deliquescent in moist air.

Change the Identification to read:

Identification

(1) A solution of Sodium Thiosulfate (1 in 10) responds to the Qualitative Tests for thiosulfate.

(2) A solution of Sodium Thiosulfate (1 in 10) responds to the Qualitative Tests for sodium salt.

Change the Loss on drying to read:

Loss on drying 32.0 – 37.0% (1 g, in vacuum, 40 – 45°C, 16 hours).

Change the Assay to read:

Assay Weigh accurately about 0.4 g of Sodium Thiosulfate, previously dried, dissolve in 30 mL of water, and titrate with 0.05 mol/L iodine VS (indicator: 1 mL of starch TS).

Each mL of 0.05 mol/L iodine VS
= 15.81 mg of $Na_2S_2O_3$

Tegafur

テガフール

Add the following next to Identification:

pH Dissolve 0.5 g of Tegafur in 50 mL of water: the pH of this solution is between 4.2 and 5.2.

Testosterone Propionate

プロピオン酸テストステロン

Change the Description to read:

Description Testosterone Propionate occurs as white to pale yellow crystals or crystalline powder.

It is freely soluble in methanol and in ethanol (95), and practically insoluble in water.

Change the Identification to read:

Identification

(1) Determine the absorption spectrum of a solution of Testosterone Propionate in ethanol (95) (1 in 100,000) as directed under the Ultraviolet-visible Spectrophotometry, and compare the spectrum with the Reference Spectrum or the spectrum of a solution of Testosterone Propionate Reference Standard prepared in the same manner as the sample solution: both spectra exhibit similar intensities of absorption at the same wavelengths.

(2) Determine the infrared absorption spectrum of Testosterone Propionate, previously dried, as directed in the potassium bromide disk method under the Infrared Spectrophotometry, and compare the spectrum with the Reference Spectrum or the spectrum of Testosterone Propionate Reference Standard: both spectra exhibit similar intensities of absorption at the same wave numbers.

Change the Optical rotation to read:

Optical rotation $[\alpha]_D^{20}$: +83 – +90° (after drying, 0.1 g, ethanol (95), 10 mL, 100 mm).

Change the Purity to read:

Purity Other steroids—Dissolve 40 mg of Testosterone Propionate in 2 mL of ethanol (95), and use this solution as the sample solution. Pipet 1 mL of this solution, add ethanol (95) to make exactly 100 mL, and use this solution as the standard solution. Perform the test with these solutions as directed under the Thin-layer Chromatography. Spot 10 μL each of the sample solution and the standard solution on a plate of silica gel with fluorescent indicator for thin-layer chromatography. Develop the plate with a mixture of chloroform and diethylamine (19:1) to a distance of about 15 cm, and air-dry the plate. Examine under ultraviolet light (main wavelength: 254 nm): the spot other than the principal

spot from the sample solution is not more intense than the spot from the standard solution.

Change the Assay to read:

Assay Weigh accurately each about 10 mg of Testosterone Propionate and Testosterone Propionate Reference Standard, previously dried, and dissolve in methanol to make exactly 100 mL. To exactly 5 mL of these solutions add exactly 5 mL of the internal standard solution and methanol to make 20 mL, and use these solutions as the sample solution and the standard solution. Perform the test with 5 μL each of the sample solution and the standard solution as directed under the Liquid Chromatography according to the following conditions, and determine the ratios, Q_T and Q_S, of the peak area of testosterone propionate to that of the internal standard.

$$\text{Amount (mg) of } C_{22}H_{32}O_3 = W_S \times \frac{Q_T}{Q_S}$$

W_S: Amount (mg) of Testosterone Propionate Reference Standard

Internal standard solution—A solution of Progesterone in methanol (9 in 100,000).

Operating conditions—

Detector: An ultraviolet absorption photometer (wavelength: 241 nm).

Column: A stainless steel column 4.6 mm in inside diameter and 15 cm in length, packed with octadecylsilanized silica gel for liquid chromatography (5 μm in particle diameter).

Column temperature: A constant temperature of about 35°C.

Mobile phase: A mixture of acetonitrile and water (7:3).

Flow rate: Adjust the flow rate so that the retention time of testosterone propionate is about 10 minutes.

System suitability—

System performance: When the procedure is run with 5 μL of the standard solution under the above operating conditions, the internal standard and testosterone propionate are eluted in this order with the resolution between these peaks being not less than 9.

System repeatability: When the test is repeated 6 times with 5 μL of the standard solution under the above operating conditions, the relative standard deviation of the ratio of the peak area of testosterone propionate to that of the internal standard is not more than 1.0%.

Testosterone Propionate Injection

プロピオン酸テストステロン注射液

Change the origin/limits of content to read:

Testosterone Propionate Injection is an oily solution for injection.

It contains not less than 92.5% and not more than 107.5% of the labeled amount of testosterone propionate ($C_{22}H_{32}O_3$: 344.49).

Change the Identification to read:

Identification Dissolve the residue obtained as directed in the procedure in the Assay in exactly 20 mL of methanol, and use this solution as the sample solution. Separately, dissolve 1 mg of Testosterone Propionate Reference Standard in 10 mL of methanol, and use this solution as the standard solution. Perform the test with these solutions as directed under the Thin-layer Chromatography. Spot 10 μL each of the sample solution and the standard solution on a plate of silica gel with fluorescent indicator for thin-layer chromatography. Develop the plate with a mixture of chloroform and diethylamine (19:1) to a distance of about 15 cm, and air-dry the plate. Examine under ultraviolet light (main wavelength: 254 nm): the *R*f values of the principal spot with the sample solution and of the spot with the standard solution are not different each other.

Add the following next to Identification:

Actual volume It meets the requirements of Injections.

Foreign insoluble matter Perform the test according to Method 1: it meets the requirements of the Foreign Insoluble Matter Test for Injections.

Sterility Perform the test according to the Membrane filtration method: it meets the requirements of the Sterility Test.

Change the Assay to read:

Assay

(i) Chromatographic tube A glass tube about 1 cm in inside diameter and about 18 cm in length, with a glass filter (G3) at the lower end.

(ii) Chromatographic column To about 2 g of silica gel for liquid chromatography add 5 mL of dichloromethane, and mix gently. Transfer and wash into the chromatographic tube with the aid of dichloromethane, allow to elute the dichloromethane through the column, and put a filter paper on the upper end of the silica gel.

(iii) Standard solution Weigh accurately about 10 mg of Testosterone Propionate Reference Standard, previously dried at 105°C for 4 hours, and dissolve in methanol to make exactly 100 mL. Pipet 5 mL of this solution, add exactly 5 mL of the internal standard solution and methanol to make exactly 20 mL.

(iv) Sample stock solution To exactly a volume of Testosterone Propionate Injection, equivalent to about 20 mg of testosterone propionate ($C_{22}H_{32}O_3$), add dichloromethane to make exactly 20 mL.

(v) Procedure Transfer exactly 2 mL of the sample stock solution into the chromatographic column, and elute to the upper surface of the silica gel. Wash the inner surface of the chromatographic tube with 15 mL of

dichloromethane, elute to the upper surface of the silica gel, and discard the effluent. Elute 15 mL of a mixture of dichloromethane and methanol (39:1), discard the first 5 mL of the effluent, and collect the subsequent effluent. Wash the lower part of the column with a few amount of dichloromethane, combine the washings and the effluent, and evaporate the solvent under reduced pressure. Dissolve the residue so obtained with methanol to make exactly 20 mL. Pipet 5 mL of this solution, add exactly 5 mL of the internal standard solution and methanol to make exactly 20 mL, and use this solution as the sample solution. Perform the test with 5 μL each of the sample solution and the standard solution as directed in the Assay under Testosterone Propionate.

$$\text{Amount (mg) of testosterone propionate } (C_{22}H_{32}O_3) = W_S \times \frac{Q_T}{Q_S} \times 2$$

W_S: Amount (mg) of Testosterone Propionate Reference Standard

Thiamine Hydrochloride

塩酸チアミン

Change the Purity (5) to read:

Purity

(5) Related substances—Dissolve 0.10 g of Thiamine Hydrochloride in 100 mL of the mobile phase, and use this solution as the sample solution. Pipet 1 mL of the sample solution, add the mobile phase to make exactly 100 mL, and use this solution, as the standard solution. Perform the test with exactly 10 μL each of the sample solution and the the standard solution as directed under the Liquid Chromatography according to the following conditions, and determine the area of each peak by the automatic integration method: the total area of the peaks other than thiamine is not larger than the peak area of thiamine from the standard solution.

Operating conditions—

Detector, column, column temperature, mobile phase, and flow rate: Proceed as directed in the operating conditions in the Assay.

Time span of measurement: About 3 times as long as the retention time of thiamine.

System suitability—

Test for required detectability: To exactly 5 mL of the standard solution add water to make exactly 50 mL. Confirm that the peak area of thiamine obtained from 10 μL of this solution is equivalent to 7 to 13% of that of thiamine obtained from 10 μL of the standard solution.

System performance: Proceed as directed in the system suitability in the Assay.

System repeatability: When the test is repeated 6 times with 10 μL of the standard solution under the above operating conditions, the relative standard deviation of the peak area of thiamine is not more than 1.0%.

Change the Assay to read:

Assay Weigh accurately about 0.1 g each of Thiamine Hydrochloride and Thiamine Hydrochloride Reference Standard (previously determine the water content), and dissolve them in the mobile phase to make exactly 50 mL. To 10 mL each of the solutions, accurately measured, add exactly 5 mL each of the internal standard solution, add the mobile phase to make 50 mL, and use these solutions as the sample solution and the standard solution. Perform the test with 10 μL each of the sample solution and the standard solution as directed under the Liquid Chromatography according to the following conditions, and calculate the ratios, Q_T and Q_S, of the peak area of thiamine to that of the internal standard.

$$\text{Amount (mg) of } C_{12}H_{17}ClN_4OS.HCl = W_S \times \frac{Q_T}{Q_S}$$

W_S: Amount (mg) of Thiamine Hydrochloride Reference Standard, calculated on the anhydrous basis

Internal standard solution—A solution methyl benzoate in methanol (1 in 50).

Operating conditions—

Detector: An ultraviolet absorption photometer (wavelength: 254 nm).

Column: A stainless steel column 4.6 mm in inside diameter and 15 cm in length, packed with octadecylsilanized silica gel for liquid chromatography (5 μm in particle diameter).

Column temperature: A constant temperature of about 25°C.

Mobile phase: Dissolve 1.1 g of sodium 1-octanesulfonate in 1000 mL of diluted acetic acid (100) (1 in 100). To 600 mL of this solution add 400 mL of a mixture of methanol and acetonitrile (3:2).

Flow rate: Adjust the flow rate so that the retention time of thiamine is about 12 minutes.

System suitability—

System performance: When the procedure is run with 10 μL of the standard solution under the above operating conditions, thiamine and the internal standard are eluted in this order with the resolution between these peaks being not less than 6.

System repeatability: When the test is repeated 6 times with 10 μL of the standard solution under the above operating conditions, the relative standard deviation of the ratio of the peak area of thiamine to that of the internal standard is not more than 1.0%.

Thiamylal Sodium

チアミラールナトリウム

Change the origin/limits of content to read:

Thiamylal Sodium contains not less than 97.5% and not more than 101.0% of $C_{12}H_{17}N_2NaO_2S$, calculated on the dried basis.

Change the Description to read:

Description Thiamylal Sodium occurs as light yellow crystals or powder.

It is very soluble in water, and freely soluble in ethanol (95).

The pH of a solution of Thiamylal Sodium (1 in 10) is between 10.0 and 11.0.

It is hygroscopic.

It is gradually decomposed by light.

Its solution in ethanol (95) (1 in 10) shows no optical rotation.

Change the Identification to read:

Identification

(1) Determine the absorption spectrum of a solution of Thiamylal Sodium in ethanol (95) (7 in 1,000,000) as directed under the Ultraviolet-visible Spectrophotometry, and compare the spectrum with the Reference Spectrum: both spectra exhibit similar intensities of absorption at the same wavelengths.

(2) Determine the infrared absorption spectrum of Thiamylal Sodium, previously dried, as directed in the potassium bromide disk method under the Infrared Spectrophotometry, and compare the spectrum with the Reference Spectrum: both spectra exhibit similar intensities of absorption at the same wave numbers.

(3) A solution of Thiamylal Sodium (1 in 10) responds to the Qualitative Tests for sodium salt.

Change the Purity (3) to read:

Purity

(3) Related substances—Dissolve 0.10 g of Thiamylal Sodium in 10 mL of ethanol (95), and use this solution as the sample solution. Pipet 1 mL and 3 mL of the sample solution, add ethanol (95) to make exactly 200 mL, and use these solutions as the standard solution (1) and the standard solution (2). Perform the test with these solutions as directed under the Thin-layer Chromatography. Spot 10 μL each of the sample solution, the standard solution (1) and the standard solution (2) on a plate of silica gel for thin-layer chromatography, develop with a mixture of toluene, methanol and ethyl acetate (40:7:3) to a distance of about 12 cm, and air-dry the plate. Allow the plate to stand in iodine vapor for a night: the spot appeared around *R*f 0.1 obtained with the sample solution is not more intense than the spot with the standard solution (2), and the spot other than the principal spot, the spot at origin and the spot mentioned above obtained with the sample solution is not more intense than the spot with the standard solution (1).

Change the Assay to read:

Assay Weigh accurately about 0.25 g of Thiamylal Sodium, dissolve in 50 mL of methanol and 5 mL of dilute hydrochloric acid, and add methanol to make exactly 100 mL. Pipet 10 mL of this solution, and add methanol to make exactly 100 mL. Pipet 5 mL of this solution, add exactly 10 mL of the internal standard solution and the mobile phase to make 200 mL, and use this solution as the sample solution. Separately, weigh accurately about 23 mg of Thiamylal Reference Standard, previously dried at 105°C for 1 hour, dissolve in 50 mL of methanol and 0.5 mL of dilute hydrochloric acid, and add methanol to make exactly 100 mL. Pipet 5 mL of this solution, add exactly 10 mL of the internal standard solution and the mobile phase to make 200 mL, and use this solution as the standard solution. Perform the test with 20 μL each of the sample solution and the standard solution as directed under the Liquid Chromatography according to the following conditions, and determine the ratios, Q_T and Q_S, of the peak area of thiamylal to that of the internal standard.

$$\text{Amount (mg) of } C_{12}H_{17}N_2NaO_2S = W_S \times \frac{Q_T}{Q_S} \times 10 \times 1.0864$$

W_S: Amount (mg) of Thiamylal Reference Standard

Internal standard solution—A solution of phenyl benzoate in methanol (3 in 500).

Operating conditions—

Detector: An ultraviolet absorption photometer (wavelength: 289 nm).

Column: A stainless steel column about 4 mm in inside diameter and about 15 cm in length, packed with octadecylsilanized silica gel for liquid chromatography (5 μm in particle diameter).

Column temperature: A constant temperature of about 25°C.

Mobile phase: A mixture of methanol and 0.05 mol/L acetic acid-sodium acetate buffer solution, pH 4.6 (13:7).

Flow rate: Adjust the flow rate so that the retention time of thiamylal is about 6 minutes.

System suitability—

System performance: When the procedure is run with 20 μL of the standard solution under the above operating conditions, thiamylal and the internal standard are eluted in this order with the resolution between these peaks being not less than 12.

System repeatability: When the test is repeated 6 times with 20 μL of the standard solution under the above operating conditions, the relative standard deviation of the ratio of the peak area of thiamylal to that of the internal standard is not more than 1.0%.

Change to read:

Thiamylal Sodium for Injection

注射用チアミラールナトリウム

Thiamylal Sodium for Injection is a preparation for injection which is dissolved before use.

It contains not less than 93.0% and not more than 107.0% of the labeled amount of thiamylal sodium ($C_{12}H_{17}N_2NaO_2S$: 276.33).

Method of preparation Prepare as directed under Injections, with 100 parts of Thiamylal Sodium and 7 parts of Dried Sodium Carbonate in mass.

Description Thiamylal Sodium for Injection occurs as light yellow crystals, powder or masses.

It is hygroscopic.

It is gradually decomposed by light.

Identification **(1)** To 1.0 g of Thiamylal Sodium for Injection add 20 mL of ethanol (95), shake vigorously, and filter. Dissolve the precipitate so obtained in 1 mL of water, and add 1 mL of barium chloride TS: a white precipitate is produced. Centrifuge this solution, take off the supernatant liquid, and to the precipitate add dilute hydrochloric acid dropwise: the precipitate dissolves with effervescence.

(2) To 50 mg of Thiamylal Sodium for Injection add 100 mL of ethanol (95), shake vigorously, and filter. To 3 mL of the filtrate add ethanol (95) to make 200 mL. Determine the absorption spectrum of this solution as directed under the Ultraviolet-visible Spectrophotometry: it exhibits maxima between 236 nm and 240 nm, and between 287 nm and 291 nm.

pH The pH of a solution obtained by dissolving 1.0 g of Thiamylal Sodium for Injection in 40 mL of water is between 10.5 and 11.5.

Purity Related substances—To 0.10 g of Thiamylal Sodium for Injection add 10 mL of ethanol (95), shake vigorously, filter, and use the filtrate as the sample solution. Proceed as diected in the Purity (3) under Thiamylal Sodium.

Bacterial endotoxins Less than 1.0 EU/mg.

Mass variation It meets the requirements of the Mass Variation Test.

Foreign insoluble matter Perform the test according to Method 2: it meets the requirements of the Foreign Insoluble Matter Test for Injections.

Insoluble particulate matter Perform the test according to Method 1: it meets the requirements of the Insoluble Particulate Matter Test for Injections.

Sterility Perform the test according to the Membrane filtration method: it meets the requirements of the Sterility Test.

Assay Open carefully 10 containers of Thiamylal Sodium for Injection, dissolve the contents with water, wash out the inside of each container with water, combine them, and add water to make exactly V mL so that each mL contains about 5 mg of thiamylal sodium ($C_{12}H_{17}N_2NaO_2S$). Pipet 5 mL of this solution, and add 0.5 mL of dilute hydrochloric acid and methanol to make exactly 100 mL. Pipet 5 mL of this solution, add exactly 10 mL of the internal standard solution and the mobile phase to make 200 mL, and use this solution as the sample solution. Proceed the test with the sample solution as directed in the Assay under Thiamylal Sodium.

Amount (mg) of thiamylal sodium ($C_{12}H_{17}N_2NaO_2S$) in 1 container

$$= W_S \times \frac{Q_T}{Q_S} \times \frac{V}{50} \times 1.0864$$

W_S: Amount (mg) of Thiamylal Reference Standard

Internal standard solution—A solution of phenyl benzoate in methanol (3 in 500).

Containers and storage Containers—Hermetic containers.

Storage—Light-resistant.

Add the following:

Tiaramide Hydrochloride Tablets

塩酸チアラミド錠

Tiaramide Hydrochloride Tablets contain not less than 95.0% and not more than 105.0% of the labeled amount of tiaramide ($C_{15}H_{18}ClN_3O_3S$: 355.84).

Method of preparation Prepare as directed under Tablets, with Tiaramide Hydrochloride.

Identification **(1)** Determine the absorption spectrum of the sample solution obtained in the Assay as directed under the Ultraviolet-visible Spectrophotometry: it exhibits maxima between 285 nm and 289 nm, and between 292 nm and 296 nm.

(2) To a quantity of powdered Tiaramide Hydrochloride Tablets, equivalent to 0.1 g of tiaramide according to the labeled amount, add 10 mL of diluted ethanol (99.5) (7 in 10), shake well, filter, and use the filtrate as the sample solution. Separately, dissolve 0.11 g of tiaramide hydrochloride for assay in 10 mL of diluted ethanol (99.5) (7 in 10), and use this solution as the standard solution. Perform the test with these solutions as directed under the Thin-layer Chromatography. Spot 20 μL each of the sample solution and the standard solution on a plate of silica gel for thin-layer chromatography, develop with a mixture of 1-butamol, water and acetic acid (100) (4:2:1) to a distance of about 10 cm, and dry the plate at 100°C for 30 minutes. Spray evenly Dragendorff's TS for spraying followed by diluted nitric acid (1 in 50) on the plate: the principal spot obtained with the sample solution and the spot with the standard solution are yellow-red in color and have the same Rf value.

Content uniformity Perform the test according to the following method: it meets the requirements of the Content uniformity Test.

To 1 tablet of Tiaramide Hydrochloride Tablets add a volume of 0.1 mol/L hydrochloric acid TS, equivalent to 3/5 volume of V mL which makes a solution so that each mL contains about 1 mg of tiaramide ($C_{15}H_{18}ClN_3O_3S$) according to the labeled amount, shake for 60 minutes, then add 0.1 mol/L hydrochloric acid TS to make exactly V mL, and filter. Discard the first 20 mL of the filtrate, pipet 5 mL of the subsequent filtrate, add water to make exactly 100 mL, and use this solution as the sample solution. Separately, weigh accurately about 55 mg of tiaramide hydrochloride for assay, previously dried at 105°C for 3 hours, and dissolve in 0.1 mol/L hydrochloric acid TS to make exactly 50 mL. Pipet 5 mL of this solution, add water to make exactly 100 mL, and use this solution as the standard solution. Determine the absorbances, A_T and A_S, of the sample solution and the standard solution at 294 nm as directed under the Ultraviolet-visible Spectrophotometry.

$$\text{Amount (mg) of tiaramide } (C_{15}H_{18}ClN_3O_3S) = W_S \times \frac{A_T}{A_S} \times \frac{V}{50} \times 0.907$$

W_S: Amount (mg) of tiaramide hydrochloride for assay

Dissolution Perform the test with 1 tablet of Tiaramide Hydrochloride Tablets at 50 revolutions per minute according to Method 2 under the Dissolution Test, using 900 mL of water as the dissolution medium. Withdraw 20 mL or more of the dissolution medium 15 minutes after starting the test for a 50-mg tablet or 30 minutes after starting the test for a 100-mg tablet, and filter through a membrane filter with pore size of not more than 0.5 μm. Discard the first 10 mL of the filtrate, pipet V mL of the subsequent filtrate, add water to make exactly V' mL so that each mL contains about 56 μg of tiaramide ($C_{15}H_{18}ClN_3O_3S$) according to the labeled amount, and use this solution as the sample solution. Separately, weigh accurately about 15 mg of tiaramide hydrochloride for assay, previously dried at 105°C for 3 hours, and dissolve in water to make exactly 50 mL. Pipet 5 mL of this solution, add water to make exactly 25 mL, and use this solution as the standard solution. Determine the absorbances, A_T and A_S, at 294 nm of the sample solution and the standard solution as directed under the Ultraviolet-visible Spectrophotometry: the dissolution rates for a 50-mg tablet in 15 minutes and a 100-mg tablet in 30 minutes are not less than 80%, respectively.

$$\text{Dissolution rate (\%) with respect to the labeled amount of tiaramide } (C_{15}H_{18}ClN_3O_3S) = W_S \times \frac{A_T}{A_S} \times \frac{V'}{V} \times \frac{1}{C} \times 360 \times 0.907$$

W_S: Amount (mg) of tiaramide hydrochloride for assay
C: Labeled amount (mg) of tiaramide ($C_{15}H_{18}ClN_3O_3S$) in 1 tablet

Assay Weigh accurately the mass of more than 20 Tiaramide Hydrochloride Tablets, and powder. Weigh accurately an amount of the powder, equivalent to about 0.1 g of tiaramide ($C_{15}H_{18}ClN_3O_3S$), add 60 mL of 0.1 mol/L hydrochloric acid TS, shake for 30 minutes, add 0.1 mol/L hydrochloric acid TS to make exactly 100 mL, and filter. Discard the first 20 mL of the filtrate, pipet 5 mL of the subsequent filtrate, add water to make exactly 100 mL, and use this solution as the sample solution. Separately, weigh accurately about 0.11 g of tiaramide hydrochloride for assay, previously dried at 105°C for 3 hours, and dissolve in 0.1 mol/L hydrochloric acid TS to make exactly 100 mL. Pipet 5 mL of this solution, add water to make exactly 100 mL, and use this solution as the standard solution. Determine the absorbances, A_T and A_S, of the sample solution and the standard solution at 294 nm as directed under the Ultraviolet-visible Spectrophotometry.

$$\text{Amount (mg) of tiaramide } (C_{15}H_{18}ClN_3O_3S) = W_S \times \frac{A_T}{A_S} \times 0.907$$

W_S: Amount (mg) of tiaramide hydrochloride for assay

Containers and storage Containers—Tight containers.

Tinidazole

チニダゾール

Change the origin/limits of content to read:

Tinidazole, when dried, contains not less than 98.5% and not more than 101.0% of $C_8H_{13}N_3O_4S$.

Change the Description to read:

Description Tinidazole occurs as a light yellow, crystalline powder.

It is soluble in acetic anhydride and in acetone, sparingly soluble in methanol, slightly soluble in ethanol (99.5), and very slightly soluble in water.

Change the Identification to read:

Identification

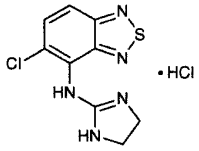
(1) Determine the absorption spectrum of a solution of Tinidazole in methanol (1 in 50,000) as directed under the Ultraviolet-visible Spectrophotometry, and compare the spectrum with the Reference Spectrum: both spectra exhibit similar intensities of absorption at the same wavelengths.

(2) Determine the infrared absorption spectrum of Tinidazole as directed in the potassium bromide disk method under the Infrared Spectrophotometry, and compare the spectrum with the Reference Spectrum: both spectra exhibit similar intensities of absorption at the same wave numbers.

Add the following:

Tizanidine Hydrochloride

塩酸チザニジン



$C_9H_8ClN_5S.HCl$: 290.17

5-Chloro-4-[(4,5-dihydro-1*H*-imidazol-2-yl)amino]benzo[*c*][1,2,5]thiadiazole monohydrochloride [*64461-82-1*]

Tizanidine Hydrochloride, when dried, contains not less than 99.0% and not more than 101.0% of $C_9H_8ClN_5S.HCl$.

Description Tizanidine Hydrochloride occurs as a white to light yellowish white crystalline powder.

It is soluble in water, slightly soluble in ethanol (99.5), and practically insoluble in acetic anhydride and in acetic acid (100).

Melting point: about 290°C (with decomposition).

Identification **(1)** Determine the absorption spectrum of a solution of Tizanidine Hydrochloride in diluted 1 mol/L ammonia TS (1 in 10) (1 in 125,000) as directed under the Ultraviolet-visible Spectrophotometry, and compare the spectrum with the Reference Spectrum: both spectra exhibit similar intensities of absorption at the same wavelengths.

(2) Determine the infrared absorption spectrum of Tizanidine Hydrochloride as directed in the potassium chloride disk method under the Infrared Spectrophotometry, and compare the spectrum with the Reference Spectrum: both spectra exhibit similar intensities of absorption at the same wave numbers.

(3) A solution of Tizanidine Hydrochloride (1 in 50) responds to the Qualitative Tests for chloride.

Purity **(1)** Heavy metals—Proceed with 1.0 g of Tizanidine Hydrochloride according to Method 3, and perform the test. Prepare the control solution with 2.0 mL of Standard Lead Solution (not more than 20 ppm).

(2) Related substances—Dissolve 60 mg of Tizanidine Hydrochloride in 10 mL of a mixture of water and acetonitrile (17:3), and use this solution as the sample solution. Pipet 1 mL of the sample solution, add the mixture of water and acetonitrile (17:3) to make exactly 200 mL, and use this solution as the standard solution. Perform the test with exactly 10 μL of the sample solution and the standard solution as directed under the Liquid Chromatography according to the following conditions, and determine each peak area by the automatic integration method: the area of the peak other than tizanidine is not larger than 1/5 times the peak area of tizanidine with the standard solution.

Operating conditions—

Detector: An ultraviolet absorption photometer (wavelength: 230 nm for about 3 minutes after sample injection and 318 nm subsequently).

Column: A stainless steel column 4.6 mm in inside diameter and 12.5 cm in length, packed with octadecylsilanized silica gel for liquid chromatography (5 μm in particle diameter).

Column temperature: A constant temperature of about 25°C.

Mobile phase A: A mixture of water and formic acid (200:1), adjusted to pH 8.5 with ammonia water (28).

Mobile phase B: A mixture of acetonitrile and the mobile phase A (4:1).

Flowing of the mobile phase: Control the gradient by mixing the mobile phases A and B as directed in the following table.

Time after injection of sample (min)	Mobile phase A (%)	Mobile phase B (%)
0 – 10	81 → 68	19 → 32
10 – 13	68	32
13 – 26	68 → 10	32 → 90
26 – 28	10	90

Flow rate: Adjust the flow rate so that the retention time of tizanidine is about 7 minute.

Time span of measurement: About 4 times as long as the retention time of tizanidine after the solvent peak.

System suitability—

Test for required detectability: Measure exactly 2 mL of the standard solution, and add the mixture of water and acetonitrile (17:3) to make exactly 10 mL. Confirm that the peak area of tizanidine obtained with 10 μL of this solution is equivalent to 14 to 26% of that with 10 μL of the standard solution.

System performance: Dissolve 2 mg each of Tizanidine Hydrochloride and *p*-toluenesulfonic acid monohydrate in 100 mL of the mixture of water and acetonitrile (17:3). When the procedure is run with 10 μL of this solution under the above operating conditions, *p*-toluenesulfonic acid and tizanidine are eluted in this order with the resolution between these peaks being not less than 10.

System repeatability: When the test is repeated 6 times with 10 μL of the standard solution under the above operating conditions, the relative standard deviation of the peak area of tizanidine is not more than 2.0%.

Loss on drying Not more than 0.2% (1 g, 105°C, 3 hours).

Residue on ignition Not more than 0.1% (1 g).

Assay Weigh accurately about 0.2 g of Tizanidine Hydrochloride, previously dried, dissolve in 60 mL of a mixture of acetic anhydride and acetic acid (100) (7:3) with the aid of warming. After cooling, titrate with 0.1 mol/L perchloric acid VS (potentiometric titration). Perform a blank determination in the same manner, and make any necessary correction.

Each mL of 0.1 mol/L perchloric acid VS
= 29.02 mg of $C_9H_8ClN_5S.HCl$

Containers and storage Containers—Well-closed containers.

Tranexamic Acid

トラネキサム酸

Change the origin/limits of content to read:

Tranexamic Acid, when dried, contains not less than 98.0% and not more than 101.0% of $C_8H_{15}NO_2$.

Change the Description to read:

Description Tranexamic Acid occurs as white crystals or crystalline powder.

It is freely soluble in water, and practically insoluble in ethanol (99.5).

Change the Identification to read:

Identification Determine the infrared absorption spectrum of Tranexamic Acid as directed in the potassium bromide disk method under the Infrared Spectrophotometry, and compare the spectrum with the Reference Spectrum or the spectrum of Tranexamic Acid Reference Standard: both spectra exhibit similar intensities of absorption at the same wave numbers.

Add the following next to Identification:

pH The pH of a solution prepared by dissolving 1.0 g of Tranexamic Acid in 20 mL of water is between 7.0 and 8.0.

Change the Purity to read:

Purity **(1)** Clarity and color of solution—Dissolve 1.0 g of Tranexamic Acid in 10 mL of water: the solution is clear and colorless.

(2) Chloride—Perform the test with 1.0 g of Tranexamic Acid. Prepare the control solution with 0.40 mL of 0.01 mol/L hydrochloric acid VS (not more than 0.014%).

(3) Heavy metals—Dissolve 2.0 g of Tranexamic Acid in water to make 20 mL, and use this solution as the sample stock solution. To 12 mL of the sample stock solution add 2 mL of hydrochloric acid-ammonium acetate buffer solution, pH 3.5, mix, add 1.2 mL of thioacetamide TS , mix immediately, and use this solution as the sample solution. Separately, proceed in the same manner as above with a mixture of 1 mL of Standard Lead Solution, 2 mL of the sample stock solution and 9 mL of water, and use the solution so obtained as the standard solution. Separately, proceed in the same manner with a mixture of 10 mL of water and 2 mL of the sample stock solution, and use the solution so obtained as the control solution. Conform that the color of the standard solution is slightly darker than that of the control solution. Compare the sample solution and the standard solution 2 minutes after they are prepared: the color of the sample solution is not more intense than that of the standard solution (not more than 10 ppm).

(4) Arsenic—Prepare the test solution by dissolving 1.0 g of Tranexamic Acid in 10 mL of water, and perform the test (not more than 2 ppm).

(5) Related substances—Dissolve 0.20 g of Tranexamic Acid in water to make exactly 20 mL, and use this solution as the sample solution. Pipet 5 mL of the sample solution, and add water to make exactly 100 mL. Pipet 1 mL of this solution, add water to make exactly 10 mL, and use this solution as the standard solution. Perform the test with exactly 20 μL each of the sample solution and the standard solution as directed under the Liquid Chromatography according to the following conditions, and determine each peak area by the automatic integration method: the area multiplied by relative response factor 1.2 of the peak, having the relative retention time of about 1.5 with respect to tranexamic acid, is not more than 2/5 of the peak area of tranexamic acid from the standard solution, and the area of the peak, having the relative retention time of about 2.1 with respect to tranexamic acid, is not more than 1/5 of the peak area of tranexamic acid from the standard solution. The area of each peak other than tranexamic acid and other than the peaks mentioned above is not more than 1/5 of the peak area of tranexamic acid from the standard solution. For this comparison, use the area of the peaks, having the relative retention time of about 1.1 and about 1.3, after multiplying by their relative response factors 0.005 and 0.006, respectively. The total area of the peaks other than tranexamic acid is not more than the peak area of tranexamic acid from the standard solution.

Operating conditions—

Detector, column, column temperature, mobile phase, and flow rate: Proceed as directed in the operating conditions in the Assay.

Time span of measurement: About 3 times as long as the retention time of tranexamic acid after the solvent peak.

System suitability—

Test for required detectability: To exactly 5 mL of the standard solution add water to make exactly 25 mL. Confirm that the peak area of tranexamic acid obtained from 20 μL of this solution is equivalent to 14 to 26% of that from 20 μL of the standard solution.

System performance: Proceed as directed in the system suitability in the Assay.

System repeatability: When the test is repeated 6 times with 20 μL of the standard solution under the above operating conditions, the relative standard deviation of the peak area of tranexamic acid is not more than 7%.

Change the Assay to read:

Assay Weigh accurately about 50 mg each of Tranexamic Acid and Tranexamic Acid Reference Standard, previously dried, dissolve in water to make exactly 25 mL, and use these solutions as the sample solution and the standard solution.

Perform the test with exactly 20 μL each of the sample solution and the standard solution as directed under the Liquid Chromatography according to the following conditions, and determine the peak areas, A_T and A_S, of tranexamic acid.

$$\text{Amount (mg) of } C_8H_{15}NO_2 = W_S \times \frac{A_T}{A_S}$$

W_S: Amount (mg) of Tranexamic Acid Reference Standard

Operating conditions—

Detector: An ultraviolet absorption photometer (wavelength: 220 nm).

Column: A stainless steel column 6.0 mm in inside diameter and 25 cm in length, packed with octadecylsilanized silica gel for liquid chromatography (5 μm in particle diameter).

Column temperature: A constant temperature of about 25°C.

Mobile phase: Dissolve 11.0 g of sodium dihydrogen phosphate in 500 mL of water, and add 5 mL of triethylamine and 1.4 g of sodium lauryl sulfate. Adjust the pH to 2.5 with phosphoric acid or diluted phosphoric acid (1 in 10), add water to make 600 mL, and add 400 mL of methanol.

Flow rate: Adjust the flow rate so that the retention time of tranexamic acid is about 20 minutes.

System suitability—

System performance: To 5 mL of the standard solution add 1 mL of a solution of 4-(aminomethyl)benzoic acid (1 in 10,000) and water to make 50 mL. When the procedure is run with 20 μL of this solution under the above operating conditions, tranexamic acid and 4-(aminomethyl)benzoic acid are eluted in this order with the resolution between these peaks being not less than 5.

System repeatability: When the test is repeated 6 times with 20 μL of the standard solution under the above operating conditions, the relative standard deviation of the peak area of tranexamic acid is not more than 0.6%.

Add the following:

Tranexamic Acid Capsules

トラネキサム酸カプセル

Tranexamic Acid Capsules contain not less than 95.0% and not more than 105.0% of the labeled amount of tranexamic acid ($C_8H_{15}NO_2$: 157.21).

Method of preparation Prepare as directed under Capsules, with Tranexamic Acid.

Identification Take an amount of powdered contents of Tranexamic Acid Capsules, equivalent to 0.5 g of Tranexamic Acid according to the labeled amount, add 50 mL of water, shake well, and filter. To 5 mL of the filtrate add 1 mL of ninhydrin TS, and heat for 3 minutes: a dark purple color develops.

Mass variation It meets the requirements of the Mass Variation Test.

Dissolution Being specified separately.

Assay Weigh accurately the mass of the contents of not less than 20 Tranexamic Acid Capsules, and powder. Weigh accurately an amount of the powder, equivalent to about 0.1 g of tranexamic acid ($C_8H_{15}NO_2$), add 30 mL of water, shake well, and add water to make exactly 50 mL. Centrifuge, filter the supernatant liquid through a membrane filter with pore size of not more than 0.45 μm, discard the first 10 mL of the filtrate, and use the subsequent filtrate as the sample solution. Separately, weigh accurately about 50 mg of Tranexamic Acid Reference Standard, previously dried at 105°C for 2 hours, dissolve in water to make exactly 25 mL, and use this solution as the standard solution. Perform the test with exactly 30 μL each of the sample solution and the standard solution as directed under the Liquid Chromatography according to the following conditions, and determine the peak areas, A_T and A_S, of tranexamic acid.

$$\text{Amount (mg) of tranexamic acid } (C_8H_{15}NO_2) = W_S \times \frac{A_T}{A_S} \times 2$$

W_S: Amount (mg) of Tranexamic Acid Reference Standard

Operating conditions—

Detector, column, and mobile phase: Proceed as directed in the operating conditions in the Assay under Tranexamic Acid.

Column temperature: A constant temperature of about 35°C.

Flow rate: Adjust the flow rate so that the retention time of tranexamic acid is about 16 minutes.

System suitability—

System performance: To 5 mL of the standard solution add 1 mL of a solution of 4-(aminomethyl)benzoic acid (1 in 10,000) and water to make 50 mL. When the procedure is run with 30 μL of this solution under the above operating conditions, tranexamic acid and 4-(aminomethyl)benzoic acid are eluted in this order with the resolution between these peaks being not less than 3.

System repeatability: When the test is repeated 6 times with 30 μL of the standard solution under the above operating conditions, the relative standard deviation of the peak area of tranexamic acid is not more than 1.0%.

Containers and storage Containers—Tight containers.

Add the following:

Tranexamic Acid Injection

トラネキサム酸注射液

Tranexamic Acid Injection is an aqueous injection. It contains not less than 95.0% and not more than 105.0% of the labeled amount of tranexamic acid ($C_8H_{15}NO_2$: 157.21).

Method of preparation Prepare as directed under Injections, with Tranexamic Acid.

Description Tranexamic Acid Injection is a clear and colorless liquid.

Identification To a volume of Tranexamic Acid Injection, equivalent to 50 mg of Tranexamic Acid according to the labeled amount, add water to make 5 mL, add 1 mL of ninhydrin TS, and heat: a dark purple color develops.

pH 7.0 – 8.0

Bacterial endotoxins Not more than 0.12 EU/mg.

Actual volume It meets the requirements of Injections.

Foreign insoluble matter Perform the test according to Method 1: it meets the requirements of the Foreign Insoluble Matter Test for Injections.

Insoluble particulate matter Perform the test according to Method 1: it meets the requirements of the Insoluble Particulate Matter Test for Injections.

Sterility Perform the test according to the Membrane filtration method: it meets the requirements of the Sterility Test.

Assay Take accurately a volume of Tranexamic Acid Injection, equivalent to about 0.1 g of tranexamic acid ($C_8H_{15}NO_2$), add water to make exactly 50 mL, and use this solution as the sample solution. Separately, weigh accurately about 50 mg of Tranexamic Acid Reference Standard, previously dried at 105°C for 2 hours, dissolve in water to make exactly 25 mL, and use this solution as the standard solution. Perform the test with exactly 30 μL each of the sample solution and the standard solution as directed under the Liquid Chromatography according to the following conditions, and determine the peak areas, A_T and A_S, of tranexamic acid.

$$\text{Amount (mg) of tranexamic acid } (C_8H_{15}NO_2) = W_S \times \frac{A_T}{A_S}$$

W_S: Amount (mg) of Tranexamic Acid Reference Standard

Operating conditions—

Detector, column, and mobile phase: Proceed as directed in the operating conditions in the Assay under Tranexamic Acid.

Column temperature: A constant temperature of about 35°C.

Flow rate: Adjust the flow rate so that the retention time of tranexamic acid is about 16 minutes.

System suitability—

System performance: To 5 mL of the standard solution add 1 mL of a solution of 4-(aminomethyl)benzoic acid (1 in 10,000) and water to make 50 mL. When the procedure is run with 30 μL of this solution under the above operating conditions, tranexamic acid and 4-(aminomethyl)benzoic acid are eluted in this order with the resolution between these peaks being not less than 3.

System repeatability: When the test is repeated 6 times with 30 μL of the standard solution under the above operating conditions, the relative standard deviation of the peak area of tranexamic acid is not more than 1.0%.

Containers and storage Containers—Hermetic containers.

Add the following:

Tranexamic Acid Tablets

トラネキサム酸錠

Tranexamic Acid Tablets contain not less than 95.0% and not more than 105.0% of the labeled amount of tranexamic acid ($C_8H_{15}NO_2$: 157.21).

Method of preparation Prepare as directed under Tablets, with Tranexamic Acid.

Identification To an amount of powdered Tranexamic Acid Tablets, equivalent to 0.5 g of Tranexamic Acid according to the labeled amount, add 50 mL of water, shake well, and filter. To 5 mL of the filtrate add 1 mL of ninhydrin TS, and heat for 3 minutes: a dark purple color develops.

Mass variation It meets the requirements of the Mass Variation Test.

Dissolution Being specified separately.

Assay Weigh accurately the mass of not less than 20 Tranexamic Acid Tablets, and powder. Weigh accurately a quantity of the powder, equivalent to about 5 g of tranexamic acid ($C_8H_{15}NO_2$), add 150 mL of water, disintegrate the tablets completely with the aid of ultrasonic waves, and add water to make exactly 200 mL. Centrifuge, pipet 4 mL of the supernatant liquid, and add water to make exactly 50 mL. Filter through a membrane filter with pore size of not more than 0.45 μm, discard the first 10 mL of the filtrate, and use the subsequent filtrate as the sample solution. Separately, weigh accurately about 50 mg of Tranexamic Acid Reference Standard, previously dried at 105°C for 2 hours, dissolve in water to make exactly 25 mL, and use this solution as the standard solution. Perform the test with exactly 30 μL each of the sample solution and the standard solution as directed under the Liquid Chromatography according to the following conditions, and determine the peak areas, A_T and A_S, of

tranexamic acid.

$$\text{Amount (mg) of tranexamic acid } (C_8H_{15}NO_2) = W_S \times \frac{A_T}{A_S} \times 100$$

W_S: Amount (mg) of Tranexamic Acid Reference Standard

Operating conditions—

Detector, column, and mobile phase: Proceed as directed in the operating conditions in the Assay under Tranexamic Acid.

Column temperature: A constant temperature of about 35°C.

Flow rate: Adjust the flow rate so that the retention time of tranexamic acid is about 16 minutes.

System suitability—

System performance: To 5 mL of the standard solution add 1 mL of a solution of 4-(aminomethyl)benzoic acid (1 in 10,000) and water to make 50 mL. When the procedure is run with 30 μL of this solution under the above operating conditions, tranexamic acid and 4-(aminomethyl)benzoic acid are eluted in this order with the resolution between these peaks being not less than 3.

System repeatability: When the test is repeated 6 times with 30 μL of the standard solution under the above operating conditions, the relative standard deviation of the peak area of tranexamic acid is not more than 1.0%.

Containers and storage Containers—Tight containers.

Trichlormethiazide

トリクロルメチアジド

Change the origin/limits of the content to read:

Trichlormethiazide, when dried, contains not less than 97.5% and not more than 102.0% of $C_8H_8Cl_3N_3O_4S_2$.

Change the Description to read:

Description Trichlormethiazide occurs as a white powder.

It is freely soluble in *N*,*N*-dimethylformamide and in acetone, slightly soluble in acetonitrile and in ethanol (95), and practically insoluble in water.

A solution of Trichlormethiazide in acetone (1 in 50) shows no optical rotation.

Melting point: about 270°C (with decomposition).

Change the Identification to read:

Identification

(1) Determine the absorption spectrum of a solution of Trichlormethiazide in ethanol (95) (3 in 250,000) as directed under the Ultraviolet-visible Spectrophotometry, and compare the spectrum with the Reference Spectrum or the spectrum of a solution of Trichlormethiazide Reference Standard prepared in the same manner as the sample solution: both spectra exhibit similar intensities of absorption at the same wavelengths.

(2) Determine the infrared absorption spectrum of Trichlormethiazide as directed in the potassium bromide disk method under the Infrared Spectrophotometry, and compare the spectrum with the Reference Spectrum or the spectrum of Trichlormethiazide Reference Standard: both spectra exhibit similar intensities of absorption at the same wave numbers.

(3) Perform the test with Trichlormethiazide as directed under the Flame Coloration Test (2): a green color appears.

Change the Purity (4) and (5) to read:

(4) Arsenic—Prepare the test solution with 0.6 g of Trichlormethiazide according to Method 5, using 20 mL of *N*,*N*-dimethylformamide, and perform the test (not more than 3.3 ppm).

(5) Related substances—Dissolve 25 mg of Trichlormethiazide in 50 mL of acetonitrile, and use the solution as the sample solution. Perform the test with 10 μL of the sample solution as directed under the Liquid Chromatography according to the following conditions, determine each peak area by the automatic integration method, and calculate the amount of related substances by the area percentage method: the amount of 4-amino-6-chlorobenzene-1,3-disulfonamide, having the relative retention time of about 0.3 with respect to trichlormethiazide, is not more than 2.0%, and the total amount of the related substances is not more than 2.5%.

Operating conditions—

Detector: An ultraviolet absorption photometer (wavelength: 268 nm).

Column: A stainless steel column 4.6 mm in inside diameter and 15 cm in length, packed with phenylsilanized silica gel for liquid chromatography (5 μm in particle diameter).

Column temperature: A constant temperature of about 25°C.

Mobile phase A: A mixture of diluted phosphoric acid (1 in 1000) and acetonitrile (3:1).

Mobile phase B: A mixture of acetonitrile and diluted phosphoric acid (1 in 1000) (3:1).

Flowing of the mobile phase: Control the gradient by mixing the mobile phases A and B as directed in the following table.

Time after injection of sample (min)	Mobile phase A (vol%)	Mobile phase B (vol%)
0 – 10	100	0
10 – 20	100 → 0	0 → 100

Flow rate: 1.5 mL per minute

Time span of measurement: About 2.5 times as long as the retention time of trichlormethiazide after the solvent peak.

System suitability—

Test for required detectability: To exactly 1 mL of the

sample solution add acetonitrile to make exactly 50 mL, and use this solution as the solution for system suitability test. Pipet 1 mL of the solution, and add acetonitrile to make exactly 20 mL. Confirm that the peak area of trichlormethiazide obtained from 10 μL of this solution is equivalent to 3.5 to 6.5% of that of trichlormethiazide obtained from 10 μL of the solution for system suitability test.

System performance: To 5 mL of the solution for system suitability test add 5 mL of water, and warm in a water bath at 60°C for 30 minutes. When the procedure is run with 10 μL of this solution, after cooling, under the above operating conditions, 4-amino-6-chlorobenzene-1,3-disulfonamide and trichlormethiazide are eluted in this order, the relative retention time of 4-amino-6-chlorobenzene-1,3-disulfonamide with respect to trichlormethiazide is about 0.3, and the number of theoretical plates and the symmetry factor of the peak of trichlormethiazide are not less than 5000 and not more than 1.2, respectively.

System repeatability: When the test is repeated 3 times with 10 μL of the solution for system suitability test under the above operating conditions, the relative standard deviation of the peak area of trichlormethiazide is not more than 2.0%.

Change the Assay to read:

Assay Weigh accurately about 25 mg of Trichlormethiazide and Trichlormethiazide Reference Standard, previously dried, and dissolve separately in exactly 20 mL of the internal standard solution. To 1 mL of these solutions add acetonitrile to make 20 mL, and use these solutions as the sample solution and the standard solution. Perform the test with 10 μL each of the sample solution and the standard solution as directed under the Liquid Chromatography according to the following conditions, and determine the ratios, Q_T and Q_S, of the peak area of trichlormethiazide to that of the internal standard.

$$\text{Amount (mg) of } C_8H_8Cl_3N_3O_4S_2 = W_S \times \frac{Q_T}{Q_S}$$

W_S: Amount (mg) of Trichlormethiazide Reference Standard

Internal standard solution—A solution of 3-nitrophenol in acetonitrile (1 in 800).

Operating conditions—

Detector: An ultraviolet absorption photometer (wavelength: 268 nm).

Column: A stainless steel column 4.6 mm in inside diameter and 15 cm in length, packed with phenylsilanized silica gel for liquid chromatography (5 μm in particle diameter).

Column temperature: A constant temperature of about 25°C.

Mobile phase: A mixture of diluted phosphoric acid (1 in 1000) and acetonitrile (3:1).

Flow rate: Adjust the flow rate so that the retention time of trichlormethiazide is about 8 minutes.

System suitability—

System performance: When the procedure is run with 10 μL of the standard solution under the above operating conditions, the internal standard and trichlormethiazide are eluted in this order with the resolution between these peaks being not less than 2.0.

System repeatability: When the test is repeated 6 times with 10 μL of the standard solution under the above operating conditions, the relative standard deviation of the ratio of the peak area of trichlormethiazide to that of the internal standard is not more than 1.0%.

Add the following:

Trichlormethiazide Tablets

トリクロルメチアジド錠

Trichlormethiazide Tablets contain not less than 93.0% and not more than 107.0% of the labeled amount of trichlormethiazide ($C_8H_8Cl_3N_3O_4S_2$: 380.66).

Method of preparation Prepare as directed under Tablets, with Trichlormethiazide.

Identification To an amount of pulverized Trichlormethiazide Tablets, equivalent to 4 mg of Trichlormethiazide according to the labeled amount, add 10 mL of acetone, shake vigorously for 5 minutes, centrifuge, and use the supernatant liquid as the sample solution. Separately, dissolve 4 mg of Trichlormethiazide Reference Standard in 10 mL of acetone, and use this solution as the standard solution. Perform the test with these solutions as directed under the Thin-layer Chromatography. Spot 5 μL each of the sample solution and the standard solution on a plate of silica gel with fluorescent indicator for thin-layer chromatography. Develop the plate with a mixture of ethyl acetate, hexane and methanol (10:4:1) to a distance of about 10 cm, and air-dry the plate. Examine under ultraviolet light (main wavelength: 254 nm): the principal spots from the sample solution and the standard solution show the same *R*f value.

Purity Related substances—Pulverize a suitable amount of Trichlormethiazide Tablets in an agate mortar. Take an amount of the powder, equivalent to 10 mg of Trichlormethiazide according to the labeled amount, add 20 mL of acetonitrile, shake vigorously for 15 minutes, centrifuge, and use the supernatant liquid as the sample solution. Perform the test with 10 μL of the sample solution as directed under the Liquid Chromatography according to the following conditions, determine each peak area by the automatic integration method, and calculate the amount of each related substance by the area percentage method: the amount of 4-amino-6-chlorobenzene-1,3-disulfoneamide, having the relative retention time of about 0.3 with respect to trichlormethiazide, is not more than 4.0%, and the total amount of the peaks other than trichlormethiazide is not more than 5.0%.

Operating conditions—

Detector: An ultraviolet absorption photometer (wavelength: 268 nm).

Column: A stainless steel column 4.6 mm in inside diameter and 15 cm in length, packed with phenylsilanized silica gel for liquid chromatography (5 μm in particle diameter).

Column temperature: A constant temperature of about 25°C.

Mobile phase A: A mixture of diluted phosphoric acid (1 in 1000) and acetonitrile (3:1).

Mobile phase B: A mixture of acetonitrile and diluted phosphoric acid (1 in 1000) (3:1).

Flowing of the mobile phase: Control the gradient by mixing the mobile phases A and B as directed in the following table.

Time after injection of sample (min)	Mobile phase A (%)	Mobile phase B (%)
0 – 10	100	0
10 – 20	100 → 0	0 → 100

Flow rate: 1.5 mL/minute

Time span of measurement: About 2.5 times as long as the retention time of trichlormethiazide after the solvent peak.

System suitability—

Test for required detectability: Dissolve 25 mg of Trichlormethiazide in 50 mL of acetonitrile. Pipet 1 mL of this solution, add acetonitrile to make exactly 50 mL, and use this solution as the solution for system suitability test. Pipet 1 mL of the solution for system suitability test, and add acetonitrile to make exactly 20 mL. Confirm that the peak area of trichlormethiazide obtained from 10 μL of this solution is equivalent to 3.5 to 6.5% of that obtained from 10 μL of the solution for system suitability test.

System performance: To 5 mL of the solution for system suitability test add 5 mL of water, and warm in a water bath of 60°C for 30 minutes. When the procedure is run with 10 μL of this solution, after cooling, under the above operating conditions, 4-amino-6-chlorobenzene-1,3-disulfonamide and trichlormethiazide are eluted in this order, the relative retention time of 4-amino-6-chlorobenzene-1,3-disulfonamide with respect to trichlormethiazide is about 0.3, and the number of theoretical plates and the symmetry factor of the peak of trichlormethiazide are not less than 5000 and not more than 1.2, respectively.

System repeatability: When the test is repeated 3 times with 10 μL of the solution for system suitability test under the above operating conditions, the relative standard deviation of the peak area of trichlormethiazide is not more than 2.0%.

Content uniformity Perform the test according to the following method: it meets the requirements of the Content Uniformity Test. To one tablet of Trichlormethiazide Tablets add 5 mL of diluted phosphoric acid (1 in 50) to disintegrate. Add exactly an amount of the internal standard solution, equivalent to 10 mL per 2 mg of trichlormethiazide ($C_8H_8Cl_3N_3O_4S_2$) according to the labeled amount, add acetonitrile to make 25 mL, shake vigorously for 15 minutes, and centrifuge. To an amount of the supernatant liquid add the mobile phase to make a solution so that it contains about 40 μg of trichlormethiazide ($C_8H_8Cl_3N_3O_4S_2$) in each mL, and use this solution as the sample solution. Separately, weigh accurately about 20 mg of Trichlormethiazide Reference Standard, previously dried at 105°C for 3 hours, and dissolve in acetonitrile to make exactly 100 mL. Pipet 5 mL of this solution, add exactly 5 mL of the internal standard solution, add 10 mL of acetonitrile and 5 mL of diluted phosphoric acid (1 in 50), and use this solution as the standard solution. Perform the test with 20 μL each of the sample solution and the standard solution as directed under the Liquid Chromatography according to the conditions described in Assay, and determine the ratios, Q_T and Q_S, of the peak area of trichlormethiazide to that of the internal standard.

$$\text{Amount (mg) of trichlormethiazide } (C_8H_8Cl_3N_3O_4S_2) = W_S \times \frac{Q_T}{Q_S} \times C \times \frac{1}{20}$$

W_S: Amount (mg) of Trichlormethiazide Reference Standard

C: Labeled amount (mg) of trichlormethiazide ($C_8H_8Cl_3N_3O_4S_2$) per tablet

Internal standard solution—A solution of 3-nitrophenol in acetonitrile (1 in 5000).

Dissolution Perform the test with 1 tablet of Trichlormethiazide Tablets at 50 revolutions per minute according to Method 2 under the Dissolution Test, using 900 mL of water as the dissolution medium. Withdraw 20 mL or more of the dissolution medium 15 minutes after starting the test, and filter through a membrane filter with pore size of not more than 0.45 μm. Discard the first 10 mL of the filtrate, pipet the subsequent V mL of the filtrate, add diluted phosphoric acid (1 in 50) to make exactly V' mL so that each mL contains about 1.1 μg of trichlormethiazide ($C_8H_8Cl_3N_3O_4S_2$) according to the labeled amount, and use this solution as the sample solution. Separately, weigh accurately about 22 mg of Trichlormethiazide Reference Standard, previously dried at 105°C for 3 hours, and dissolve in acetonitrile to make exactly 200 mL. Pipet 2 mL of this solution, add diluted phosphoric acid (1 in 50) to make exactly 200 mL, and use this solution as the standard solution. Perform the test with exactly 40 μL each of the sample solution and the standard solution as directed under the Liquid Chromatography according to the following conditions, and determine the peak areas, A_{Ta} and A_{Sa}, of trichlormethiazide obtained with the sample solution and the standard solution, and the area, A_{Tb}, of the peak, having the relative retention time of about 0.3 with respect to trichlormethiazide, obtained with the sample solution. The dissolution rate in 15 minutes is not less than 75%.

Dissolution rate (%) with respect to the labeled amount of trichlormethiazide ($C_8H_8Cl_3N_3O_4S_2$)

$$= W_S \times \frac{A_{Ta} + A_{Tb} \times 0.95}{A_{Sa}} \times \frac{V'}{V} \times \frac{1}{C} \times \frac{9}{2}$$

W_S: Amount (mg) of Trichlormethiazide Reference Standard

C: Labeled amount (mg) of trichlormethiazide ($C_8H_8Cl_3N_3O_4S_2$) per tablet

Operating conditions—

Proceed as directed in the operating conditions in the Assay.

System suitability—

System performance: Dissolve 25 mg of Trichlormethiazide in 50 mL of acetonitrile. To 1 mL of this solution add acetonitrile to make 50 mL. To 5 mL of this solution add 5 mL of water, and heat at 60°C in a water bath for 30 minutes. After cooling, when the procedure is run with 10 μL of this solution under the above operating conditions, 4-amino-6-chlorobenzene-1,3-disulfonamide and trichlormethiazide are eluted in this order, the relative retention time of 4-amino-6-chlorobenzene-1,3-disulfonamide with respect to trichlormethiazide is about 0.3, and the number of theoretical plates and the symmetry factor of the peak of trichlormethiazide are not less than 5000 and not more than 1.2, respectively.

System repeatability: When the test is repeated 6 times with 40 μL of the standard solution under the above operating conditions, the relative standard deviation of the peak area of trichlormethiazide is not more than 2.0%.

Assay Weigh accurately the mass of not less than 20 Trichlormethiazide Tablets, and pulverize the tablets in an agate mortar. Weigh accurately an amount of the powder, equivalent to about 2 mg of trichlormethiazide ($C_8H_8Cl_3N_3O_4S_2$) according to the labeled amount, add 5 mL of diluted phosphoric acid (1 in 50) and exactly 10 mL of the internal standard solution, add 10 mL of acetonitrile, shake vigorously for 15 minutes, and centrifuge. To 2 mL of the supernatant liquid add 2 mL of the mobile phase, and use this solution as the sample solution. Separately, weigh accurately about 40 mg of Trichlormethiazide Reference Standard, previously dried at 105°C for 3 hours, and dissolve in acetonitrile to make exactly 200 mL. Pipet 5 mL of this solution, add exactly 5 mL of the internal standard solution, add 10 mL of acetonitrile and 5 mL of diluted phosphoric acid (1 in 50), and use this solution as the standard solution. Perform the test with 20 μL each of the sample solution and the standard solution as directed under the Liquid Chromatography according to the following conditions, and determine the ratios, Q_T and Q_S, of the peak area of trichlormethiazide to that of the internal standard.

Amount (mg) of trichlormethiazide ($C_8H_8Cl_3N_3O_4S_2$)

$$= W_S \times \frac{Q_T}{Q_S} \times \frac{1}{20}$$

W_S: Amount (mg) of Trichlormethiazide Reference Standard

Internal standard solution—A solution of 3-nitrophenol in acetonitrile (1 in 5000).

Operating conditions—

Detector: An ultraviolet absorption photometer (wavelength: 268 nm).

Column: A stainless steel column 4.6 mm in inside diameter and 15 cm in length, packed with phenylsilanized silica gel for liquid chromatography (5 μm in particle diameter).

Column temperature: A constant temperature of about 25°C.

Mobile phase: A mixture of diluted phosphoric acid (1 in 1000) and acetonitrile (3:1).

Flow rate: Adjust the flow rate so that the retention time of trichlormethiazide is about 8 minutes.

System suitability—

System performance: When the procedure is run with 20 μL of the standard solution under the above operating conditions, the internal standard and trichlormethiazide are eluted in this order with the resolution between these peaks being not less than 2.0.

System repeatability: When the test is repeated 6 times with 20 μL of the standard solution under the above operating conditions, the relative standard deviation of the ratio of the peak area of trichlormethiazide to that of the internal standard is not more than 1.0%.

Containers and storage Containers—Tight containers.

Vasopressin Injection

バソプレシン注射液

Change the paragraph (i) under Purity to read:

Purity

(i) Standard stock solution: Dissolve 200 oxytocin Units of Posterior Pituitary Reference Standard, according to the labeled Units, in exactly 10 mL of diluted acetic acid (100) (1 in 400). Pipet 1 mL of this solution, and add diluted acetic acid (100) (1 in 400) to make exactly 10 mL. Store in a cold place, avoiding freezing. Use within 6 months from the date of preparation.

Change the paragraph (ii) under Assay to read:

Assay

(ii) Standard stock solution: Dissolve 2000 vasopressin Units of Posterior Pituitary Reference Standard, according to the labeled Units, in exactly 100 mL of diluted acetic acid (100) (1 in 400). Pipet 1 mL of this solution, and add diluted acetic acid (100) (1 in 400) to make exactly 10 mL. Store in a cold place, avoiding freezing. Use within 6 months from the date of preparation.

Vinblastine Sulfate

硫酸ビンブラスチン

Change to read except the structural formula, chemical name and origin/limits of content:

Description Vinblastine Sulfate occurs as a white to pale yellow powder.

It is soluble in water, sparingly soluble in methanol, and practically insoluble in ethanol (99.5).

It is hygroscopic.

Optical rotation $[\alpha]_D^{20}$: -28 – $-35°$ (0.20 g calculated on the dried basis, methanol, 10 mL, 100 mm).

Identification **(1)** Determine the absorption spectrum of a solution of Vinblastine Sulfate (1 in 50,000) as directed under the Ultraviolet-visible Spectrophotometry, and compare the spectrum with the Reference Spectrum or the spectrum of a solution of Vinblastine Sulfate Reference Standard prepared in the same manner as the sample solution: both spectra exhibit similar intensities of absorption at the same wavelengths.

(2) Determine the infrared absorption spectrum of Vinblastine Sulfate as directed in the potassium bromide disk method under the Infrared Spectrophotometry, and compare the spectrum with the Reference Spectrum or the spectrum of Vinblastine Sulfate Reference Standard: both spectra exhibit similar intensities of absorption at the same wave numbers.

(3) A solution of Vinblastine Sulfate (1 in 100) responds to the Qualitative Tests for sulfate.

pH Dissolve 15 mg of Vinblastine Sulfate in 10 mL of water: the pH of this solution is between 3.5 and 5.0.

Purity **(1)** Clarity and color of solution—Dissolve 50 mg of Vinblastine Sulfate in 10 mL of water: the solution is clear and colorless.

(2) Related substances—Dissolve about 4 mg of Vinblastine Sulfate in 10 mL of water, and use this solution as the sample solution. Pipet 1 mL of the sample solution, add water to make exactly 25 mL, and use this solution as the standard solution. Perform the test with exactly 200 μL each of the sample solution and the standard solution as directed under the Liquid Chromatography according to the following conditions, and determine each peak area of these solutions by the automatic integration method: the area of peak other than the main peak is not larger than 1/4 of the peak area of vinblastine from the standard solution, and the total area of the peaks other than the main peak is not larger than 3/4 of the peak area of vinblastine from the standard solution.

Operating conditions—

Detector, column, column temperature, mobile phase, and flow rate: Proceed as directed in the operating conditions in the Assay.

Time span of measurement: About 4 times as long as the retention time of vinblastine after the solvent peak.

System suitability—

Test for required detectability: To exactly 2.5 mL of the standard solution add water to make exactly 100 mL. Confirm that the peak area of vinblastine obtained from 200 μL of this solution is equivalent to 1.7 to 3.3% of that from 200 μL of the standard solution.

System performance: Proceed as directed in the system suitability in the Assay.

System repeatability: When the test is repeated 5 times with 200 μL of the standard solution under the above operating conditions, the relative standard deviation of the peak area of vinblastine is not more than 1.5%.

Loss on drying Perform the test with about 10 mg of Vinblastine Sulfate as directed in Method 2 under the Thermal Analysis according to the following conditions: not more than 15.0%.

Operating conditions—

Heating rate: 5°C/minute

Temperature range: room temperature to 200°C

Atmospheric gas: dried Nitrogen

Flow rate of atmospheric gas: 40 mL/minute

Assay Weigh accurately about 10 mg each of Vinblastine Sulfate and Vinblastine Sulfate Reference Standard (separately determine its loss on drying), dissolve in water to make exactly 25 mL, and use these solutions as the sample solution and the standard solution, respectively. Perform the test with exactly 20 μL each of the sample solution and the standard solution as directed under the Liquid Chromatography according to the following conditions, and determine the peak areas, A_T and A_S, of vinblastine.

$$\text{Amount (mg) of } C_{46}H_{58}N_4O_9.H_2SO_4 = W_S \times \frac{A_T}{A_S}$$

W_S: Amount (mg) of Vinblastine Sulfate Reference Standard, calculated on the dried basis

Operating conditions—

Detector: An ultraviolet absorption photometer (wavelength: 262 nm).

Column: A stainless steel column 4.6 mm in inside diameter and 15 cm in length, packed with octadecylsilanized silica gel for liquid chromatography (5 μm in particle diameter).

Column temperature: A constant temperature of about 25°C.

Mobile phase: To 7 mL of diethylamine add water to make 500 mL, and adjust the pH to 7.5 with phosphoric acid. To 380 mL of this solution add 620 mL of a mixture of methanol and acetonitrile (4:1).

Flow rate: Adjust the flow rate so that the retention time of vinblastine is about 8 minutes.

System suitability—

System performance: Dissolve 10 mg each of Vinblastine Sulfate and vincristine sulfate in 25 mL of water. When the procedure is run with 20 μL of this solution under the above operating conditions, vincristine and vinblastine are eluted in this order with the resolution between these peaks being

not less than 4.

System repeatability: When the test is repeated 5 times with 20 μL of the standard solution under the above operating conditions, the relative standard deviation of the peak area of vinblastine is not more than 1.0%.

Containers and storage Containers—Tight containers.

Storage—Light-resistant, at not exceeding −20°C.

Change to read:

Vinblastine Sulfate for Injection

注射用硫酸ビンブラスチン

Vinblastine Sulfate for Injection is a preparation for injection, which is dissolved before use. It contains not less than 90.0% and not more than 110.0% of the labeled amount of vinblastine sulfate ($C_{46}H_{58}N_4O_9.H_2SO_4$: 909.05).

Method of preparation Prepare as directed under Injections, with Vinblastine Sulfate.

Description Vinblastine Sulfate for Injection occurs as white to pale yellow, light masses or powder.

It is freely soluble in water.

The pH of a solution (1 in 1000) is 3.5 – 5.0.

Identification Proceed as directed in the Identification (1) under Vinblastine Sulfate.

Purity Related substances—Dissolve 4 mg of Vinblastine Sulfate for Injection in 10 mL of water, and use this solution as the sample solution. Pipet 1 mL of the sample solution, add water to make exactly 25 mL, and use this solution as the standard solution. Perform the test with exactly 200 μL each of the sample solution and the standard solution as directed under the Liquid Chromatography according to the following conditions, and determine each peak area by the automatic integration method: the area of the peak other than the main peak from the sample solution is not larger than 1/2 of the peak area of vinblastine from the standard solution, and the total area of the peaks other than the main peak is not larger than 2 times the peak area of vinblastine from the standard solution.

Operating conditions—

Perform as directed in the operating conditions in Purity (2) under Vinblastine Sulfate.

System suitability—

Perform as directed in the system suitability in Purity (2) under Vinblastine Sulfate.

Bacterial endotoxins Less than 10 EU/mg.

Content uniformity Perform the test according to the following method: it meets the requirements of the Content Uniformity Test. Open a container of Vinblastine Sulfate for Injection, add an exact amount of water to make a solution so that each mL contains about 0.4 mg of vinblastine sulfate ($C_{46}H_{58}N_4O_9.H_2SO_4$) according to the labeled amount, and use this solution as the sample solution. Separately, weigh accurately about 10 mg of Vinblastine Sulfate Reference Standard (separately determine its loss on drying in the same manner as for Vinblastine Sulfate), dissolve in water to make exactly 25 mL, and use this solution as the standard solution. Proceed as directed in the Assay under Vinblastine Sulfate.

Amount (mg) of vinblastine sulfate ($C_{46}H_{58}N_4O_9.H_2SO_4$)

$$= W_S \times \frac{A_T}{A_S}$$

W_S: Amount (mg) of Vinblastine Sulfate Reference Standard, calculated on the dried basis

Foreign insoluble matter Perform the test according to Method 2: it meets the requirements of the Foreign Insoluble Matter Test for Injections.

Insoluble particulate matter Perform the test according to Method 1: it meets the requirements of the Insoluble Particulate Matter Test for Injections.

Sterility Perform the test according to the Membrane filtration method: it meets the requirements of the Sterility Test.

Assay Take an amount of Vinblastine Sulfate for Injection, equivalent to 0.10 g of vinblastine sulfate ($C_{46}H_{58}N_4O_9.H_2SO_4$), dissolve each content with a suitable amount of water, transfer into a 100-mL volumetric flask, wash each container with water, transfer the washings into the volumetric flask, and add water to make exactly 100 mL. Pipet 10 mL of this solution, add water to make exactly 25 mL, and use this solution as the sample solution. Separately, weigh accurately about 10 mg of Vinblastine Sulfate Reference Standard (separately determine its loss on drying in the same manner as for Vinblastine Sulfate), dissolve in water to make exactly 25 mL, and use this solution as the standard solution. Proceed as directed in the Assay under Vinblastine Sulfate.

Amount (mg) of vinblastine sulfate ($C_{46}H_{58}N_4O_9.H_2SO_4$)

$$= W_S \times \frac{A_T}{A_S}$$

W_S: Amount (mg) of Vinblastine Sulfate Reference Standard, calculated on the dried basis

Containers and storage Containers—Hermetic containers, and colored containers may be used.

Storage—Light-resistant, at 2 to 8°C.

General Rules for Crude Drugs

Change the paragraph 1 to read:

1. Crude drugs in the monographs include medicinal parts obtained from plants or animals, cell inclusions and secretes separated from the origins, their extracts, and minerals. General Rules for Crude Drugs and the Crude Drugs in General Tests, Processes and Apparatus are applicable to the followings:

Acacia, Achyranthes Root, Agar, Agar Powder, Akebia Stem, Alisma Rhizome, Aloe, Alpinia Officinarum Rhizome, Amomum Seed, Anemarrhena Rhizome, Angelica Dahurica Root, Apricot Kernel, Areca, Artemisia Capillaris Flower, Asiasarum Root, Asparagus Tuber, Astragalus Root, Atractylodes Lancea Rhizome, Atractylodes Rhizome, Bear Bile, Bearberry Leaf, Belladonna Root, Benzoin, Bitter Cardamon, Bitter Orange Peel, Bupleurum Root, Burdock Fruit, Calumba, Capsicum, Cardamon, Cassia Seed, Catalpa Fruit, Chrysanthemum Flower, Chuling, Cimicifuga Rhizome, Cinnamon Bark, Citrus Unshiu Peel, Clematis Root, Clove, Cnidium Monnieri Fruit, Cnidium Rhizome, Coix Seed, Condurango, Coptis Rhizome, Cornus Fruit, Corydalis Tuber, Cyperus Rhizome, Digenea, Dioscorea Rhizome, Ephedra Herb, Epimedium Herb, Eucommia Bark, Evodia Fruit, Fennel, Forsythia Fruit, Fritillaria Bulb, Gambir, Gardenia Fruit, Gastrodia Tuber, Gentian, Geranium Herb, Ginger, Ginseng, Glehnia Root, Glycyrrhiza, Gypsum, Hemp Fruit, Hoelen, Honey, Houttuynia Herb, Immature Orange, Imperata Rhizome, Ipecac, Japanese Angelica Root, Japanese Gentian, Japanese Valerian, Jujube, Jujube Seed, Lindera Root, Lithospermum Root, Longgu, Lonicera Leaf and Stem, Loquat Leaf, Lycium Bark, Lycium Fruit, Magnolia Bark, Magnolia Flower, Mallotus Bark, Mentha Herb, Moutan Bark, Mulberry Bark, Notopterygium Rhizome, Nuphar Rhizome, Nux Vomica, Ophiopogon Tuber, Oriental Bezoar, Oyster Shell, Panax Rhizome, Peach Kernel, Peony Root, Perilla Herb, Pharbitis Seed, Phellodendron Bark, Picrasma Wood, Pinellia Tuber, Plantago Herb, Plantago Seed, Platycodon Root, Polygala Root, Polygonum Root, Powdered Acacia, Powdered Alisma Rhizome, Powdered Aloe, Powdered Amomum Seed, Powdered Atractylodes Lancea Rhizome, Powdered Atractylodes Rhizome, Powdered Calumba, Powdered Capsicum, Powdered Cinnamon Bark, Powdered Clove, Powdered Cnidium Rhizome, Powdered Coix Seed, Powdered Coptis Rhizome, Powdered Cyperus Rhizome, Powdered Dioscorea Rhizome, Powdered Fennel, Powdered Gambir, Powdered Gardenia Fruit, Powdered Gentian, Powdered Geranium Herb, Powdered Ginger, Powdered Ginseng, Powdered Glycyrrhiza, Powdered Hoelen, Powdered Ipecac, Powdered Japanese Angelica Root, Powdered Japanese Gentian, Powdered Japanese Valerian, Powdered Magnolia Bark, Powdered Moutan Bark, Powdered Oyster Shell, Powdered Panax Rhizome, Powdered Peach Kernel, Powdered Peony Root, Powdered Phellodendron Bark, Powdered Picrasma Wood, Powdered Platycodon Root, Powdered Polygala Root, Powdered Polypourus Sclerotium, Powdered Processed Aconite Root, Powdered Rhubarb, Powdered Rose Fruit, Powdered Scutellaria Root, Powdered Senega, Powdered Senna Leaf, Powdered Smilax Rhizome, Powdered Sophora Root, Powdered Sweet Hydrangea Leaf, Powdered Swertia Herb, Powdered Tragacanth, Powdered Zanthoxylum Fruit, Processed Aconite Root, Processed Ginger, Prunella Spike, Pueraria Root, Red Ginseng, Rehmannia Root, Rhubarb, Rice Starch, Rose Fruit, Rosin, Safflower, Saffron, Saposhnikovia Root, Sappan Wood, Saussurea Root, Schisandra Fruit, Schizonepeta Spike, Scopolia Rhizome, Scutellaria Root, Senega, Senna Leaf, Sinomenium Stem, Smilax Rhizome, Sophora Root, Sweet Hydrangea Leaf, Swertia Herb, Termeric, Toad Venom, Tragacanth, Tribulus Fruit, Trichosanthes Root, Uncaria Thorn, Zanthoxylum Fruit, Zedoary.

Official Monographs for Part II

Delete the following Monographs:

Absorbent Cotton

Purified Absorbent Cotton

Sterile Absorbent Cotton

Sterile Purified Absorbent Cotton

Absorbent Gauze

Sterile Absorbent Gauze

Adhesive Plaster

Powdered Aloe

アロエ末

Change the origin/limits of content to read:

Powdered Aloe is the powder of Aloe.

It contains not less than 4.0% of barbaloin, calculated on the basis of dried material.

Change the Loss on drying to read:

Loss on drying Not more than 12.0%.

Add the following next to Extract content:

Component determination Weigh accurately about 0.1 g of Powdered Aloe, add 40 mL of methanol, and heat under a reflex condenser on a water bath for 30 minutes. After cooling, filter, and add methanol to the filtrate to make exactly 50 mL. Pipet 5 mL of the solution, add methanol to make exactly 10 mL, and use this solution as the sample solution. Separately, weigh accurately about 10 mg of barbaloin for component determination, previously dried in a desiccator (in vacuum, phosphorus (V) oxide) for 24 hours, add 40 mg of oxalic acid dihydrate, and dissolve in methanol to make exactly 100 mL. Pipet 5 mL of the solution, add methanol to make exactly 10 mL, and use this solution as the standard solution. Perform the test with exactly 5 μL each of the sample solution and the standard solution as directed under the Liquid Chromatography according to the following conditions, and determine the peak areas of barbaloin, A_T and A_S, of both solutions.

$$\text{Amount (mg) of barbaloin} = W_S \times \frac{A_T}{A_S} \times 2$$

W_S: Amount (mg) of barbaloin for component determination

Operating conditions—

Detector: An ultraviolet absorption photometer (wavelength: 360 nm).

Column: A stainless steel column about 6 mm in inside diameter and about 15 cm in length, packed with octadecylsilanized silica gel for liquid chromatography (5 μm in particle diameter).

Column temperature: A constant temperature of about 30°C.

Mobile phase: A mixture of water, acetonitrile and acetic acid (100) (74:26:1).

Flow rate: Adjust the flow rate so that the retention time of barbaloin is about 12 minutes.

System suitability—

System performance: To about 10 mg of barbaloin for component determination add 40 mg of oxalic acid dihydrate, and dissolve in methanol to make exactly 100 mL. Pipet 5 mL of the solution, add 1 mL of a solution of ethenzamide in methanol (1 in 2000) and methanol to make exactly 10 mL. When the procedure is run with 5 μL of this solution under the above operating conditions except the wavelength of 300 nm, barbaloin and ethenzamide are eluted in this order with the resolution between these peaks being not less than 2.0.

System repeatability: When the test is repeated 6 times with 5 μL of the standard solution under the above operating conditions, the relative standard deviation of the peak area of barbaloin is not more than 1.5%.

Benzyl Alcohol

ベンジルアルコール

Change to read except the structural formula and chemical name:

Benzyl Alcohol contains not less than 98.0% and not more than 100.5% of C_7H_8O.

The label states, where applicable, that it is suitable for use in the manufacture of injection forms.

Description Benzyl Alcohol is a clear, colorless oily liquid.

It is miscible with ethanol (95), with fatty oils and with volatile oils.

It is soluble in water.

Specific gravity d_{20}^{20}: 1.043 – 1.049

Identification Determine the infrared absorption spectrum of Benzyl Alcohol as directed in the liquid film method under the Infrared Spectrophotometry, and compare the spectrum with the Reference Spectrum: both spectra exhibit similar intensities of absorption at the same wave numbers.

Refractive index n_D^{20}: 1.538 – 1.541

Purity **(1)** Clarity and color of solution—Dissolve 2.0 mL of Benzyl Alcohol in 60 mL of water: the solution is clear and colorless.

(2) Acid—To 10 mL of Benzyl Alcohol add 10 mL of neutralized ethanol, 2 drops of phenolphthalein TS and 1.0 mL of 0.1 mol/L sodium hydroxide VS: the color of the solution is red.

(3) Benzaldehyde and other related substances—Use Benzyl Alcohol as the sample solution. Separately, weigh exactly 0.750 g of benzaldehyde and 0.500 g of cyclohexylmethanol, and add Benzyl Alcohol to make exactly 25 mL. Pipet 1 mL of this solution, add exactly 2 mL of ethylbenzene internal standard solution and exactly 3 mL of dicyclohexyl internal standard solution, then add Benzyl Alcohol to make exactly 20 mL, and use this solution as the standard solution (1). Perform the test with 0.1 μL each of the sample solution and the standard solution (1) as directed under the Gas Chromatography according to the following conditions: no peaks of ethylbenzene and dicyclohexyl appear on the chromatogram obtained with the sample solution. When 0.1 μL of the standard solution (1) is injected, adjust the sensitivity of the detector so that the peak height of ethylbenzene is not more than 30% of the full scale of the recorder. The peak area of benzaldehyde obtained with the sample solution is not more than the deference between the peak areas of benzaldehyde of the sample solution and the standard solution (1) (0.15%), and the peak area of cyclohexylmethanol with the sample solution is not more than the deference between the peak areas of cyclohexylmethanol of the sample solution and the standard solution (1) (0.10%). The total area of the peaks having smaller retention time than benzyl alcohol and other than benzaldehyde and cyclohexylmethanol obtained with the sample solution is not more than 4 times the peak area of ethylbenzene with the standard solution (1) (0.04%). The total area of the peaks having larger retention time than benzaldehyde obtained with the sample solution is not more than the peak area of cyclohexylmethanol with the standard solution (1) (0.3%). For these calculations the peak areas less than 1/100 times the peak area of ethylbenzene with the standard solution (1) are excluded.

Benzyl Alcohol labeled that it is suitable for use in the manufacture of injection forms meets the following requirements.

Use Benzyl Alcohol as the sample solution. Separately, weigh exactly 0.250 g of benzaldehyde and 0.500 g of cyclohexylmethanol, and add Benzyl Alcohol to make exactly 25 mL. Pipet 1 mL of this solution, add exactly 2 mL of the ethylbenzene internal standard solution and exactly 2 mL of the dicyclohexyl internal standard solution, then add Benzyl Alcohol to make exactly 20 mL, and use this solution as the standard solution (2). Perform the test with 0.1 μL each of the sample solution and the standard solution (2) as directed under the Gas Chromatography according to the following conditions: no peaks of ethylbenzene and dicyclohexyl appear on the chromatogram obtained with the sample solution. When 0.1 μL of the standard solution (2) is injected, adjust the sensitivity of the detector so that the peak height of ethylbenzene is not more than 30% of the full scale of the recorder. The peak area of benzaldehyde of obtained with the sample solution is not more than the deference between the peak areas of benzaldehyde of the sample solution and the standard solution (2) (0.05%), and the peak area of cyclohexylmethanol with the sample solution is not more than the deference between the peak areas of cyclohexylmethanol of the sample solution and the standard solution (2) (0.10%). The total area of the peaks having smaller retention time than benzyl alcohol and other than benzaldehyde and cyclohexylmethanol obtained with the sample solution is not more than 2 times the peak area of ethylbenzene with the standard solution (2) (0.02%). The total area of the peaks having larger retention time than benzaldehyde obtained with the sample solution is not more than the peak area of cyclohexylmethanol with the standard solution (2) (0.2%). For these calculation the peak areas less than 1/100 times the peak area of ethylbenzene with the standard solution (2) are excluded.

Ethylbenzene internal standard solution: Dissolve exactly 0.100 g of ethylbenzene in Benzyl Alcohol to make exactly 10 mL. Pipet 2 mL of this solution, and add Benzyl Alcohol to make exactly 20 mL.

Dicyclohexyl internal standard solution: Dissolve exactly 2.000 g of dicyclohexyl in Benzyl Alcohol to make exactly 10 mL. Pipet 2 mL of this solution, and add Benzyl Alcohol to make exactly 20 mL.

Operating conditions—

Detector: A hydrogen flame-ionization detector

Column: A fused silica column 0.33 mm in inside diameter and 30 m in length, coated inside with polyethylene glycol 20M for gas chromatography in 0.5 μm thickness.

Column temperature: Raise the temperature at a rate of 5°C per minutes from 50°C to 220°C, and maintain at 220°C for 35 minutes.

Temperature of injection port: A constant temperature of about 200°C.

Temperature of detector: A constant temperature of about 310°C.

Carrier gas: Helium

Flow rate: Adjust the flow rate so that the retention time of benzyl alcohol is between 24 and 28 minutes.

Split ratio: Splitless

System suitability—

System performance: When the procedure is run with the

standard solution (1) under the above operating conditions, the relative retention times of ethylbenzene, dicyclohexyl, benzaldehyde and cyclohexylmethanol with respect to benzyl alcohol are about 0.28, about 0.59, about 0.68 and about 0.71, respectively, and the resolution between the peaks of benzaldehyde and cyclohexylmethanol is not less than 3.0. In the case of Benzyl Alcohol labeled to use for injection, proceed with the standard solution (2) instead of the standard solution (1).

(4) Peroxide value—Dissolve 5 g of Benzyl Alcohol in 30 mL of a mixture of acetic acid (100) and chloroform (3:2) in a 250-mL glass-stoppered conical flask. Add 0.5 mL of potassium iodide saturated solution, shake exactly for 1 minute, add 30 mL of water, and titrate with 0.01 mol/L sodium thiosulfate VS until the blue color of the solution disappears after addition of 10 mL of starch TS near the end point where the solution is a pale yellow color. Perform a blank determination in the same manner. Calculate the amount of peroxide by the following formula: not more than 5.

$$\text{Amount (mEq/kg) of peroxide} = \frac{(V_1 - V_0)}{W} \times 10$$

V_1: Volume (mL) of 0.01 mol/L sodium thiosulfate VS consumed in the test
V_0: Volume (mL) of 0.01 mol/L sodium thiosulfate VS consumed in the blank
W: Amount (g) of the sample

(5) Residue on evaporation—Perform the test after conformation that the sample meets the requirement of the peroxide value. Evaporate to dryness 10.0 g of Benzyl Alcohol on a water bath, dry the residue at 105°C for 1 hour, and allow to cool in a desiccator: not more than 5 mg.

Assay Weigh accurately about 0.9 g of Benzyl Alcohol, add exactly 15.0 mL of a mixture of pyridine and acetic anhydride (7:1), and heat on a water bath under a reflux condenser for 30 minutes. Cool, add 25 mL of water, and titrate the excess acetic acid with 1 mol/L sodium hydroxide VS (indicator: 2 drops of phenolphthalein TS). Perform a blank determination.

Each mL of 1 mol/L sodium hydroxide VS
= 108.1 mg of C_7H_8O

Containers and storage Containers—Tight containers.
Storage—Light-resistant.

Carmellose Calcium

カルメロースカルシウム

Change the Description to read:

Description Carmellose Calcium occurs as a white to yellowish white powder.

It is practically insoluble in ethanol (95) and in diethyl ether.

It swells with water to form a suspension.

The pH of a suspension, obtained by shaking 1.0 g of Carmellose Calcium with 100 mL of water, is between 4.5 and 6.0.

It is hygroscopic.

Change the Identification (1) and (4) to read:

Identification (1) Shake thoroughly 0.1 g of Carmellose Calcium with 10 mL of water, followed by 2 mL of sodium hydroxide TS, allow to stand for 10 minutes, and use this solution as the sample solution. To 1 mL of the sample solution add water to make 5 mL. To 1 drop of this solution add 0.5 mL of chromotropic acid TS, and heat in a water bath for 10 minutes: a red-purple color develops.

(4) Ignite 1 g of Carmellose Calcium to ash, dissolve the residue in 10 mL of water and 6 mL of acetic acid (31), and filter, if necessary. Boil the filtrate, cool, and neutralize with ammonia TS: the solution responds to the Qualitative Tests (1) and (3) for calcium salt.

Change the Purity to read:

Purity (1) Alkali—Shake thoroughly 1.0 g of Carmellose Calcium with 50 mL of freshly boiled and cooled water, and add 2 drops of phenolphthalein TS: no red color develops.

(2) Chloride—Shake thoroughly 0.80 g of Carmellose Calcium with 50 mL of water, add 10 mL of sodium hydroxide TS to dissolved, add water to make 100 mL, and use this solution as the sample solution. Heat 20 mL of the sample solution with 10 mL of 2 mol/L nitric acid on a water bath until a flocculent precipitate is produced. After cooling, centrifuge, and take out the supernatant liquid. Wash the precipitate with three 10-mL portions of water by centrifuging each time, combine the supernatant and the washings, and add water to make 100 mL. Take 25 mL of this solution, and add 1 mL of nitric acid and water to make 50 mL. Perform the test using this solution as the test solution. Prepare the control solution with 0.40 mL of 0.01 mol/L hydrochloric acid VS (not more than 0.36%).

(3) Sulfate—Heat 10 mL of the sample solution obtained in (2) with 1 mL of hydrochloric acid in a water bath until a flocculent precipitate is produced. Cool, centrifuge, and take out the supernatant liquid. Wash the precipitate with three 10-mL portions of water by centrifuging each time, combine the supernatant and the washings, and add water to make 100 mL. Perform the test with 25 mL this solution as the test solution. Prepare the control solution with 0.42 mL of 0.005 mol/L sulfuric acid VS. To the test solution and the control solution add 1 mL of 3 mol/L hydrochloric acid and 3 mL of barium chloride TS, then add water to make 50 mL, and mix. Allow to stand for 10 minutes, and compare the turbidity of these solutions: the turbidity obtained with the test solution is not more than that obtained with the control solution (not more than 1.0%).

(4) Heavy metals—Proceed with 1.0 g of Carmellose Calcium according to Method 2, and perform the test. Prepare the control solution with 2.0 mL of Standard Lead

Solution (not more than 20 ppm).

Change the Residue on ignition to read:

Residue on ignition 10.0 – 20.0% (after drying, 1 g).

Cellulose Acetate Phthalate

酢酸フタル酸セルロース

Add the following commonly used name:

Cellacefate

Change the origin/limits of the content to read:

Cellulose Acetate Phthalate is a reaction product of phthalic anhydride and partially acetylated cellulose.

Cellulose Acetate Phthalate, calculated on the anhydrous and free acid-free basis, contains not less than 21.5% and not more than 26.0% of acetyl group (-COCH3: 43.04), and not less than 30.0% and not more than 36.0% of carboxybenzoyl group ($-COC_6H_4COOH$: 149.12).

Change the Description to read:

Description Cellulose Acetate Phthalate occurs as a white powder or grain.

It is freely soluble in acetone, and practically insoluble in water, in methanol and in ethanol (99.5).

Change the Identification to read:

Identification Determine the infrared absorption spectrum of Cellulose Acetate Phthalate as directed in the potassium bromide disk method under the Infrared Spectrophotometry, and compare the spectrum with the Reference Spectrum or spectrum of Cellulose Acetate Phthalate Reference Standard: both spectra exhibit similar intensities of absorption at the same wave numbers.

Change the Water to read:

Water Not more than 5.0% (1 g, volumetric titration, direct titration, using a mixture of ethanol (99.5) and dichloromethane (3:2) instead of methanol for Karl Fischer method).

Change the Assay (2) to read:

Assay

(2) Acetyl group—Weigh accurately about 0.1 g of Cellulose Acetate Phthalate, put in a glass-stoppered conical flask, add exactly 25 mL of 0.1 mol/L sodium hydroxide VS, and boil for 30 minutes under a reflux condenser. After cooling, add 5 drops of phenolphthalein TS, and titrate with 0.1 mol/L hydrochloric acid VS. Perform a blank determination.

$$\text{Content (\%) of free acids and bound acetyl group } (C_2H_3O) = \frac{A}{W} \times 0.4304$$

A: Amount (mL) of 0.1 mol/L sodium hydroxide consumed, corrected by the blank determination

W: Amount (g) of the test sample, calculated on the anhydrous basis

$$\text{Content (\%) of acetyl group } (C_2H_3O) = \frac{P - 0.5182 \times B}{100 - B} \times 100 - 0.5772 \times C$$

B: Amount (%) of free acids obtained in Purity (2) Free acids

C: Content (%) of carboxybenzoyl group

P: Content (%) of free acids and bound acetyl group (C_2H_3O)

Chrysanthemum Flower

キクカ

Change the Extract content to read:

Extract content Dilute ethanol-extract: not less than 30.0%.

Add the following:

Cnidium Monnieri Fruit

Cnidii Monnieris Fructus

ジャショウシ

Cnidium Monnieri Fruit is the fruit of *Cnidium monnieri* Cusson (*Umbelliferae*).

Description Elliptical cremocarp, often each mericarp separated; 2 – 3 mm in length, 1 – 2 mm in width; externally light brown to brown, each mericarp usually with five winged longitudinal ridges; inner surface of mericarp almost flat.

Odor, characteristic; it gives characteristic aroma, later a slight sensation of numbness on chewing.

Under a microscope, a transverse section reveals one oil canal between longitudinal ridges, usually two oil canals in the inner part of mericarp facing to gynophore; longitudinal ridges composed of slightly lignified parenchymatous cells, with vascular bundles in the base; epidermal cells and parenchymatous cells of longitudinal ridges contain solitary crystals of calcium oxalate; parenchymatous cells of albumen contain oil drops and aleurone grains, and occasionally starch grains.

Identification To 1 g of pulverized Cnidium Monnieri Fruit add 10 mL of ethyl acetate, shake for 10 minutes, filter, and use the filtrate as the sample solution. Separately,

dissolve 1 mg of osthole for thin-layer chromatography in 2 mL of methanol, and use this solution as the standard solution. Perform the test with these solutions as directed under the Thin-layer Chromatography. Spot 5 μL each of the sample solution and the standard solution on a plate of silica gel for thin-layer chromatography, develop the plate with a mixture of hexane and ethyl acetate (2:1) to a distance of about 10 cm, and air-dry the plate. Examine under ultraviolet light (main wavelength: 365 nm): one of the spot among the several spots from the sample solution has the same color tone and the *R*f value with the blue-white fluorescent spot from the standard solution.

Loss on drying Not more than 12.0% (6 hours).

Total ash Not more than 17.0%.

Acid-insoluble ash Not more than 6.0%.

Extract content Not less than 8.0% (dilute ethanol-soluble extract).

Change to read:

Corn Starch

Amylum Maydis

トウモロコシデンプン

Corn Starch consists of starch granules derived from the ripen seeds of *Zea mays* Linné (*Gramineae*).

Description Corn Starch occurs as white to pale yellowish white masses or powder.

It is practically insoluble in water and in ethanol (99.5).

Identification **(1)** Under a microscope, Corn Starch, preserved in a mixture of water and glycerin (1:1), appears as irregularly polygonal simple grains usually 2 – 23 μm in diameter, or irregularly orbicular or spherical simple grains 25 – 35 μm in diameter; hilum appears as distinct cave or 2 – 5 radial clefts; concentric striation absent; a black cross, its intersection point on hilum, is observed when grains are put between two polarizing prisms fixed at right angle to each other.

(2) To 1 g of Corn Starch add 50 mL of water, boil for 1 minute, and allow to cool: a subtle white-turbid, pasty liquid is formed.

(3) To 10 mL of the pasty liquid obtained in (2) add 0.04 mL of iodine TS: a orange-red to dark blue-purple color is formed, and the color disappears by heating.

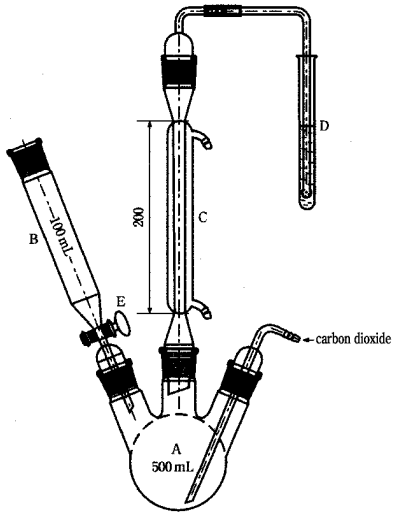
pH Put 5.0 g of Corn Starch in a non-metal vessel, add 25.0 mL of freshly boiled and cooled water, mix gently for 1 minute, and allow to stand for 15 minutes: the pH of the solution is between 4.0 and 7.0.

Purity **(1)** Iron—To 1.5 g of Corn Starch add 15 mL of 2 mol/L hydrochloric acid TS, mix, filter, and use the filtrate as the test solution. To 2.0 mL of Standard Iron Solution add water to make 20 mL, and use as the control solution. Put 10 mL each of the test solution and the control solution in test tubes, add 2 mL of a solution of citric acid (2 in 10) and 0.1 mL of mercapto acetic acid, and mix. Alkalize with ammonia solution (28) to litmus paper, add water to make 20 mL, and mix. Transfer 10 mL each of these solutions into test tubes, allow to stand for 5 minutes, and compare the color of these solutions against a white background: the color of the test solution is not darker than that of the control solution (not more than 10 ppm).

(2) Oxidizing substances—To 4.0 g of Corn Starch add 50.0 mL of water, shake for 5 minutes, and centrifuge. To 30.0 mL of the supernatant liquid add 1 mL of acetic acid (100) and 0.5 to 1.0 g of potassium iodide, shake, and allow to stand for 25 to 30 minutes at a dark place. Add 1 mL of starch TS, and titrate with 0.002 mol/L sodium thiosulfate VS until the color of the solution disappears. Perform a blank determination and make any necessary correction: the volume of 0.002 mol/L sodium thiosulfate VS consumed is not more than 1.4 mL (not more than 20 ppm, calculated as hydrogen peroxide).

(3) Sulfur dioxide—

(i) Apparatus Use as shown in the following figure.



The figures are in mm.

A: Boiling flask (500 mL)
B: Funnel (100 mL)
C: Condenser
D: Test-tube
E: Tap

(ii) Procedure Introduce 150 mL of water into the boiling flask, close the tap of the funnel, and pass carbon dioxide through the whole system at a rate of 100 ± 5 mL per minute. Pass cooling water through the condenser, and place 10 mL of hydrogen peroxide-sodium hydroxide TS in the test-tube. After 15 minutes, remove the funnel without interrupting the stream of carbon dioxide, and introduce through the opening into the flask about 25 g of Corn Starch, accurately weighed, with the aid of 100 mL of water. Apply tap grease to the outside of the connection part of the

funnel, and load the funnel. Close the tap of the funnel, pour 80 mL of 2 mol/L hydrochloric acid TS into the funnel, open the tap to introduce the hydrochloric acid into the flask, and close the tap while several mL of the hydrochloric acid remains, in order to avoid losing sulfur dioxide. Place the flask in a water bath, and heat the mixture for 1 hour. Transfer the contents of the test-tube with the aid of a little water to a wide-necked conical flask. Heat in a water bath for 15 minutes, and cool. Add 0.1 mL of bromophenol blue TS, and titrate with 0.1 mol/L sodium hydroxide VS until the color changes from yellow to violet-blue lasting for at least 20 seconds. Perform a blank determination and make any necessary correction. Calculate the amount of sulfur dioxide by applying the following formula: it is not more than 50 ppm.

$$\text{Amount (ppm) of sulfur dioxide} = \frac{V}{W} \times 1000 \times 3.203$$

W: Amount (g) of the sample
V: Amount (mL) of 0.1 mol/L sodium hydroxide VS consumed

Loss on drying Not more than 15.0% (1 g, 130°C, 90 minutes).

Reisdue on ignition Not more than 0.6% (1 g).

Containers and storage Containers—Well-closed containers.

Cornus Fruit

サンシュユ

Change the Identification to read:

Identification To 1 g of coarse cuttings of Cornus Fruit add 10 mL of methanol, shake for 5 minutes, filter, and use the filtrate as the sample solution. Separately, dissolve 1 mg of loganin for thin-layer chromatography in 2 mL of methanol, and use this solution as the standard solution. Perform the test with these solutions as directed under the Thin-layer Chromatography. Spot 10 μL each of the sample solution and the standard solution on a plate of silica gel for thin-layer chromatography. Develop with a mixture of ethyl acetate, water and formic acid (6:1:1) to a distance of about 10 cm, and air-dry the plate. Spray evenly 4-methoxybenzaldehyde-sulfuric acid TS on the plate, and heat at 105°C for 5 minutes: one of the spots from the sample solution is the same with a red-purple spot from the standard solution in color tone and *R*f value.

Delete the following Monographs:

Digitalis

Powdered Digitalis

Add the following:

Epimedium Herb

Epimedii Herba

インヨウカク

Epimedium Herb is the terrestrial part of *Epimedium pubescens* Maximowicz, *Epimedium brevicornum* Maximowicz, *Epimedium wushanense* T. S. Ying, *Epimedium sagittatum* Maximowicz, *Epimedium koreanum* Nakai, *Epimedium grandiflorum* Morren var. *thunbergianum* Nakai or *Epimedium sempervirens* Nakai (*Berberidaceae*).

Description Epimedium Herb is composed of a stem and a ternate to triternate compound leaf; leaflet ovate to broadly ovate or ovate-lanceolate, 3 – 20 cm in length, 2 – 8 cm in width, petiolule 15 – 70 mm in length, apex of leaflet acuminate, needle hair on margin 0.1 – 0.2 cm in length, base of leaflet cordate to deeply cordate, lateral leaflet asymmetry; upper surface green to greenish brown, sometimes lustrous, lower surface light green, often pilose, especially on vein densely pilose, papery or coriaceous; petiole and stem cylindrical, light yellowish brown to slightly purplish and light greenish brown, easily broken.

Odor, slight; taste, slightly bitter.

Under a microscope, a transverse section of the leaf reveals 3 – 6 vascular bundles in midvein; mesophyll composed of upper epidermis, single-layered palisade, spongy tissue and lower epidermis; leaf margins orbicular or oblong, sclerenchymatous; multi-cellular hairs on epidermis; 8 – 20 vascular bundles in petiole and 6 – 15 vascular bundles in petiolule. Under a microscope, a transverse section of the stem reveals a single to several-layered hypodermis, cortex of 4 – 10 layers of sclerenchymatous cells, vascular bundle 13 – 30 in number, oblong to obovate.

Identification To 2 g of pulverized Epimedium Herb add 20 mL of methanol, shake for 15 minutes, filter, and use the filtrate as the sample solution. Separately, dissolve 1 mg of icariin for thin-layer chromatography in 1 mL of methanol, and use this solution as the standard solution. Perform the test with these solutions as directed under the Thin-layer Chromatography. Spot 10 μL each of the sample solution and the standard solution on a plate of silica gel with fluorescent indicator for thin-layer chromatography. Develop the plate with a mixture of ethyl acetate, ethanol (99.5) and water (8:2:1) to a distance of about 10 cm, and air-dry the plate. Examine under ultraviolet light (main wavelength: 254

nm): one spot among the spots from the sample solution and a dark purple spot from the standard solution show the same color tone and the same *R*f value.

Loss on drying Not more than 12.5% (6 hours).

Total ash Not more than 8.5%.

Acid-insoluble ash Not more than 2.0%.

Extract content Not less than 17.0% (dilute ethanol-soluble extract).

Powdered Gardenia Fruit

サンシシ末

Change the origin/limits of content to read:

Powdered Gardenia Fruit is the powder of Gardenia Fruit.

It contains not less than 3.0% of geniposide, calculated on the basis of dried material.

Change the Identification (1) to read:

Identification (1) To 1.0 g of Powdered Gardenia Fruit, previously dried in a desiccator (silica gel) for 24 hours, add 100 mL of hot water, warm the mixture between 60°C and 70°C for 30 minutes with frequent shaking, and filter after cooling. To 1.0 mL of the filtrate add water to make 10 mL: the color of the resulting solution is yellow and is not lighter than that of the following control solution.

Control solution: Dissolve 9.8 mg of carbazochrome sodium sulfonate in water to make exactly 10 mL. Pipet 1 mL of this solution, and add water to make exactly 50 mL.

Add the following next to Identification:

Loss on drying Not more than 13.0%.

Add the following next to Total ash:

Component determination Weigh accurately about 0.5 g of Powdered Gardenia Fruit, transfer into a glass-stoppered centrifuge tube, add 40 mL of diluted methanol (1 in 2), shake for 15 minutes, centrifuge, and take the supernatant liquid. To the residue add 40 mL of diluted methanol (1 in 2), and perform as the same as above. Combine the extracts so obtained, and add diluted methanol (1 in 2) to make exactly 100 mL. Pipet 5 mL of the solution, add methanol to make exactly 20 mL, use this solution as the sample solution. Separately, weigh accurately about 10 mg of geniposide for component determination, previously dried in a desiccator (in vacuum, phosphorus (V) oxide) for 24 hours, and dissolve in methanol to make exactly 100 mL. Pipet 5 mL of the solution, add methanol to make exactly 10 mL, and use this solution as the standard solution. Perform the test with exactly 10 μL each of the sample solution and the standard solution as directed under the Liquid Chromatography according to the following conditions, and measure the peak areas of geniposide, A_T and A_S, of both solutions.

$$\text{Amount (mg) of geniposide} = W_S \times \frac{A_T}{A_S} \times 2$$

W_S: Amount (mg) of geniposide for component determination

Operating conditions—

Detector: An ultraviolet absorption photometer (wavelength: 240 nm).

Column: A stainless steel column 6 mm in inside diameter and 15 cm in length, packed with octadecylsilanized silica gel for liquid chromatography (5 μm in particle diameter).

Column temperature: A constant temperature of about 30°C.

Mobile phase: A mixture of water and acetonitrile (22:3).

Flow rate: Adjust the flow rate so that the retention time of geniposide is about 15 minutes.

System suitability—

System performance: Dissolve 1 mg each of geniposide for component determination and caffeine in methanol to make 15 mL. When the procedure is run with 10 μL of this solution under the above operating conditions, caffeine and geniposide are eluted in this order with the resolution between these peaks being not less than 3.5.

System repeatability: When the test is repeated 6 times with 10 μL of the standard solution under the above operating conditions, the relative standard deviation of the peak area of geniposide is not more than 1.5%.

Ginger

ショウキョウ

Change the Identification to read:

Identification To 2 g of pulverized Ginger add 5 mL of diethyl ether, shake for 10 minutes, filter, and use the filtrate as the sample solution. Separately, dissolve 1 mg of [6]-gingerol for thin-layer chromatography in 2 mL of methanol, and use this solution as the standard solution. Perform the test with these solutions as directed under the Thin-layer Chromatography. Spot 10 μL of the sample solution and the standard solution on a plate of silica gel for thin-layer chromatography. Develop the plate with a mixture of ethyl acetate and hexane (1:1) to a distance of about 10 cm, and air-dry the plate. Spray evenly 4-dimethylaminobenzaldehyde TS on the plate, heat at 105°C for 5 minutes, and allow to cool: one of the spots from the sample solution and a green spot from the standard solution show the same color tone and *R*f value.

Powdered Ginger

ショウキョウ末

Change the Identification to read:

Identification To 2 g of Powdered Ginger add 5 mL of diethyl ether, shake for 10 minutes, filter, and use the filtrate as the sample solution. Separately, dissolve 1 mg of [6]-gingerol for thin-layer chromatography in 2 mL of methanol, and use this solution as the standard solution. Perform the test with these solutions as directed under the Thin-layer Chromatography. Spot 10 μL of the sample solution and the standard solution on a plate of silica gel for thin-layer chromatography. Develop the plate with a mixture of ethyl acetate and hexane (1:1) to a distance of about 10 cm, and air-dry the plate. Spray evenly 4-dimethylaminobenzaldehyde TS on the plate, heat at 105°C for 5 minutes, and allow to cool: one of the spots from the sample solution and a green spot from the standard solution show the same color tone and *R*f value.

Chorionic Gonadotrophin

胎盤性性腺刺激ホルモン

Delete the origin/limits of content and add the following:

Method of preparation Chorionic Gonadotrophin is a dried preparation of gonad-stimulating hormone obtained from the urine of healthy pregnant women after the manufacturing process to remove or inactivate the virus. It contains not less than 2500 chorionic gonadotrophin Units per mg, and contains not less than 3000 chorionic gonadotrophin Units per mg protein.

It contains not less than 80% and not more than 125% of the labeled Units of chorionic gonadotrophin.

Change the Description to read:

Description Chorionic Gonadotrophin occurs as a white to light yellow-brown powder.

It is freely soluble in water.

Delete the Toxicity and Pyrogen, and add the following:

Bacterial endotoxins Less than 0.03 EU/unit.

Abnormal toxicity Dilute Chorionic Gonadotrophin with isotonic sodium chloride solution so that each mL of the solution contains 120 Units, and use this solution as the sample solution. Inject 5.0 mL of the sample solution into the peritoneal cavity of each of 2 or more of well-nourished, healthy guinea pigs weighing about 350 g, and observe the conditions of the animals for more than 7 days: all the animals exhibit no abnormalities.

Specific activity When calculate from the results obtained by the Assay and the following test, the specific activity is not less than 3000 chorionic gonadotrophin Units per mg protein.

(i) Sample solution—To an exactly amount of Chorionic Gonadotrophin add water to make a solution so that each mL contains about 500 Units of chorionic gonadotrophin according to the labeled amount.

(ii) Standard solution—Weigh accurately about 10 mg of bovine serum albumin, and dissolve in water to make exactly 20 mL. To a suitable volume of this solution add water to make four solutions containing exactly 300, 200, 100 and 50 μg of the albumin per mL, respectively.

(iii) Procedure—Pipet 0.5 mL each of the sample solution and the standard solutions, put them in glass test tubes about 18 mm in inside diameter and about 130 mm in length, add exactly 5 mL of alkaline copper TS, mix, and allow the tubes to stand in a water bath at 30°C for 10 minutes. Then add exactly 0.5 mL of diluted Folin's TS (1 in 2), mix, and warm in a water bath at 30°C for 20 minutes. Determine the absorbances of these solutions at 750 nm as directed under the Ultraviolet-visible Spectrophotometry using a solution obtained in the same manner with 0.5 mL of water as the blank.

Plot the absorbances of the standard solutions on the vertical axis and their protein concentrations on the horizontal axis to prepare a calibration curve, and determine the protein content of the sample solution from its absorbance by using this curve. Then calculate the amount of the protein in the sample.

Change the Assay (i) to read:

Assay (i) Test animals—Select healthy female albino rats weighing about 45 to 65 g.

Chorionic Gonadotrophin for Injection

注射用胎盤性性腺刺激ホルモン

Change the Description to read:

Description Chorionic Gonadotrophin for Injection occurs as a white to light yellow-brown powder or masses.

Add the following next to Identification:

pH Prepare a solution so that each mL of isotonic sodium chloride solution contains 2 mg of Chorionic Gonadotorophin for Injection: the pH of this solution is between 5.0 and 7.0.

Delete the Pyrogaen and add the following:

Bacterial endotoxins Less than 0.03 EU/unit.

Mass variation When calculate the acceptance value using the mean of estimated contents of the units tested as M, it

meets the requirements of the Mass Variation Test.

Foreign insoluble matter Perform the test according to Method 2: it meets the requirements of the Foreign Insoluble Matter Test for Injections.

Insoluble particulate matter Perform the test according to Method 1: it meets the requirements of the Insoluble Particulate Matter Test for Injections.

Sterility Perform the test according to the Membrane filtration method: it meets the requirements of the Sterility Test.

Serum Gonadotrophin

血清性性腺刺激ホルモン

Change the origin/limits of content to read:

Method of preparation Serum Gonadotrophin is a dried preparation of gonad-stimulating hormone, obtained from pregnant mares' serum which has adequately inspected viruses, and subjected to a suitable process for removal or inactivation of viruses. It contains not less than 2000 serum gonadotrophin Units per mg.

It contains not less than 80% and not more than 125% of the labeled Units of serum gonadotrophin.

Change the Description to read:

Description Serum Gonadotrophin occurs as a white powder.

It is freely soluble in water.

Add the following next to Purity:

Specific activity When calculated from the results obtained by the Assay and the following test, Serum Gonadotrophin containes not less than 3000 serum gonadotrophin Units per mg of protein.

(1) Standard solutions—Dissolve about 3 mg of bovine serum albumin in water to make a solution containing 500 μg of the albumin in each mL. To this solution add water to make four standard solutions so that each mL contains exactly 200 μg, 150 μg, 100 μg and 50 μg of the albumin, respectively.

(2) Sample solution—Dissolve about 1 mg of Serum Gonadotrophin in water to make a solution containing exactly 180 μg in each mL.

(3) Sodium carbonate solution—Dissolve 2 g of sodium carbonate (standard reagent) in 0.1 mol/L sodium hydroxide TS to make 100 mL.

(4) Sodium tartrate solution—Dissolve about 1 g of sodium tartrate dihydrate in water to make 100 mL.

(5) Copper (II) sulfate solution—Dissolve 0.5 g of copper (II) sulfate pentahydrate in the sodium tartrate solution to make 100 mL.

(6) Alkaline copper solution—Mix 50 mL of the sodium carbonate solution and 1 mL of the copper (II) sulfate solution. Prepare before use. Use within the day of preparation.

(7) Procedure—Pipet 0.5 mL each of the standard solutions and the sample solution in small test tubes, add 3 mL of the alkaline copper solution to them, and mix. Allow them to stand at the room temperature for not less than 10 minutes, add 0.3 mL of diluted Folin's TS (1 in 2), mix immediately, and allow to stand for not less than 30 minutes. Determine the absorbances of these solutions so obtained at 750 nm as directed under the Ultraviolet-visible Spectrophotometry using a solution, prepared in the same manner with 0.5 mL of water, as the blank. Plot the calibration curve from the absorbances obtained with the standard solutions, and determine the amount of protein in the sample solution from this curve.

$$\text{Specific activity (unit/mg protein)} = \frac{\text{units per mg, obtained in the Assay}}{\text{amount (\%) of protein in the sample}} \times 100$$

Change the Toxicity to read:

Abnormal toxicity Dissolve Serum Gonadotrophin in isotonic sodium chloride solution so that each 5 mL of the solution contains 4000 Units, and use this solution as the sample solution. Inject 5.0 mL of the sample solution into the peritoneal cavity of each of 2 or more of well-nourished, healthy guinea pigs weighing about 350 g, and inject 0.5 mL of the sample solution into the peritoneal cavity of each of 2 or more of well-nourished, healthy mice aged about 5 weeks. Observe the conditions of the animals for more than 7 days: all the animals exhibit no abnormalities.

Change the Pyrogen to read:

Bacterial endotoxins Less than 0.1 EU/unit.

Serum Gonadotrophin for Injection

注射用血清性性腺刺激ホルモン

Change the Description to read:

Description Serum Gonadotrophin for Injection occurs as white powder or masses.

Add the following next to Identification:

pH Dissolve 30 mg of Serum Gonadotrophin for Injection in 20 mL of isotonic sodium chloride solution: the pH of this solution is between 5.0 and 7.0.

Delete the Pyrogen and add the following:

Bacterial endotoxins Less than 0.1 EU/unit.

Add the following:

Lindera Root

Linderae Radix

ウヤク

Lindera Root is the root of *Lindera strychnifolia* Fernandez-Villar (*Lauraceae*).

Description Fusiform or rosary-like root, 10 – 15 cm in length, 10 – 25 mm in diameter; externally yellowish brown to brown, with a few scars of rootlets; a transverse section reveals cortex brown, xylem light yellowish brown, concentric circles and radially arranged lines brown; dense and hard in texture.

Odor, camphor-like; taste, bitter.

Under a microscope, a transverse section of the root with periderm reveals a cork layer several cells thick, partially consisting of cork stone cells; cortex parenchyma sometimes contains oil cells and fibers; in xylem, vessels-xylem fibers and rays are arranged alternately; parenchyatous cells of cortex and xylem contain sandy and columnar crystals of calcium oxalate, simple starch grains 1 – 15 μm in diameter, and 2- to 4- compound starch grains.

Identification To 3 g of pulverized Lindera Root add 40 mL of hexane, and warm under a reflux condenser on a water bath for 30 minutes. After cooling, filter, to the residue add 10 mL of ammonia TS and 30 mL of a mixture of ethyl acetate and diethyl ether (1:1), shake vigorously for 20 minutes, and centrifuge. Separate the supernatant liquid, add 10 g of anhydrous sodium sulfate, shake, and filter. Evaporate the filtrate, dissolve the residue with 0.5 mL of ethanol (99.5), and use this solution as the sample solution. Perform the test with this solution as directed under the Thin-layer Chromatography. Spot 20 μL of the sample solution on a plate of silica gel for thin-layer chromatography, develop the plate with a mixture of ethyl acetate, methanol and ammonia water (28) (10:2:1) to a distance of about 10 cm, and air-dry the plate. Spray evenly Dragendorff's TS for spraying on the plate: a yellow-brown spot appears at around *R*f 0.4.

Loss on drying Not more than 14.0% (6 hours).

Total ash Not more than 2.5%.

Extract content Not less than 6.0% (dilute ethanol-soluble extract).

Add the following:

Lonicera Leaf and Stem

Lonicerae Folium Cum Caulis

ニンドウ

Lonicera Leaf and Stem is the leaves and stems of *Lonicera japonica* Thunberg (*Caprifoliaceae*).

Description Leaves and opposite leaves on short stem; leaf, ovate and entire, with short petiole, 3 – 7 cm in length, 1 – 3 cm in width; upper surface greenish brown, lower surface light grayish green; under a magnifying glass, both surfaces pubescent. Stem, 1 – 4 mm in diameter; externally grayish yellow-brown to purplish brown, a transverse section of stem, round and hollow.

Almost odorless; taste, slightly astringent, followed by a litter bitterness.

Under a microscope, a transverse section of leaf reveals the outermost layer of upper and lower surfaces to be composed of a single-layered epidermis, uni-cellular non-glandular hairs and multi-cellular glandular hairs on epidermis; in midvein, several-layered collenchyma present beneath the epidermis and vascular bundles in the center; in mesophyll, palisade layer adjacent to upper epidermis, spongy tissue adjacent to lower epidermis; glandular hairs contain brown secretion, parenchymatous cells contain aggregate crystals of calcium oxalate, and occasionally starch grains.

Identification To 1 g of pulverized Lonicera Leaf and Stem add 5 mL of methanol, shake for 5 minutes, centrifuge, and use the supernatant liquid as the sample solution. Separately, dissolve 1 mg of chlorogenic acid for thin-layer chromatography in 2 mL of methanol, and use this solution as the standard solution (1). Separately, dissolve 1 mg of loganin for thin-layer chromatography in 2 mL of methanol, and use this solution as the standard solution (2). Perform the test with these solutions as directed under the Thin-layer Chromatography. Spot 10 μL each of the sample solution, the standard solution (1) and the standard solution (2) on a plate of silica gel for thin-layer chromatography. Develop the plate with a mixture of ethyl acetate, water and formic acid (6:1:1) to a distance of about 10 cm, and air-dry the plate. Examine under ultraviolet light (main wavelength: 365 nm): one of the spot among the several spots from the sample solution has the same color tone and *R*f value with the blue-white fluorescent spot from the standard solution (1). Spray evenly 4-methoxybenzaldehyde-sulfuric acid TS on the plate, and heat at 105°C for 5 minutes: one of the spot among the several spots from the sample solution has the same color tone and *R*f value with the red-purple spot from the standard solution (2).

Purity Stem—Lonicera Leaf and Stem does not contains the stems larger than 5 mm in diameter.

Loss on drying Not more than 12.0% (6 hours).

Total ash Not more than 9.0%.

Acid-insoluble ash Not more than 1.0%.

Extract content Not less than 12.0% (dilute ethanol-soluble extract).

Add the following:

Lycium Bark

Lycii Cortex

ジコッピ

Lycium Bark is the root bark of *Lycium chinense* Miller or *Lycium barbarum* Linné (*Solanaceae*).

Description Tubular to semitubular bark, 1 – 6 mm in thickness; externally light brown to light yellowish brown, periderm peeled easily as scale; internally grayish brown, longitudinally striate; brittle in texture; fractured surface, grayish white, not fibrous.

Odor, weak and characteristic; taste, slightly sweet at first.

Under a microscope, a transverse section reveals periderm composed of a cork layer of several layers of thin walled cork cells; in cortex parenchymatous cells containing sandy crystals of calcium oxalate sparsely distributed, occasionally a few fibers observed; parenchymatous cells contain starch grains, 1 – 10 μm in diameter; stone cells very rare.

Identification To 1.0 g of pulverized Lycium Bark add 10 mL of methanol, shake for 15 minutes, filter, and use the filtrate as the sample solution. Perform the test with this solution as directed under the Thin-layer Chromatography. Spot 10 μL of the sample solution on a plate of silica gel for thin-layer chromatography. Develop the plate with a mixture of 1-butanol, water, pyridine and acetic acid (100) (3:1:1:1) to a distance of about 10 cm, and air-dry the plate. Spray evenly Dragendorff's TS for spraying on the plate, heat at 105°C for 3 minutes, then spray evenly sodium nitrite TS: a dark brown principal spot appears at around *R*f 0.5.

Loss on drying Not more than 11.5% (6 hours).

Total ash Not more than 20.0%.

Acid-insoluble ash Not more than 3.0%.

Extract content Not less than 10.0% (dilute ethanol-soluble extract).

Add the following:

Lycium Fruit

Lycii Fructus

クコシ

Lycium Fruit is the fruit of *Lycium chinense* Miller or *Lycium barbarum* Linne (*Solanaceae*).

Description Fusiform fruit with acute apex, 6 – 20 mm in length, 3 – 8 mm in diameter, pericarp red to dark red, externally roughly wrinkled; under a magnifying glass, a transverse section of fruit reveals two locules containing numerous seeds; seed light brown to light yellowish brown, about 2 mm in a diameter, compressed reniform.

Odor, characteristic; taste, sweet, later slightly bitter.

Identification To 1.0 g of powdered Lycium Fruit add 5 mL of ethyl acetate, shake for 15 minutes, filter, and use the filtrate as the sample solution. Perform the test with this solution as directed under the Thin-layer Chromatography. Spot 20 μL of the sample solution on a plate of silica gel for thin-layer chromatography, develop the plate with a mixture of hexane and ethyl acetate (10:1) to a distance of about 10 cm, and air-dry the plate: a yellow principal spot appears at around *R*f 0.6.

Purity Foreign matter—It contains not more than 2.0% of foreign matter such as peduncle or others.

Total ash Not more than 8.0%.

Acid-insoluble ash Not more than 1.0%.

Extract content Not less than 35.0% (dilute ethanol-soluble extract).

Opium Alkaloids Hydrochlorides

塩酸アヘンアルカロイド

Change the origin/limits of content to read:

Opium Alkaloids Hydrochlorides consists of the hydrochlorides of some of the main alkaloids obtained from opium.

It contains not less than 47.0% and not more than 52.0% of morphine ($C_{17}H_{19}NO_3$: 285.34), and not less than 35.0% and not more than 41.0% of other opium alkaloids.

Change the Description to read:

Description Opium Alkaloids Hydrochlorides occur as a white to light brown powder.

It is soluble in water, and slightly soluble in ethanol (99.5).

It is colored by light.

Change the Identification (1) to read:

Identification (1) Dissolve 0.1 g of Opium Alkaloids Hydrochlorides in 10 mL of diluted ethanol (1 in 2), and use this solution as the sample solution. Separately, dissolve 60 mg of Morphine Hydrochloride, 40 mg of Noscapine Hydrochloride, 10 mg of Codein Phosphate and 10 mg of Papaverine Hydrochloride in 10 mL each of diluted ethanol (1 in 2), and use these solutions as the standard solutions (1), (2), (3) and (4), respectively. Perform the test with these solutions as directed under the Thin-layer Chromatography. Spot 20 μL each of the sample solution and the standard solutions on a plate of silica gel with fluorescent indicator for thin-layer chromatography. Develop the plate with a mixture of acetone, toluene, ethanol (99.5) and ammonia solution (28) (20:20:3:1) to a distance of about 10 cm, and air-dry the plate. Examine under ultraviolet light (main wavelength: 254 nm): each spot from the sample solution is the same in color tone and *R*f value with the corresponding spot from the standard solutions (1), (2), (3) and (4) (morphine, noscapine, codeine and papaverine).

Change the Assay to read:

Assay Weigh accurately about 0.1 g of Opium Alkaloids Hydrochlorides, and dissolve in water to make exactly 50 mL, and use this solution as the sample solution. Separately, weigh accurately about 60 mg of morphine hydrochloride for assay, dissolve in water to make exactly 50 mL, and use this solution as the standard solution. Perform the test with exactly 20 μL each of the sample solution and the standard solution as directed under the Liquid Chromatography according to the following conditions, and determine the peak areas of morphine, codeine, papaverine, thebaine, narceine and noscapine, A_{T1}, A_{T2}, A_{T3}, A_{T4}, A_{T5} and A_{T6}, from the sample solution, and the peak area of morphine, A_S, from the standard solution.

$$\text{Amount (mg) of morphine } (C_{17}H_{19}NO_3) = W_S \times \frac{A_{T1}}{A_S} \times 0.887$$

$$\text{Amount (mg) of other opium alkaloids} = W_S \times \frac{A_{T2} + A_{T3} \times 0.29 + A_{T4} \times 0.20 + A_{T5} \times 0.19 + A_{T6}}{AS} \times 0.887$$

W_S: Amount (mg) of morphine hydrochloride for assay, calculated on the anhydrous basis

The relative retention time of codine, papaverine, thebaine, narceine and noscapine with respect to morphine obtained under the following operating conditions are as follows.

Component	Relative retention time
codeine	1.1
papaverine	1.9
thebaine	2.5
narceine	2.8
noscapine	3.6

Operating conditions—

Detector: An ultraviolet absorption photometer (wavelength: 285 nm).

Column: A stainless steel column 4.6 mm in inside diameter and 15 cm in length, packed with octadecylsilanized silica gel for liquid chromatography (5 μm in particle diameter).

Column temperature: A constant temperature of about 40°C.

Mobile phase: Dissolve 1.0 g of sodium lauryl sulfate in 500 mL of diluted phosphoric acid (1 in 1000), and adjust the pH to 3.0 with sodium hydroxide TS. To 240 mL of this solution add 70 mL of tetrahydrofuran, and mix.

Flow rate: Adjust the flow rate so that the retention time of morphine is about 10 minutes.

System suitability—

System performance: Dissolve 60 mg of Morphine Hydrochloride, 10 mg of Codeine Phosphate, 10 mg of Papaverine Hydrochloride and 40 mg of Noscapine Hydrochloride in water to make 50 mL. When the procedure is run with 20 μL of this solution under the above operating conditions, morphine, codeine, papaverine and noscapine are eluted in this order with the complete separation between these peaks and with the resolution between the peaks of morphine and codeine being not less than 1.5.

System repeatability: When the test is repeated 6 times with 20 μL of the standard solution under the above operating conditions, the relative standard deviation of the peak area of morphine is not more than 1.0%.

Powdered Peony Root

シャクヤク末

Change the origin/limits of content to read:

Powdered Peony Root is the powder of Peony Root.

It contains not less than 2.0% of paeoniflorin ($C_{23}H_{28}O_{11}$: 480.46), calculated on the dried basis.

Change the Assay to read:

Assay Weigh accurately about 0.5 g of Powdered Peony Root, add 50 mL of diluted methanol (1 in 2), heat under a reflux condenser on a water bath for 30 minutes, cool, and filter. To the residue add 50 mL of diluted methanol (1 in 2), and proceed in the same manner. Combine the filtrates, add diluted methanol (1 in 2) to make exactly 100 mL, and use this solution as the sample solution. Separately, weigh accurately about 10 mg of Paeoniflorin Reference Standard (separately detarmine the water content), dissolve in diluted methanol (1 in 2) to make exactly 100 mL, and use this solution as the standard solution. Perform the test with exactly 10 μL each of the sample solution and the standard solution as directed under the Liquid Chromatography

according to the following conditions. Determine the peak areas, A_T and A_S, of paeoniflorin in each solution.

$$\text{Amount (mg) of paeoniflorin } (C_{23}H_{28}O_{11}) = W_S \times \frac{A_T}{A_S}$$

W_S: Amount (mg) of Paeoniflorin Reference Standard, calculated on the anhydrous basis

Operating conditions—

Detector: An ultraviolet absorption photometer (wavelength: 232 nm).

Column: A stainless steel column 4.6 mm in inside diameter and 15 cm in length, packed with octadecylsilanized silica gel for liquid chromatography (5 μm in particle diameter).

Column temperature: A constant temperature of about 20°C.

Mobile phase: A mixture of water, acetonitrile and phosphoric acid (850:150:1).

Flow rate: Adjust the flow rate so that the retention time of paeoniflorin is about 10 minutes.

System suitability—

System performance: Dissolve 1 mg each of Paeoniflorin Reference Standard and albiflorin in diluted methanol (1 in 2) to make 10 mL. When the procedure is run with 10 μL of this solution under the above operating conditions, albiflorin and paeoniflorin are eluted in this order with the resolution between these peaks being not less than 2.5.

System repeatability: When the test is repeated 6 times with the standard solution under the above operating conditions, the relative standard deviation of the peak area of paeoniflorin is not more than 1.5%.

Change to read:

Potato Starch

Amylum Solani

バレイショデンプン

Potato Starch consists of starch granules derived from the tuber of *Solanum tuberosum* Linné (*Solanaceae*).

Description Potato Starch occurs as a white powder.

It is practically insoluble in water and in ethanol (99.5).

Identification **(1)** Under a microscope, Potato Starch, preserved in a mixture of water and glycerin (1:1), appears as unevenly ovoid or pyriform simple grains usually 30 – 100 μm, often more than 100 μm in diameter, or spherical simple grains 10 – 35 μm in diameter, rarely 2- to 4-compound grains; ovoid or pyriform simple grains with eccentric hilum, spherical simple grains with non-centric or slightly eccentric hilum; striation distinct in all grains; a black cross, its intersection point on hilum, is observed when grains are put between two polarizing prisms fixed at right angle to each other.

(2) To 1 g of Potato Starch add 50 mL of water, boil for 1 minute, and allow to cool: a subtle white-turbid, pasty liquid is formed.

(3) To 1 mL of the pasty liquid obtained in (2) add 0.05 mL of iodine TS: a orange-red to dark blue-purple color is formed, and the color disappears by heating.

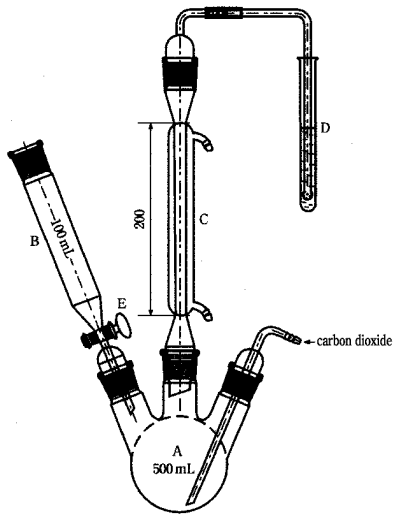
pH Put 5.0 g of Potato Starch in a non-metal vessel, add 25.0 mL of freshly boiled and cooled water, mix gently for 1 minute, and allow to stand for 15 minutes: the pH of the solution is between 5.0 and 8.0.

Purity **(1)** Iron—To 1.5 g of Potato Starch add 15 mL of 2 mol/L hydrochloric acid TS, mix, filter, and use the filtrate as the test solution. To 2.0 mL of Standard Iron Solution add water to make 20 mL, and use as the control solution. Put 10 mL each of the test solution and the control solution in test tubes, add 2 mL of a solution of citric acid (2 in 10) and 0.1 mL of mercapto acetic acid, and mix. Alkalize with ammonia solution (28) to litmus paper, add water to make 20 mL, and mix. Transfer 10 mL each of these solutions into test tubes, allow to stand for 5 minutes, and compare the color of these solutions against a white background: the color of the test solution is not darker than that of the control solution (not more than 10 ppm).

(2) Oxidizing substances—To 4.0 g of Potato Starch add 50.0 mL of water, shake for 5 minutes, and centrifuge. To 30.0 mL of the supernatant liquid add 1 mL of acetic acid (100) and 0.5 to 1.0 g of potassium iodide, shake, and allow to stand for 25 to 30 minutes at a dark place. Add 1 mL of starch TS, and titrate with 0.002 mol/L sodium thiosulfate VS until the color of the solution disappears. Perform a blank determination and make any necessary correction: the volume of 0.002 mol/L sodium thiosulfate VS consumed is not more than 1.4 mL (not more than 20 ppm, calculated as hydrogen peroxide).

(3) Sulfur dioxide—

(i) Apparatus Use as shown in the following figure.



The figures are in mm.

A: Boiling flask (500 mL)
B: Funnel (100 mL)
C: Condenser
D: Test-tube
E: Tap

(ii) Procedure Introduce 150 mL of water into the boiling flask, close the tap of the funnel, and pass carbon dioxide through the whole system at a rate of 100 ± 5 mL per minute. Pass cooling water through the condenser, and place 10 mL of hydrogen peroxide-sodium hydroxide TS in the test-tube. After 15 minutes, remove the funnel without interrupting the stream of carbon dioxide, and introduce through the opening into the flask about 25 g of Potato Starch, accurately weighed, with the aid of 100 mL of water. Apply tap grease to the outside of the connection part of the funnel, and load the funnel. Close the tap of the funnel, pour 80 mL of 2 mol/L hydrochloric acid TS into the funnel, open the tap to introduce the hydrochloric acid into the flask, and close the tap while several mL of the hydrochloric acid remains, in order to avoid losing sulfur dioxide. Place the flask in a water bath, and heat the mixture for 1 hour. Transfer the contents of the test-tube with the aid of a little water to a wide-necked conical flask. Heat in a water bath for 15 minutes, and cool. Add 0.1 mL of bromophenol blue TS, and titrate with 0.1 mol/L sodium hydroxide VS until the color changes from yellow to violet-blue lasting for at least 20 seconds. Perform a blank determination and make any necessary correction. Calculate the amount of sulfur dioxide by applying the following formula: it is not more than 50 ppm.

$$\text{Amount (ppm) of sulfur dioxide} = \frac{V}{W} \times 1000 \times 3.203$$

W: Amount (g) of the sample
V: Amount (mL) of 0.1 mol/L sodium hydroxide VS consumed

Loss on drying Not more than 20.0% (1 g, 130°C, 90 minutes).

Residue on ignition Not more than 0.6% (1 g).

Containers and storage Containers—Well-closed containers.

Add the following:

Processed Aconite Root

Processi Aconiti Radix

ブシ

Processed Aconite Root is the tuberous root of *Aconitum carmichaeli* Debeaux or *Aconitum japonicum* Thunberg (*Ranunculaceae*) prepared by the following processes.

Process 1: Autoclaving. [Processed Aconite Root 1]

Process 2: Heating or autoclaving after rinsing in the salt, rock salt or calcium chloride water. [Processed Aconite Root 2]

Process 3: Treating with lime after rinsing in the salt water. [Processed Aconite Root 3]

There are three kinds of Processed Aconite Root based on their preparing processes as shown above.

Processed Aconite Root 1, Processed Aconite Root 2 and Processed Aconite Root 3 contain the total alkaloid [as benzoylaconine ($C_{32}H_{45}NO_{10}$: 603.70)] of not less than 0.7% and not more than 1.5%, not less than 0.1% and not more than 0.6%, and not less than 0.5% and not more than 0.9%, calculated on the dried bases, respectively.

The label indicates the treating process.

Description

Processed Aconite Root 1: Cut pieces irregularly polygonal, less than 10 mm in diameter; externally dark grayish brown to blackish brown; hard in texture; cut surface flat, light brown to dark brown, usually horny and lustrous.

Odor, weak and characteristic.

Under a microscope, transverse and longitudinal sections reveal pitted, scaraliform, reticulate and spiral vessels; starch grains in parenchymatous cells usually gelatinized but sometimes not gelatinized; starch grains, simple, spherical or ellipsoid, 2 – 25 μm in diameter, or 2- to a dozen or so- compound, hilum of starch grain distinct.

Processed Aconite Root 2: Nearly obconical root, 15 – 30 mm in length, 12 – 16 mm in diameter, slices cut longitudinally or transversely, 20 – 60 mm in length, 15 – 40 mm in width, and 200 – 700 μm in thickness, or cut pieces irregularly polygonal, less than 12 mm in diameter; externally light brown to dark brown or yellowish brown; hard in texture, usually without wrinkles; cut surface flat, light brown to dark brown or yellowish white to light yellowish brown, usually horny, semi-transparent and lustrous.

Odor, weak and characteristic.

Under a microscope, transverse and longitudinal sections reveal metaderm, primary cortex, endodermis, secondary cortex, cambium, and xylem; primary cortex contains oblong to oblong-square sclerenchymatous cells, 30 – 75 μm in short axis, 60 – 150 μm in long axis; endodermis single layered, endodermal cells elongated in tangential direction; cambium, star shaped or irregular polygons to orbicular; a group of vessel in xylem v-shaped; sometimes isolated ring of cambium appears in secondary cortex or in pith; vessels, pitted, scaraliform, reticulate and spiral; starch grains in parenchymatous cells gelatinized.

Processed Aconite Root 3: Cut pieces irregularly polygonal, less than 5 mm in diameter; externally grayish brown; hard in texture; cut surface flat, light grayish brown to grayish white, not lustrous.

Odor, weak and characteristic.

Under a microscope, transverse and longitudinal sections reveal pitted, scaraliform, reticulate and spiral vessels; starch grains, simple, spherical or ellipsoid, 2 – 25 μm in diameter, or 2- to a dozen or so- compound, hilum of starch grain distinct.

Identification To 3 g of pulverized Processed Aconite Root add 20 mL of diethyl ether and 2 mL of ammonia TS, shake for 10 minutes, and centrifuge. Evaporate the ether layer to dryness under reduced pressure, dissolve the residue in 1 mL of diethyl ether, and use this solution as the sample solution. Separately, dissolve 1 mg of benzoylmesaconine hydrochloride for thin-layer chromatography in 10 mL of ethanol (99.5), and use this solution as the standard solution. Perform the test with these solutions as directed under the Thin-layer Chromatography. Spot 10 μL each of the sample solution and the standard solution on a plate of silica gel for thin-layer chromatography, develop the plate with a mixture of ethyl acetate, ethanol (99.5) and ammonia water (28) (40:3:2) to a distance of about 10 cm, and air-dry the plate. Spray evenly Dragendorff's TS for spraying on the plate, air-dry the plate, and spray evenly sodium nitrite TS: one of the spot among the several spots from the sample solution has the same color tone and *R*f value with the yellow-brown spot from the standard solution.

Purity Aconitum diester alkaloids (aconitine, jesaconitine, hypaconitine and mesaconitine)—Weigh accurately about 0.5 g of pulverized Processed Aconite Root, put in a glass-stoppered centrifuge tube, suspend in 3.0 mL of water by shaking, and add 1.0 mL of ammonia TS and 20 mL of diethyl ether. Stopper tightly the tube, shake for 30 minutes, centrifuge, and separate the ether layer. To the residue add 1.0 mL of ammonia TS and 20 mL of diethyl ether, and repeat the above process two times. Combine all extracts, evaporate to dryness under reduced pressure at not more than 40°C, and dissolve the residue with exactly 10 mL of a mixture of phosphate buffer solution for aconite root and acetonitrile (1:1). Centrifuge this solution, and use the supernatant liquid as the sample solution. Perform the test with exactly 20 μL each of the sample solution and the aconitum diester alkaloids standard solution as directed under the Liquid Chromatography according to the following conditions, and determine the heights of the peaks corresponding to aconitine, jesaconitine, hypaconitine and mesaconitine, H_{TA} and H_{SA}, H_{TJ} and H_{SJ}, H_{TH} and H_{SH}, and H_{TM} and H_{SM}, respectively, and calculate the amounts of them by the following formulae: the amounts of aconitine, jesaconitine, hypaconitine and mesaconitine per g calculated on the dried basis are not more than 60 μg, 60 μg, 280 μg and 140 μg, respectively, and the total amount of them is not more than 450 μg.

$$\text{Amount } (\mu g) \text{ of aconitine } (C_{34}H_{47}NO_{11}) = \frac{C_{SA}}{W} \times \frac{H_{TA}}{H_{SA}} \times 10$$

$$\text{Amount } (\mu g) \text{ of jesaconitine } (C_{35}H_{49}NO_{12}) = \frac{C_{SJ}}{W} \times \frac{H_{TJ}}{H_{SJ}} \times 10$$

$$\text{Amount } (\mu g) \text{ of hypaconitine } (C_{33}H_{45}NO_{10}) = \frac{C_{SH}}{W} \times \frac{H_{TH}}{H_{SH}} \times 10$$

$$\text{Amount } (\mu g) \text{ of mesaconitine } (C_{33}H_{45}NO_{11}) = \frac{C_{SM}}{W} \times \frac{H_{TM}}{H_{SM}} \times 10$$

C_{SA}: Concentration (μg/mL) of aconitine for purity in the aconitum diester alkaloids standard solution for purity

C_{SJ}: Concentration (μg/mL) of jesaconitine for purity in the aconitum diester alkaloids standard solution for purity

C_{SH}: Concentration (μg/mL) of hypaconitine for purity in the aconitum diester alkaloids standard solution for purity

C_{SM}: Concentration (μg/mL) of mesaconitine for purity in the aconitum diester alkaloids standard solution for purity

W: Amount (g) of the sample, calculated on the dried basis

Operating conditions—

Detector: An ultraviolet absorption photometer (wavelength: 231 nm for aconitine, hypaconitine and mesaconitine; 254 nm for jesaconitine).

Column: A stainless steel column 4.6 mm in inside diameter and 15 cm in length, packed with octadecylsilanized silica gel for liquid chromatography (5 μm in particle diameter).

Column temperature: A constant temperature of about 40°C.

Mobile phase: A mixture of phosphate buffer solution for aconite root and tetrahydrofuran (183:17).

Flow rate: Adjust the flow rate so that the retention time of mesaconitine is about 31 minutes.

System suitability—

System performance: When the procedure is run with 20 μL of the aconitum diester alkaloids standard solution for purity under the above operating conditions, using 254

nm, mesaconitine, hypaconitine, aconitine and jesaconitine are eluted in this order, and each resolution between their peaks is not less than 1.5, respectively.

System repeatability: To 1 mL of aconitum diester alkaloids standard solution for purity add a mixture of phosphate buffer solution for aconite root and acetonitrile (1:1) to make 10 mL. When the test is repeated 6 times with 20 μL of this solution under the above operating conditions, using 231 nm, the relative standard deviation of the peak height of mesaconitine is not more than 1.5%.

Loss on drying Not more than 15.0% (6 hours).

Total ash

Processed Aconite Root 1: Not more than 4.0%.
Processed Aconite Root 2: Not more than 12.0%.
Processed Aconite Root 3: Not more than 19.0%.

Acid-insoluble ash Not more than 0.9%.

Assay Weigh accurately about 2 g of pulverized Processed Aconite Root, put in a glass-stoppered centrifuge tube, and add 1.6 mL of ammonia TS and 20 mL of diethyl ether. Stopper tightly the tube, shake for 30 minutes, centrifuge, and separate the ether layer. To the residue add 0.8 mL of ammonia TS and 20 mL of diethyl ether, and proceed as above. Repeat this process more three times. Combine all extracts, evaporate to dryness under reduced pressure, dissolve the residue in 5 mL of ethanol (99.5), add 30 mL of freshly boiled and cooled water, and titrate with 0.01 mol/L hydrochloric acid VS until the color of the solution changes from green to gray-blue through blue-green (indicator: 3 drops of methyl red-methylene blue TS). Perform a blank determination and make any necessary correction.

Each mL of 0.01 mol/L hydrochloric acid VS
= 6.037 mg of total alkaloid [as benzoylaconine ($C_{32}H_{45}NO_{10}$: 603.70)]

Add the following:

Powdered Processed Aconite Root

Processi Aconiti Radix Pulverata

ブシ末

Powdered Processed Aconite Root is the powder of Processed Aconite Root prepared by Process 1 or Process 2, or the powder of the tuberous root of *Aconitum carmichaeli* Debeaux or *Aconitum japonicum* Thunberg (*Ranunculaceae*) prepared by Process 1.

Occasionally, it contains Corn Starch or Lactose.

Process 1: Autoclaving. [Powdered Processed Aconite Root 1]

Process 2: Heating or autoclaving after rinsing in the salt, rock salt or calcium chloride water. [Powdered Processed Aconite Root 2]

There are two kinds of Powdered Processed Aconite Root based on their preparing processes as shown above.

Powdered Processed Aconite Root 1 and Powdered Processed Aconite Root 2 contain the total alkaloid [as benzoylaconine ($C_{32}H_{45}NO_{10}$: 603.70)] of not less than 0.4% and not more than 1.2%, and not less than 0.1% and not more than 0.3%, calculated on the dried bases, respectively.

The label indicates the treating process.

Description

Powdered Processed Aconite Root 1: Powdered Processed Aconite Root 1 occurs as a light grayish brown powder. It has a characteristic odor.

Under a microscope, Powered Processed Aconite Root 1 reveals gelatinized starch masses or starch grains and parenchymatous cells containing them, fragments of reddish brown metaderm, fragments of pitted, scaraliform, reticulate and spiral vessels; also square to oblong-square sclerenchymatous cells, 30 – 150 μm in diameter, 100 – 250 μm in length, cell wall of sclerenchymatous cells, 6 – 12 μm in thickness; starch grains of ''Processed Aconite Root'', simple, spherical or ellipsoid, 2 – 25 μm in diameter, or 2- to a dozen or so- compound, hilum of starch grain distinct.

Powdered Processed Aconite Root 2: Powdered Processed Aconite Root 2 occurs as a light yellowish white powder. It has a characteristic odor.

Under a microscope, Powered Processed Aconite Root 2 reveals gelatinized starch masses and parenchymatous cells containing them, fragments of reddish brown metaderm, fragments of pitted, scaraliform, reticulate and spiral vessels; also square to oblong-square sclerenchymatous cells, 30 – 150 μm in diameter, 100 – 250 μm in length, cell wall of sclerenchymatous cells, 6 – 12 μm in thickness.

Identification To 3 g of Powdered Processed Aconite Root add 2 mL of ammonia TS and 20 mL of diethyl ether, shake for 10 minutes, and centrifuge. Evaporate the ether layer to dryness under reduced pressure, dissolve the residue in 1 mL of diethyl ether, and use this solution as the sample solution. Separately, dissolve 1 mg of benzoylmesaconine hydrochloride for thin-layer chromatography in 10 mL of ethanol (99.5), and use this solution as the standard solution. Perform the test with these solutions as directed under the Thin-layer Chromatography. Spot 10 μL each of the sample solution and the standard solution on a plate of silica gel for thin-layer chromatography, develop the plate with a mixture of ethyl acetate, ethanol (99.5) and ammonia water (28) (40:3:2) to a distance of about 10 cm, and air-dry the plate. Spray evenly Dragendorff's TS for spraying on the plate, air-dry the plate, and spray evenly sodium nitrite TS: one of the spot among the several spots from the sample solution has the same color tone and *R*f value with the yellow-brown spot from the standard solution.

Purity Aconitum diester alkaloids (aconitine, jesaconitine, hypaconitine and mesaconitine)—Weigh accurately about 0.5 g of Powdered Processed Aconite Root, put in a glass-stoppered centrifuge tube, suspend in 3.0 mL of water by

shaking, and add 1.0 mL of ammonia TS and 20 mL of diethyl ether. Stopper tightly the tube, shake for 30 minutes, centrifuge, and separate the ether layer. To the residue add 1.0 mL of ammonia TS and 20 mL of diethyl ether, and repeat the above process two times. Combine all extracts, evaporate to dryness under reduced pressure at not more than 40°C, and dissolve the residue with exactly 10 mL of a mixture of phosphate buffer solution for aconite root and acetonitrile (1:1). Centrifuge this solution, and use the supernatant liquid as the sample solution. Perform the test with exactly 20 μL each of the sample solution and the aconitum diester alkaloids standard solution as directed under the Liquid Chromatography according to the following conditions, and determine the heights of the peaks corresponding to aconitine, jesaconitine, hypaconitine and mesaconitine, H_{TA} and H_{SA}, H_{TJ} and H_{SJ}, H_{TH} and H_{SH}, and H_{TM} and H_{SM}, respectively, and calculate the amounts of them by the following formulae: the amounts of aconitine, jesaconitine, hypaconitine and mesaconitine per g calculated on the dried basis are not more than 55 μg, 40 μg, 55 μg and 120 μg, respectively, and the total amount of them is not more than 230 μg.

$$\text{Amount } (\mu g) \text{ of aconitine } (C_{34}H_{47}NO_{11}) = \frac{C_{SA}}{W} \times \frac{H_{TA}}{H_{SA}} \times 10$$

$$\text{Amount } (\mu g) \text{ of jesaconitine } (C_{35}H_{49}NO_{12}) = \frac{C_{SJ}}{W} \times \frac{H_{TJ}}{H_{SJ}} \times 10$$

$$\text{Amount } (\mu g) \text{ of hypaconitine } (C_{33}H_{45}NO_{10}) = \frac{C_{SH}}{W} \times \frac{H_{TH}}{H_{SH}} \times 10$$

$$\text{Amount } (\mu g) \text{ of mesaconitine } (C_{33}H_{45}NO_{11}) = \frac{C_{SM}}{W} \times \frac{H_{TM}}{H_{SM}} \times 10$$

C_{SA}: Concentration (μg/mL) of aconitine for purity in the aconitum diester alkaloids standard solution for purity

C_{SJ}: Concentration (μg/mL) of jesaconitine for purity in the aconitum diester alkaloids standard solution for purity

C_{SH}: Concentration (μg/mL) of hypaconitine for purity in the aconitum diester alkaloids standard solution for purity

C_{SM}: Concentration (μg/mL) of mesaconitine for purity in the aconitum diester alkaloids standard solution for purity

W: Amount (g) of the sample, calculated on the dried basis

Operating conditions—

Detector: An ultraviolet absorption photometer (wavelength: 231 nm for aconitine, hypaconitine and mesaconitine; 254 nm for jesaconitine).

Column: A stainless steel column 4.6 mm in inside diameter and 15 cm in length, packed with octadecylsilanized silica gel for liquid chromatography (5 μm in particle diameter).

Column temperature: A constant temperature of about 40°C.

Mobile phase: A mixture of phosphate buffer solution for aconite root and tetrahydrofuran (183:17).

Flow rate: Adjust the flow rate so that the retention time of mesaconitine is about 31 minutes.

System suitability—

System performance: When the procedure is run with 20 μL of the aconitum diester alkaloids standard solution for purity under the above operating conditions, using 254 nm, mesaconitine, hypaconitine, aconitine and jesaconitine are eluted in this order, and each resolution between their peaks is not less than 1.5, respectively.

System repeatability: To 1 mL of aconitum diester alkaloids standard solution for purity add a mixture of phosphate buffer solution for aconite root and acetonitrile (1:1) to make 10 mL. When the test is repeated 6 times with 20 μL of this solution under the above operating conditions, using 231 nm, the relative standard deviation of the peak height of mesaconitine is not more than 1.5%.

Loss on drying Not more than 11.0% (6 hours).

Total ash

Powdered Processed Aconite Root 1: Not more than 4.0%.

Powdered Processed Aconite Root 2: Not more than 7.0%.

Acid-insoluble ash Not more than 0.7%.

Assay Weigh accurately about 2 g of Powdered Processed Aconite Root, put in a glass-stoppered centrifuge tube, and add 1.6 mL of ammonia TS and 20 mL of diethyl ether. Stopper tightly the tube, shake for 30 minutes, centrifuge, and separate the ether layer. To the residue add 0.8 mL of ammonia TS and 20 mL of diethyl ether, and proceed as above. Repeat this process more three times. Combine all extracts, evaporate to dryness under reduced pressure, dissolve the residue in 5 mL of ethanol (99.5), add 30 mL of freshly boiled and cooled water, and titrate with 0.01 mol/L hydrochloric acid VS until the color of the solution changes from green to gray-blue through blue-green (indicator: 3 drops of methyl red-methylene blue TS). Perform a blank determination and make any necessary correction.

Each mL of 0.01 mol/L hydrochloric acid VS
= 6.037 mg of total alkaloid [as benzoylaconine ($C_{32}H_{45}NO_{10}$: 603.70)]

Add the following:

Processed Ginger

Zingiberis Processum Rhizoma

カンキョウ

Processed Ginger is the rhizome of *Zingiber officinale* Roscoe (*Zingiberaceae*), after being passed through hot water or being steamed.

Description Irregularly compressed and often branched massive rhizome; branched parts slightly curved ovoid or oblong-ovoid, 2 – 4 cm in length, and 1 – 2 cm in diameter; external surface grayish yellow to grayish yellow-brown, with wrinkles and ring node; fractured surface brown to dark brown, transparent and horny; under a magnifying glass, a transverse section reveals cortex and stele distinctly divided; vascular bundles scattered throughout the surface.

Odor, characteristic; taste, extremely pungent.

Under a microscope, a transverse section reveals cork layer, cortex and stele in this order from the outside; cortex and stele, divided by a single-layered endodermis, composed of parenchyma, vascular bundles scattered and surrounded by fiber bundles; oil cells contain yellow oil-like substances, scattered in parenchyma; parenchymatous cells contain solitary crystals of calcium oxalate, and gelatinized starch.

Identification To 2 g of pulverized Processed Ginger add 5 mL of diethyl ether, shake for 10 minutes, filter, and use the filtrate as the sample solution (1). To the residue add 5 mL of methanol, proceed in the same manner as above, and use so obtained solution as the sample solution (2). Separately, dissolve 1 mg of [6]-shogaol for thin-layer chromatography in 2 mL of methanol, and use this solution as the standard solution (1). Separately, dissolve 1 mg of Sucrose in 2 mL of methanol, and use this solution as the standard solution (2). Perform the test with these solutions as directed under the Thin-layer Chromatography. Spot 10 μL each of the sample solution (1) and the standard solution (1) on a plate of silica gel for thin-layer chromatography. Develop the plate with a mixture of ethyl acetate and hexane (1:1) to a distance of about 10 cm, and air-dry the plate. Spray evenly 4-dimethylaminobenzaldehyde TS on the plate, heat at 105°C for 5 minutes, and allow to cool: one of the spot among the several spots from the sample solution (1) has the same color tone and *R*f value with the green spot from the standard solution (1). Spot 10 μL each of the sample solution (2) and the standard solution (2) on a plate of silica gel for thin-layer chromatography, develop the plate with a mixture of 1-butanol, water and acetic acid (100) (8:5:3) to a distance of about 10 cm, and air-dry the plate. Spray evenly 1,3-naphthalenediol TS on the plate, and heat at 105°C for 5 minutes: one of the spot among the several spots from the sample solution (2) has the same color tone and *R*f value with the red-purple spot from the standard solution (2).

Loss on drying Not more than 15.0% (6 hours).

Total ash Not more than 6.5%.

Acid-insoluble ash Not more than 1.5%.

Extract content Not less than 8.0% (dilute ethanol-soluble extract).

Rhubarb

ダイオウ

Change the origin/limits of content to read:

Rhubarb is usually the rhizome of *Rheum palmatum* Linné, *Rheum tanguticum* Maximowicz, *Rheum officinale* Baillon, *Rheum coreanum* Nakai or their interspecific hybrids (*Polygonaceae*).

It contains not less than 0.25% of sennosides A ($C_{42}H_{38}O_{20}$: 862.74), calculated on the basis of dried material.

Powdered Rhubarb

ダイオウ末

Change the origin/limits of content to read:

Powdered Rhubarb is the powder of Rhubarb.

It contains not less than 0.25% of sennoside A ($C_{42}H_{38}O_{20}$: 862.74), calculated on the basis of dried materials.

Add the following:

Sappan Wood

Sappan Lignum

ソボク

Sappan Wood is the duramen of *Caesalpinia sappan* Linné (*Leguminosae*).

Description Chips, slices or short pieces of wood; yellowish red to grayish yellow-brown, sometimes with light brown to grayish white splint woods; hard in texture; a transverse section shows a pattern like annual ring.

Almost odorless; almost tasteless.

Under a microscope, a transverse section reveals ray composed of 1 – 2 rows of slender and long cells; the area between rays filled with fiber cells, and large and oblong vessels scattered there; solitary crystals of calcium oxalate in parenchymatous cells of the innermost of xylem.

Identification To 0.5 g of pulverized Sappan Wood add 10 mL of dilute ethanol, shake, and filter. To 5 mL of the filtrate add 2 to 3 drops of sodium hydroxide TS: a dark red

color develops.

Purity Put a small piece of Sappan Wood in calcium hydroxide TS: no purple-blue color develops.

Loss on drying Not more than 11.5% (6 hours).

Total ash Not more than 2.0%.

Extract content Not less than 7.0% (dilute ethanol-soluble extract).

Powdered Scutellaria Root

オウゴン末

Change the origin/limits of content to read:

Powdered Scutellaria Root is the powder of Scutellaria Root.

It contains not less than 10.0% of baicalin ($C_{21}H_{18}O_{11}$: 446.36), calculated on the basis of dried material.

Add the following:

Termeric

Curcumae Rhizoma

ウコン

Termeric is the rhizome or being removed the cork layer from it of *Curcuma longa* Linné (*Zingiberaceae*), usually after being passed through hot water.

Description Termeric is a main rhizome or a lateral rhizome; main rhizome, nearly ovoid, about 3 cm in diameter, about 4 cm in length; lateral rhizome, cylindrical, with round tips, curved, about 1 cm in diameter, 2 - 6 cm in length; both main and lateral rhizomes with cyclic nodes; rhizome with cork layer, yellowish brown, lustrous; rhizome without cork layer, dark yellowish red, with yellowish red powders on surface; hard in texture, not easily broken; transversely cut surface yellowish brown to reddish brown, lustrous like wax.

Odor, characteristic; taste, slightly bitter and stimulant, it colors a saliva yellow on chewing.

Under a microscope, a transverse section reveals the outermost layer to be composed of a cork layer 4 – 10 cells thick; sometimes a cork layer partly remains; cortex and stele, divided by a single-layered endodermis, composed of parenchyma, vascular bundles scattered; oil cells scattered in parenchyma; parenchymatous cells contain yellow substances, sandy and solitary crystals of calcium oxalate, and gelatinized starch.

Identification To 0.5 g of pulverized Termeric add 20 mL of methanol, shake for 15 minutes, filter and use the filtrate as the sample solution. Perform the test with this solution as directed under the Thin-layer Chromatography. Spot 5 μL of the sample solution on a plate of silica gel for thin-layer chromatography. Develop the plate with a mixture of ethyl acetate, hexane and acetic acid (100) (70:30:1) to a distance of about 10 cm, and air-dry the plate: a yellow spot appears at around *R*f 0.4.

Loss on drying Not more than 17.0% (6 hours).

Total ash Not more than 7.5%.

Acid-insoluble ash Not more than 1.0%.

Extract content Not less than 9.0% (dilute ethanol-soluble extract).

Add the following:

Tribulus Fruit

Tribuli Fructus

シツリシ

Tribulus Fruit is the fruit of *Tribulus terrestris* Linné (*Zygophyllaceae*).

Description Pentagonal star shaped fruit, composed of five mericarps, 7 – 12 mm in diameter, often each mericarp separated; externally grayish green to grayish brown; a pair of longer and shorter spines on surface of each mericarp, the longer spine 3 – 7 mm in length, the shorter one 2 – 5 mm in length, numerous small processes on midrib; pericarp hard in texture, cut surface light yellow; each mericarp contains 1 – 3 seeds.

Almost odorless; taste, mild at first, followed by bitterness.

Under a microscope, a transverse section reveals epicarp composed of a single-layered epidermis; mesocarp composed of parenchyma and sclerenchyma layer; endocarp composed of several-layered fiber cells; a single-layer of cell between mesocarp and endocarp contain solitary crystals of calcium oxalate; cotyledons of seed contain oil drops and aleurone grains, and occasionally starch grains.

Identification To 2 g of pulverized Tribulus Fruit add 5 mL of methanol, shake for 10 minutes, filter, and use the filtrate as the sample solution. Perform the test with this solution as directed under the Thin-layer Chromatography. Spot 10 μL of the sample solution on a plate of silica gel for thin-layer chromatography, develop the plate with a mixture of ethyl acetate and water (40:1) to a distance of about 10 cm, and air-dry the plate. Spray evenly dilute sulfuric acid on the plate, heat at 105°C for 5 minutes, and examine under ultraviolet light (main wavelength: 365 nm): a blue-white fluorescent spot appears at around *R*f 0.4.

Purity **(1)** Peduncle—Not more than 4.0%.

(2) Foreign matters—Not more than 1.0% of foreign

matters other than peduncle.

Loss on drying Not more than 11.0% (6 hours).

Total ash Not more than 13.0%.

Acid-insoluble ash Not more than 1.5%.

Extract content Not less than 8.5% (dilute ethanol-soluble extract).

Uncaria Thorn

チョウトウコウ

Change the origin/limits of the content to read:

Uncaria Thorn is, usually the prickle, of *Uncaria rhynchophylla* Miquel, *Uncaria sinensis* Haviland or *Uncaria macrophylla* Wallich (*Rubiaceae*).

Uncaria Thorn contains not less than 0.03% of total alkaloids (rhynchophylline and hirstine), calculated on the dried basis.

Change the Component determination to read:

Component determination Weigh accurately about 0.2 g of medium powdered Uncaria Thorn, transfer into a glass-stoppered centrifuge tube, add 30 mL of a mixture of methanol and dilute acetic acid (7:3), shake for 30 minutes, centrifuge, and separate the supernatant liquid. To the residue add two 10-mL portions of a mixture of methanol and dilute acetic acid (7:3), proceed in the same manner, and combine all of the supernatant liquid. To the combined liquid add a mixture of methanol and dilute acetic acid (7:3) to make exactly 50 mL, and use this as the sample solution. Separately, weigh accurately about 5 mg of rhynchophylline for component determination, previously dried in a desiccator (silica gel) for 24 hours, and dissolve in a mixture of methanol and dilute acetic acid (7:3) to make exactly 100 mL. Pipet 1 mL of this solution, add a mixture of methanol and dilute acetic acid (7:3) to make exactly 10 mL, and use this solution as the standard solution (1). Separately, dissolve 1 mg of hirsutine in 100 mL of a mixture of methanol and dilute acetic acid (7:3), and use this solution as the standard solution (2). Perform the test with exactly 20 μL each of the sample solution and the standard solution (1) and (2) as directed under the Liquid Chromatography according to the following conditions, and determine the peak areas, A_{Ta} and A_{Tb}, of rhynchophylline and hirsutine obtained from the sample solution, and the peak area, A_S, of rhynchophylline from the standard solution (1).

Amount (mg) of total alkaloids (rhynchophylline and hirstine)

$$= W_S \times \frac{A_{Ta} + 1.405A_{Tb}}{A_S} \times \frac{1}{20}$$

W_S: Amount (mg) of rhynchophylline for component determination

Operating conditions—

Detector: An ultraviolet absorption photometer (wavelength: 245 nm).

Column: A stainless steel column 4.6 mm in inside diameter and 25 cm in length, packed with octadecylsilanized silica gel for liquid chromatography (5 μm in particle diameter).

Column temperature: A constant temperature of about 40°C.

Mobile phase: Dissolve 3.85 g of ammonium acetate in 200 mL of water, add 10 mL of acetic acid (100) and water to make 1000 mL, and add 350 mL of acetonitrile.

Flow rate: Adjust the flow rate so that the retention time of rhynchophylline is about 17 minutes.

System suitability—

System performance: Dissolve 5 mg of rhynchophylline for component determination in 100 mL of a mixture of methanol and dilute acetic acid (7:3). To 5 mL of this solution add 1 mL of ammonia solution (28), and reflux for 10 minutes or warm at about 50°C for 2 hours. After cooling, to 1 mL of the solution so obtained add a mixture of methanol and dilute acetic acid (7:3) to make 5 mL. When the procedure is run with 20 μL of this solution under the above operating conditions, the peak of isorhynchophylline is appears in addition to the peak of rhynchophylline, and the resolution between these peaks is not less than 1.5.

System repeatability: When the test is repeated 6 times with 20 μL of the standard solution (1) under the above operating conditions, the relative standard deviation of the peak areas of rhynchophylline is not more than 1.5%.

Change to read:

Wheat Starch

Amylum Tritici

コムギデンプン

Wheat Starch consists of the starch granules obtained from the seeds of *Triticum aestivum* Linné (*Gramineae*).

Description Wheat Starch occurs as white masses or powder.

It is practically insoluble in water and in ethanol (99.5).

Identification (1) Under a microscope, Wheat Starch, preserved in a mixture of water and glycerin (1:1), appears as large and small sized simple grains, or quite rarely median sized simple grains; usually, large sized grains usually 10 – 60 μm in diameter, from upper view, disc like or quite rarely reniform, centric hilum and striation indistinct or hardly distinct, often cleft on marginal portion visible; from lateral view, narrowly ellipsoid or fusiform, hilum recognized as a cleft along with long axis; small sized grains 2 – 10 μm in diameter, spherical or polygonal; a black cross, its intersection point on hilum, is observed when grains are put between

two polarizing prisms fixed at right angle to each other.

(2) To 1 g of Wheat Starch add 50 mL of water, boil for 1 minute, and allow to cool: a subtle white-turbid, pasty liquid is formed.

(3) To 1 mL of the pasty liquid obtained in (2) add 0.05 mL of iodine TS: a dark blue-purple color is formed, and the color disappears by heating.

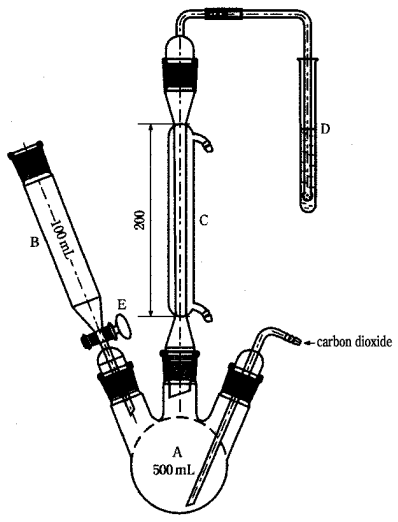
pH Put 5.0 g of Wheat Starch in a non-metal vessel, add 25.0 mL of freshly boiled and cooled water, mix gently for 1 minute, and allow to stand for 15 minutes: the pH of the solution is between 4.5 and 7.0.

Purity **(1)** Iron—To 1.5 g of Wheat Starch add 15 mL of 2 mol/L hydrochloric acid TS, mix, filter, and use the filtrate as the test solution. To 2.0 mL of Standard Iron Solution add water to make 20 mL, and use as the control solution. Put 10 mL each of the test solution and the control solution in test tubes, add 2 mL of a solution of citric acid (2 in 10) and 0.1 mL of mercapto acetic acid, and mix. Alkalize with ammonia solution (28) to litmus paper, add water to make 20 mL, and mix. Transfer 10 mL each of these solutions into test tubes, allow to stand for 5 minutes, and compare the color of these solutions against a white background: the color of the test solution is not darker than that of the control solution (not more than 10 ppm).

(2) Oxidizing substances—To 4.0 g of Wheat Starch add 50.0 mL of water, shake for 5 minutes, and centrifuge. To 30.0 mL of the supernatant liquid add 1 mL of acetic acid (100) and 0.5 to 1.0 g of potassium iodide, shake, and allow to stand for 25 to 30 minutes at a dark place. Add 1 mL of starch TS, and titrate with 0.002 mol/L sodium thiosulfate VS until the color of the solution disappears. Perform a blank determination and make any necessary correction: the volume of 0.002 mol/L sodium thiosulfate VS consumed is not more than 1.4 mL (not more than 20 ppm, calculated as hydrogen peroxide).

(3) Sulfur dioxide—

(i) Apparatus Use as shown in the following figure.



The figures are in mm.

A: Boiling flask (500 mL)
B: Funnel (100 mL)
C: Condenser
D: Test-tube
E: Tap

(ii) Procedure Introduce 150 mL of water into the boiling flask, close the tap of the funnel, and pass carbon dioxide through the whole system at a rate of 100 ± 5 mL per minute. Pass cooling water through the condenser, and place 10 mL of hydrogen peroxide-sodium hydroxide TS in the test-tube. After 15 minutes, remove the funnel without interrupting the stream of carbon dioxide, and introduce through the opening into the flask about 25 g of Wheat Starch, accurately weighed, with the aid of 100 mL of water. Apply tap grease to the outside of the connection part of the funnel, and load the funnel. Close the tap of the funnel, pour 80 mL of 2 mol/L hydrochloric TS acid into the funnel, open the tap to introduce the hydrochloric acid into the flask, and close the tap while several mL of the hydrochloric acid remains, in order to avoid losing sulfur dioxide. Place the flask in a water bath, and heat the mixture for 1 hour. Transfer the contents of the test-tube with the aid of a little water to a wide-necked conical flask. Heat in a water bath for 15 minutes, and cool. Add 0.1 mL of bromophenol blue TS, and titrate with 0.1 mol/L sodium hydroxide VS until the color changes from yellow to violet-blue lasting for at least 20 seconds. Perform a blank determination and make any necessary correction. Calculate the amount of sulfur dioxide by applying the following formula: it is not more than 50 ppm.

$$\text{Amount (ppm) of sulfur dioxide} = \frac{V}{W} \times 1000 \times 3.203$$

W: Amount (g) of the sample
V: Amount (mL) of 0.1 mol/L sodium hydroxide VS consumed

Loss on drying Not more than 15.0% (1 g, 130°C, 90 minutes).

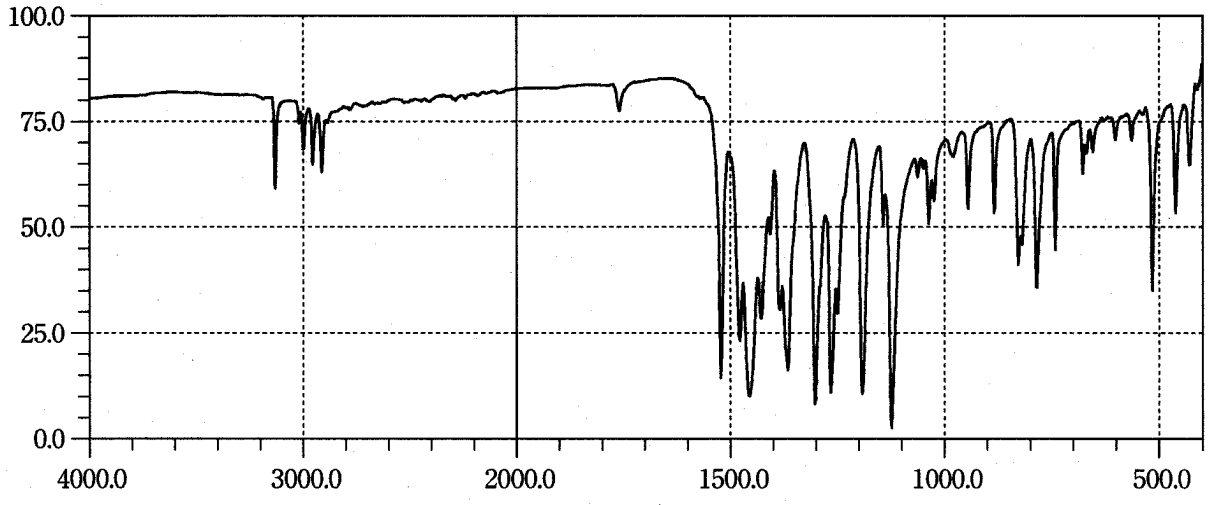
Residue on ignition Not more than 0.6% (1 g).

Containers and storage Containers—Well-closed containers.

Change to read:

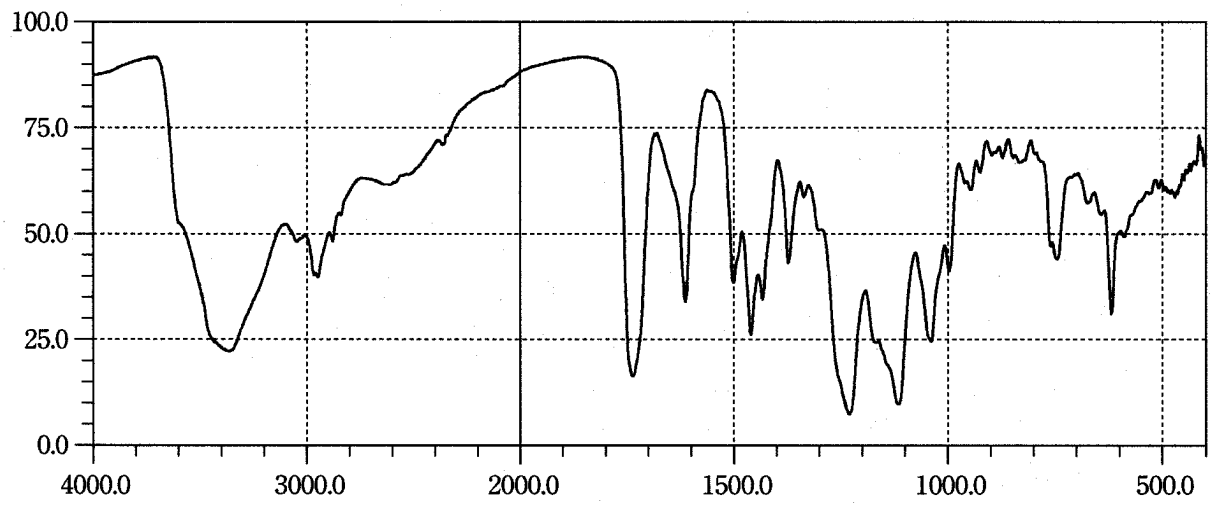
Part I

Tinidazole



Preparation of sample: Potassium bromide disk method

Vinblastine Sulfate

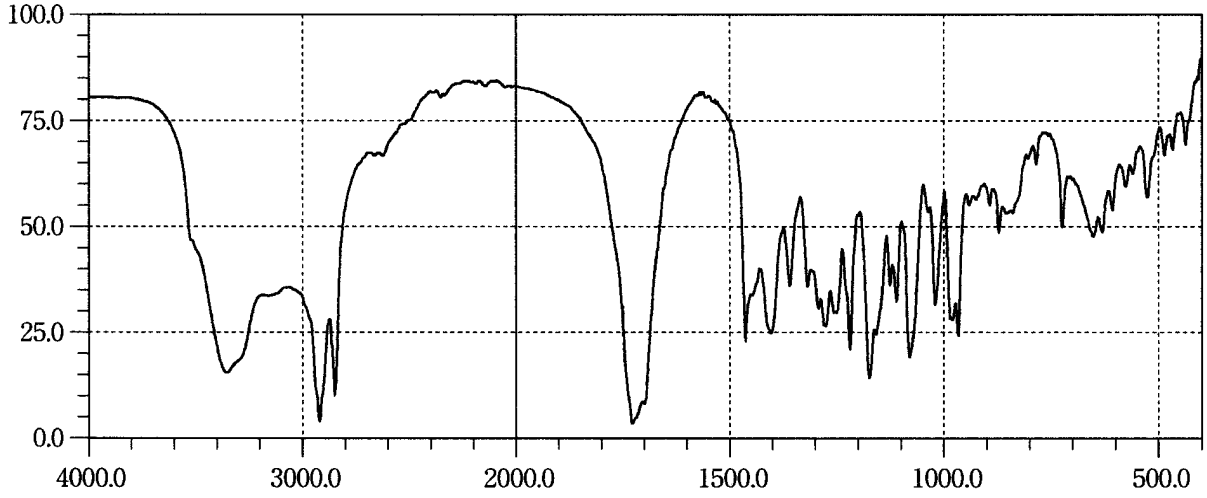


Preparation of sample: Potassium bromide disk method

Add the following:

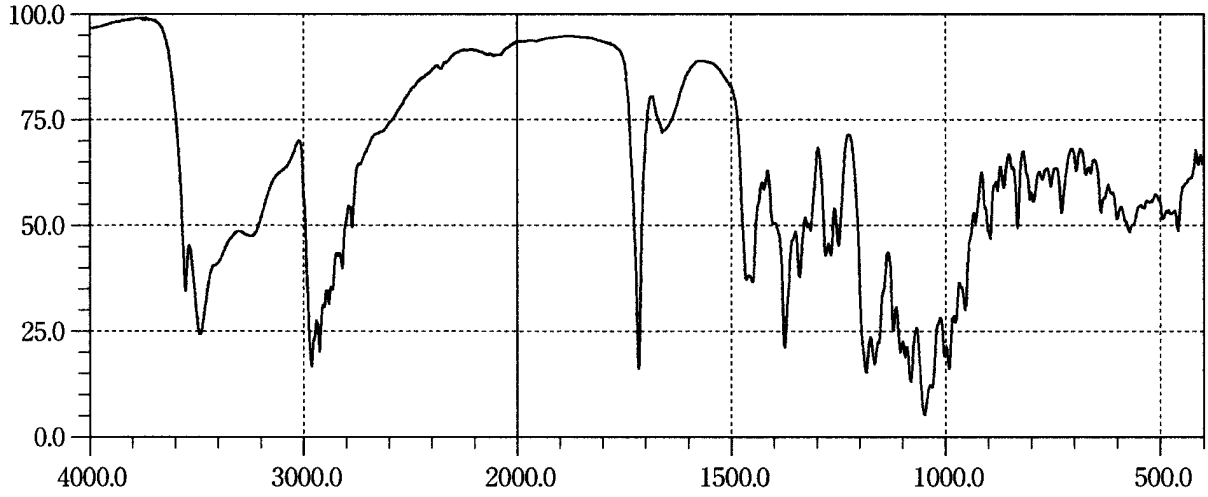
Part I

Alprostadil



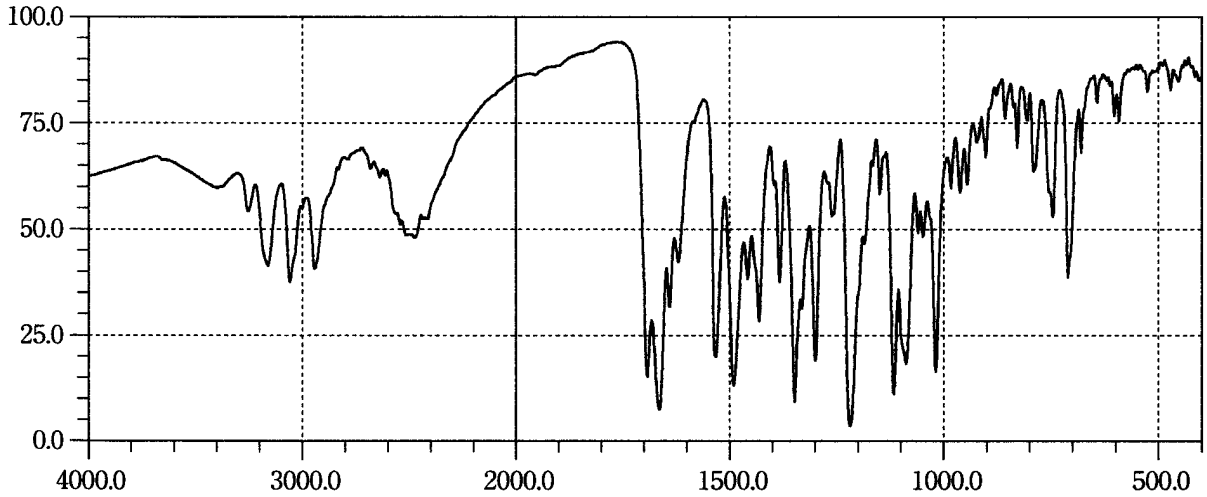
Preparation of sample: Potassium bromide disk method

Azithromycin Hydrate



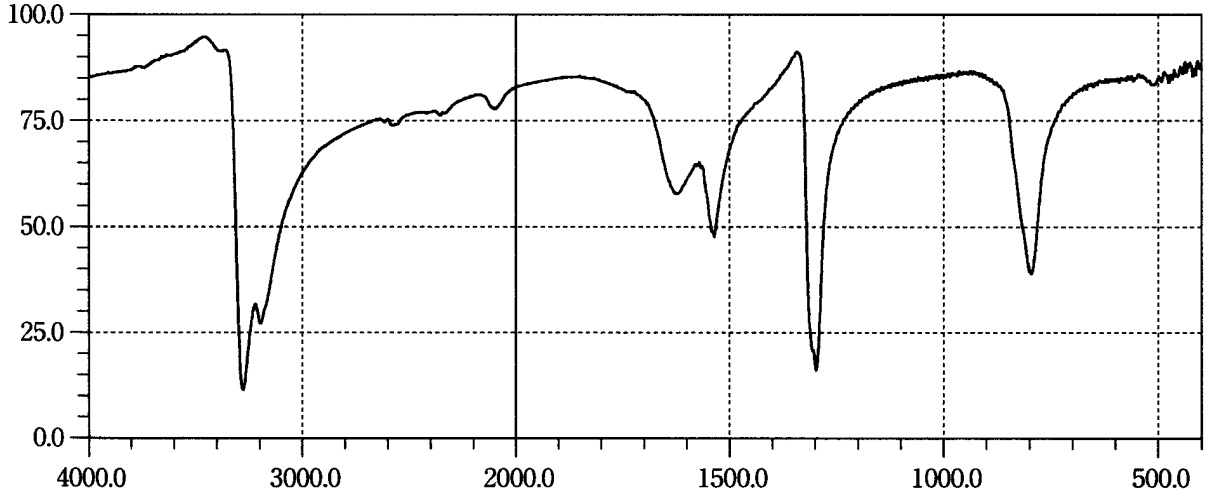
Preparation of sample: Potassium bromide disk method

Benidipine Hidrochloride



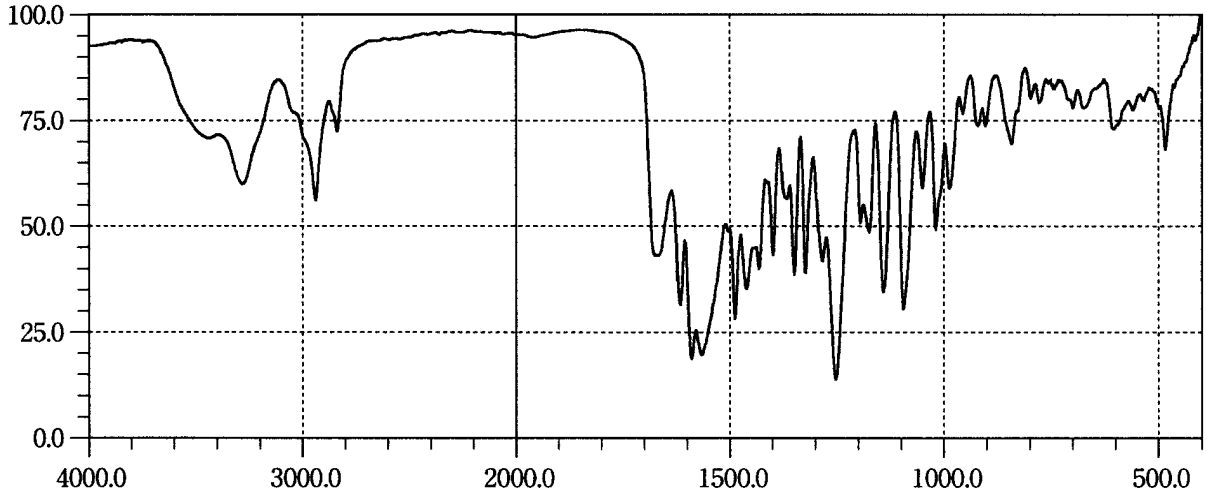
Preparation of sample: Potassium chloride disk method

Cisplatin



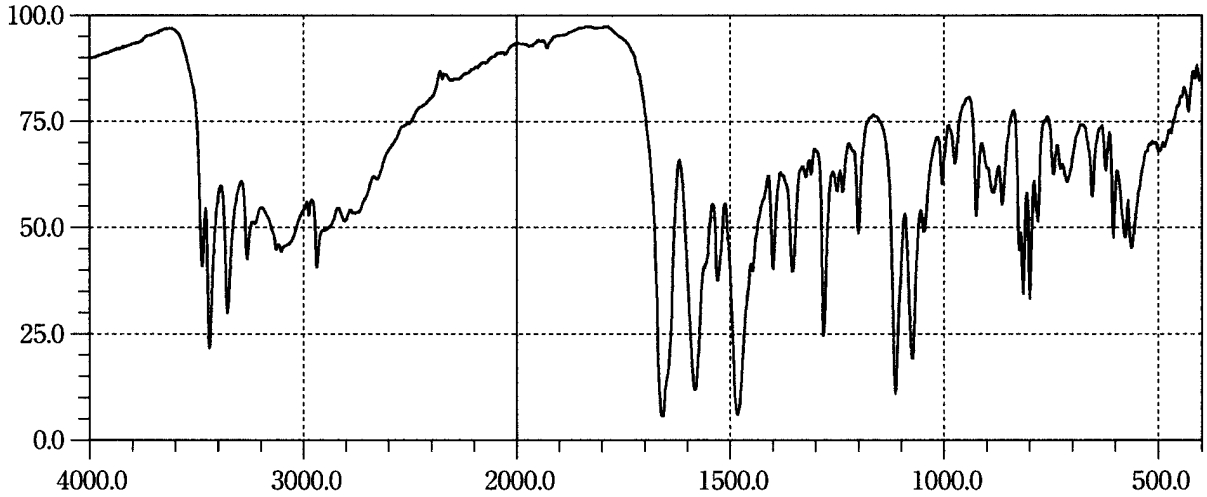
Preparation of sample: Potassium bromide disk method

Colchicine



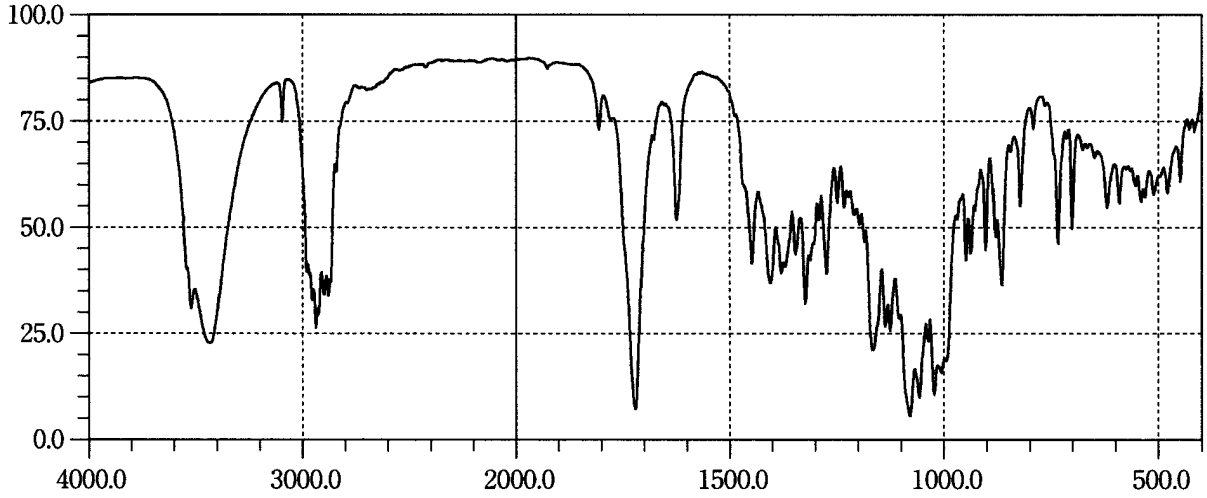
Preparation of sample: Potassium bromide disk method [To 0.5 mL of a solution of Colchicine in methanol (1 in 50) add 1 g of potassium bromide, and dry under reduced pressure (80°C, 1 hour).]

Cytarabine



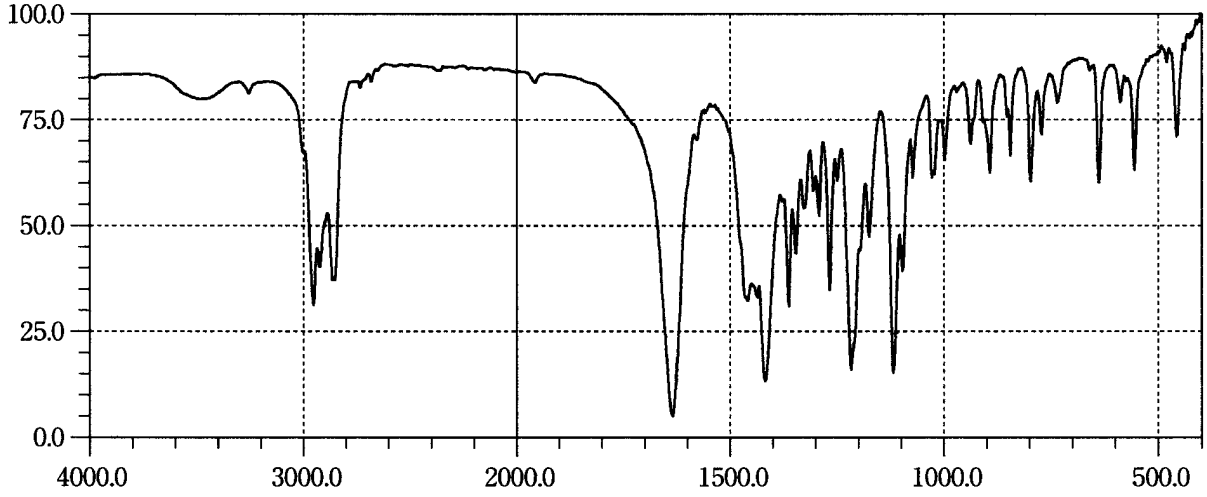
Preparation of sample: Potassium bromide disk method

Digoxin



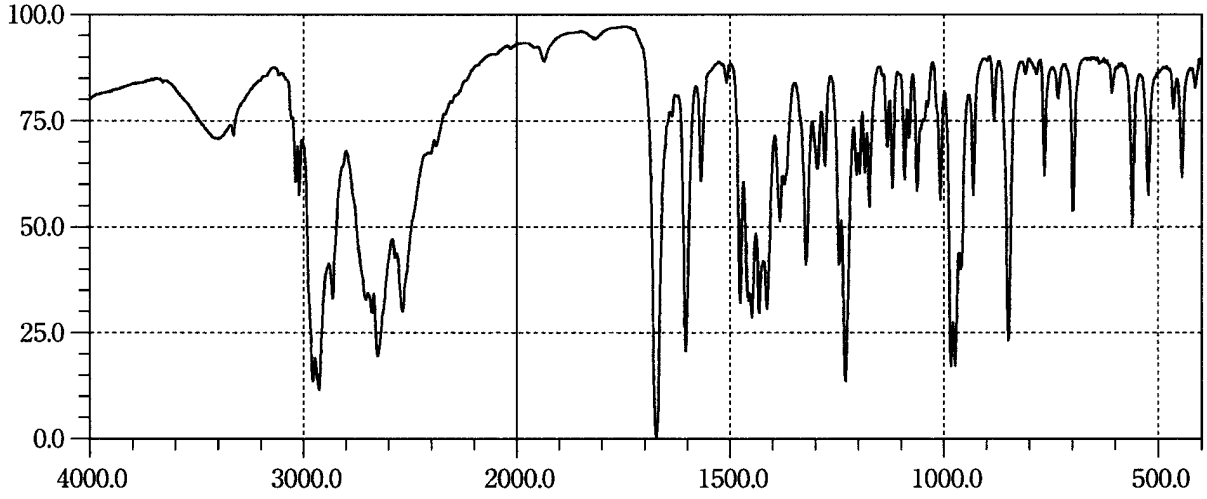
Preparation of sample: Potassium bromide disk method

Dimorpholamine



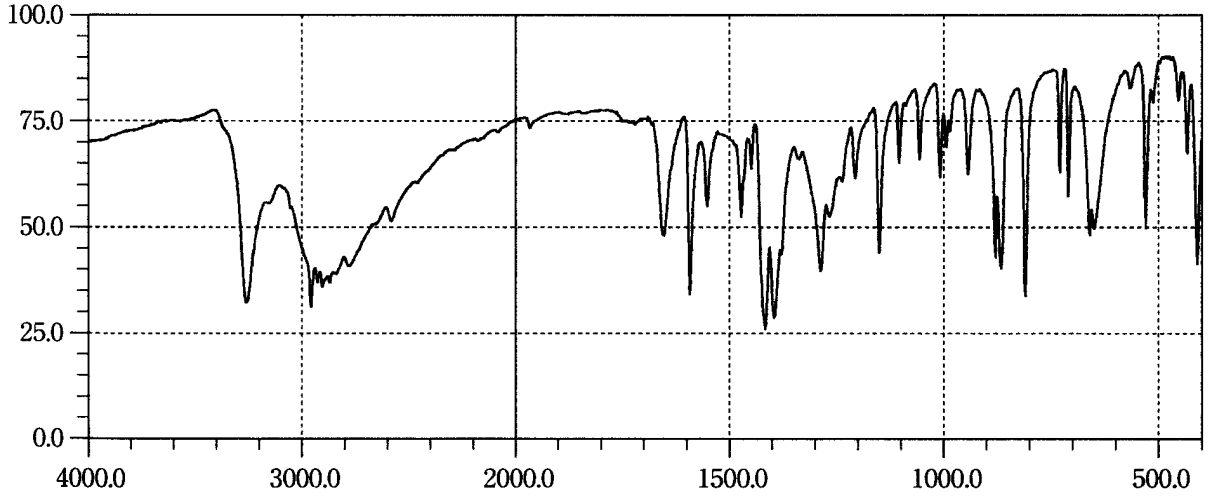
Preparation of sample: Potassium bromide disk method

Eperisone Hydrochloride



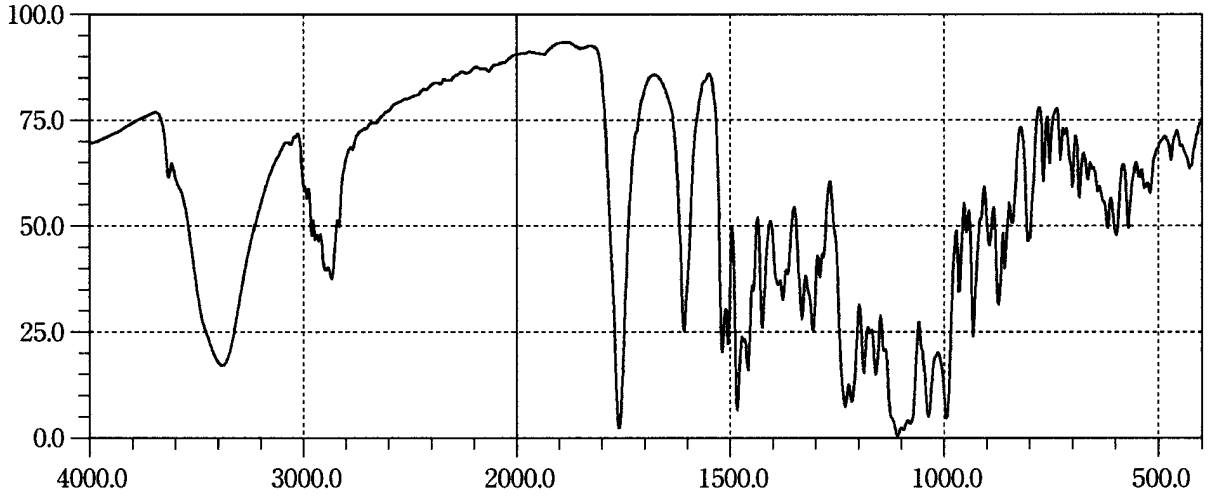
Preparation of sample: Potassium chloride disk method

Ethionamide



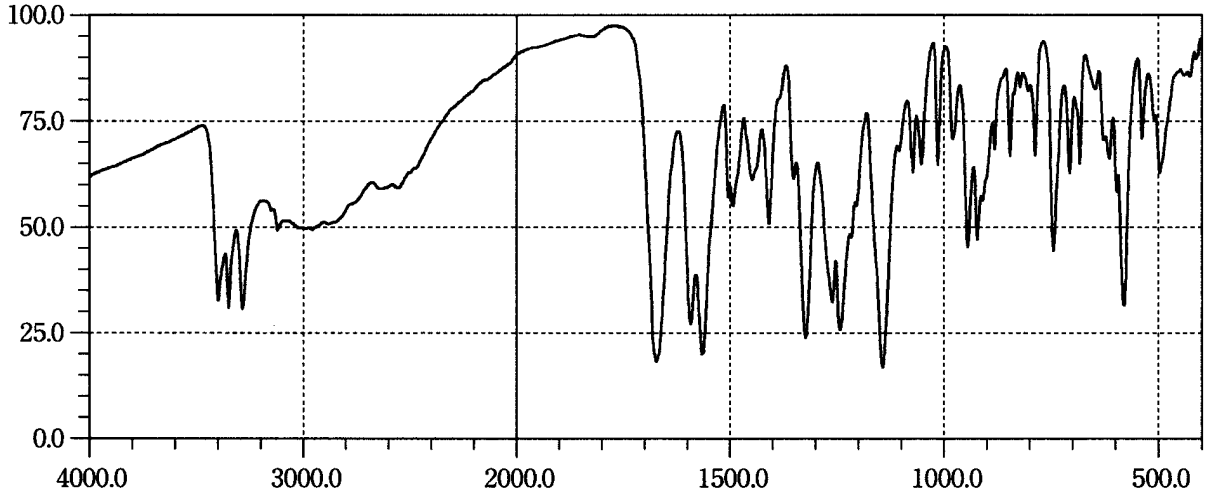
Preparation of sample: Potassium bromide disk method

Etoposide



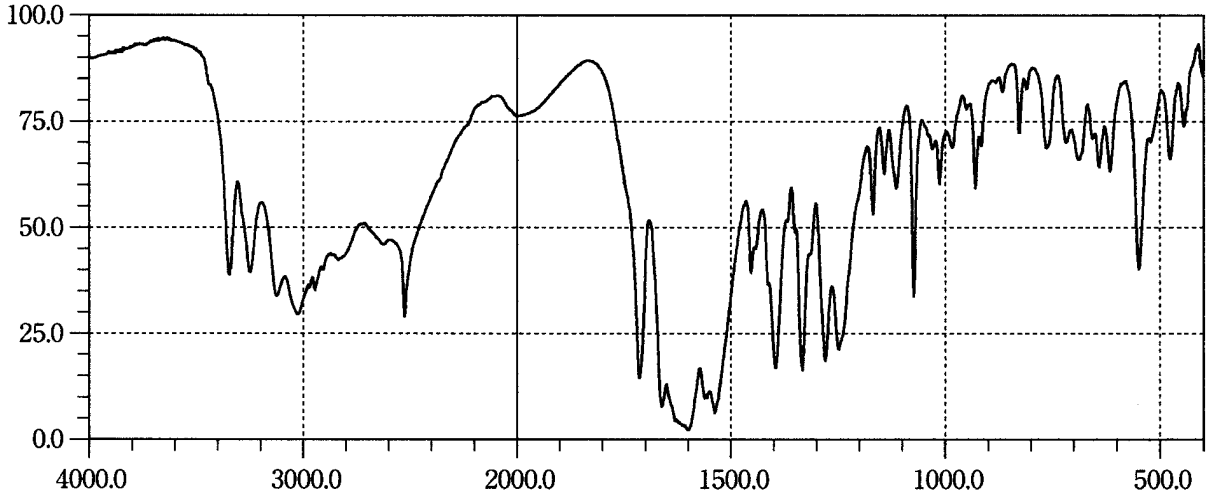
Preparation of sample: Potassium bromide disk method

Furosemide



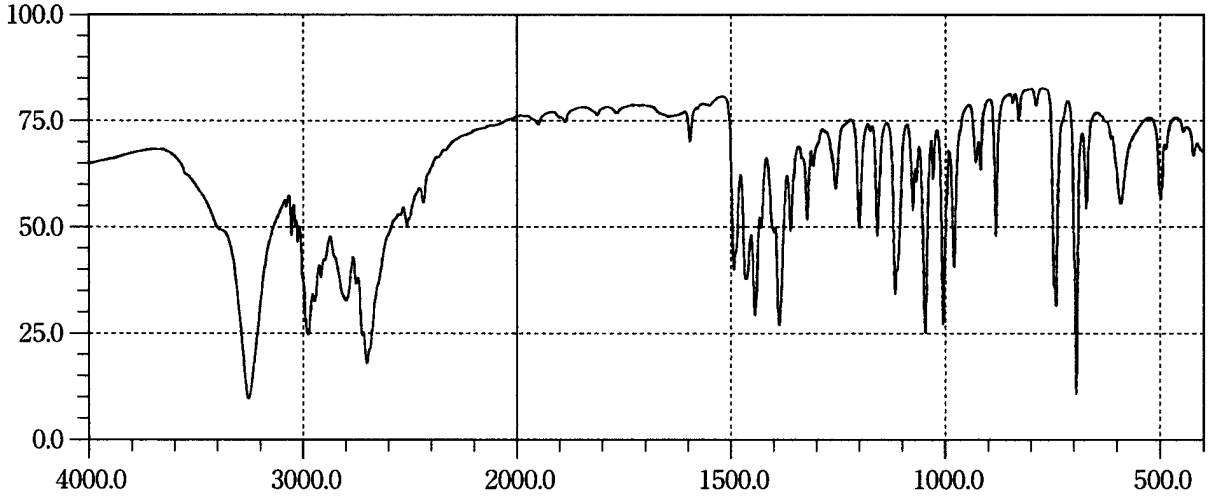
Preparation of sample: Potassium bromide disk method

Glutathione



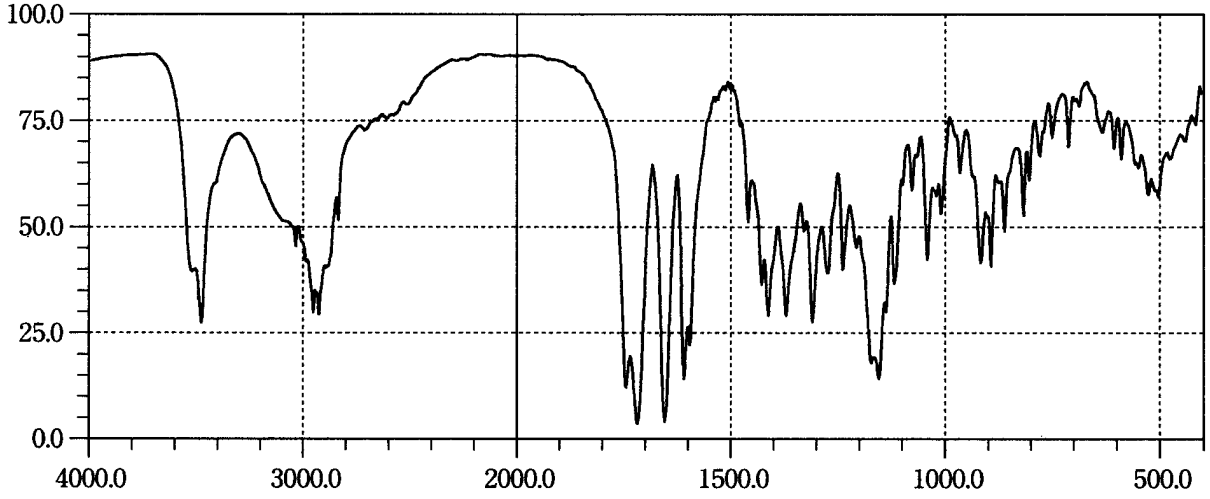
Preparation of sample: Potassium bromide disk method

***dl*-Methylephedrine Hydrochloride**



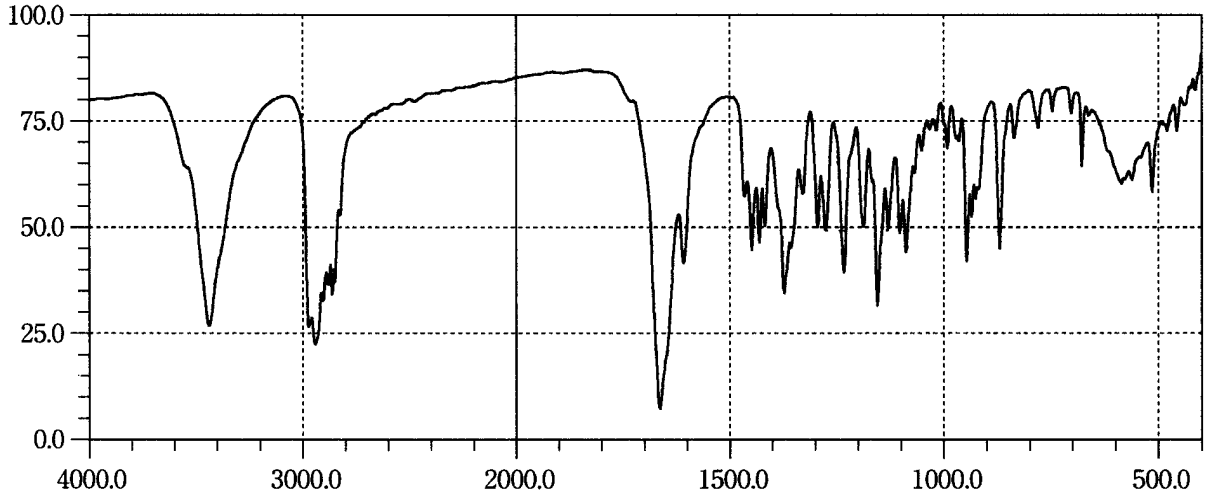
Preparation of sample: Potassium chloride disk method

Methylprednisolone Succinate



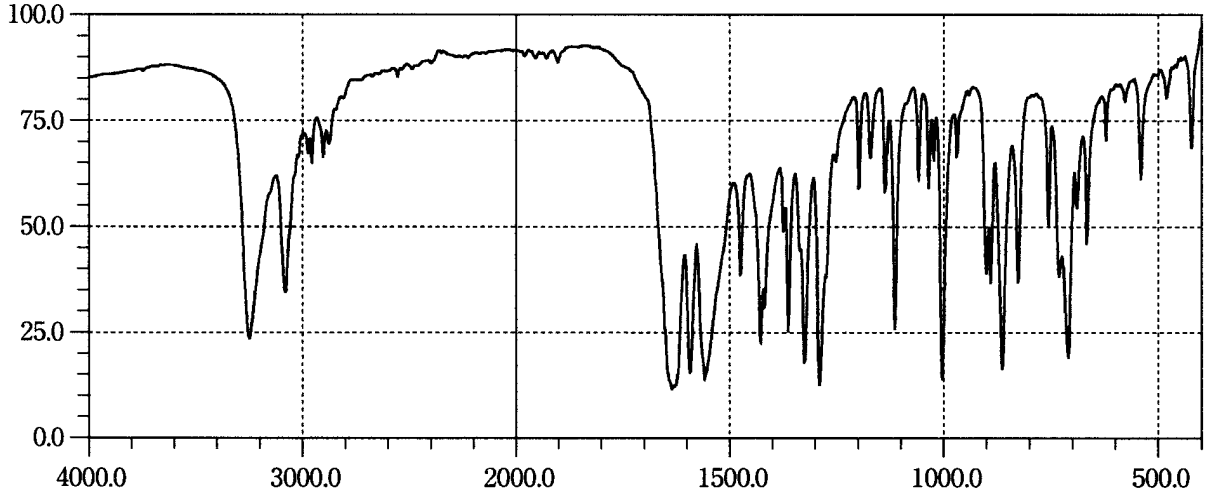
Preparation of sample: Potassium bromide disk method

Methyltestosterone



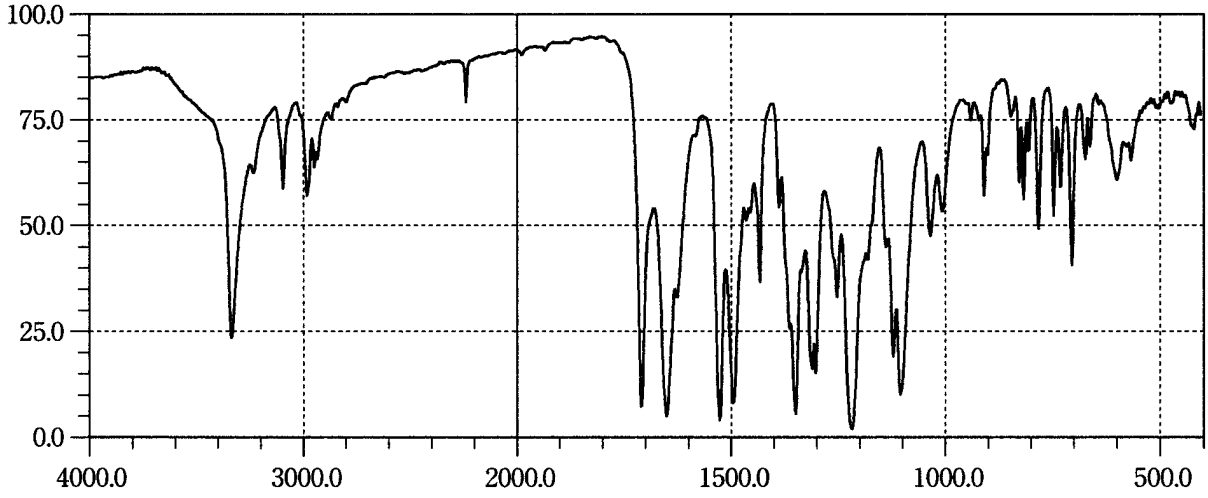
Preparation of sample: Potassium bromide disk method

Nicorandil



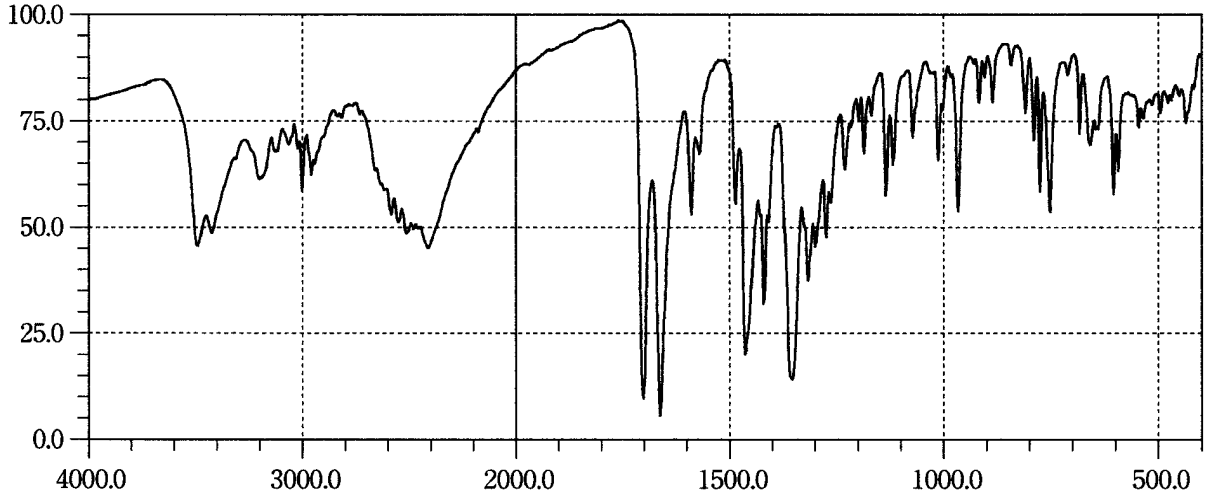
Preparation of sample: Potassium bromide disk method

Nilvadipine



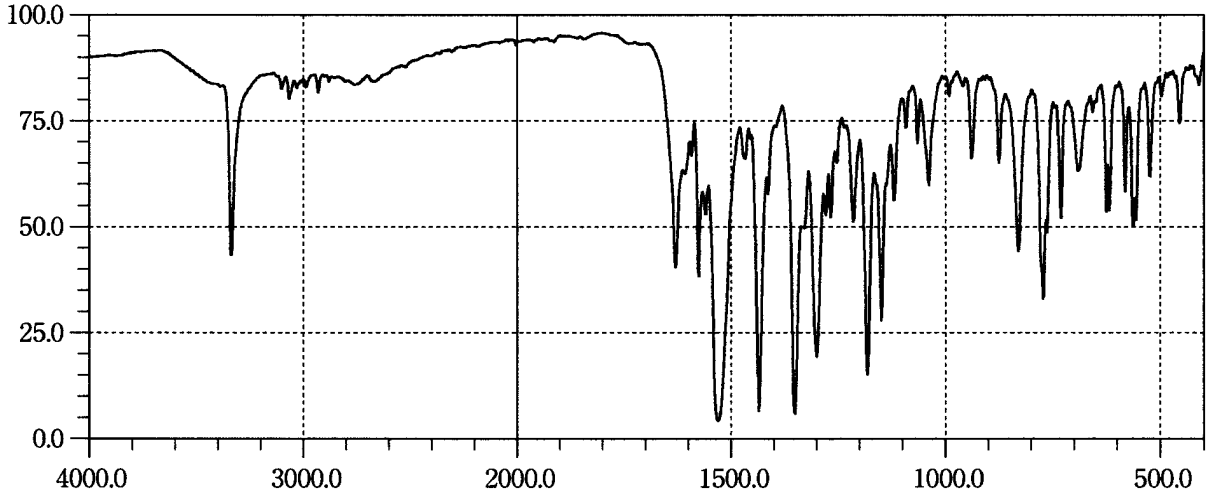
Preparation of sample: Potassium bromide disk method

Pirenzepine Hydrochloride Hydrate



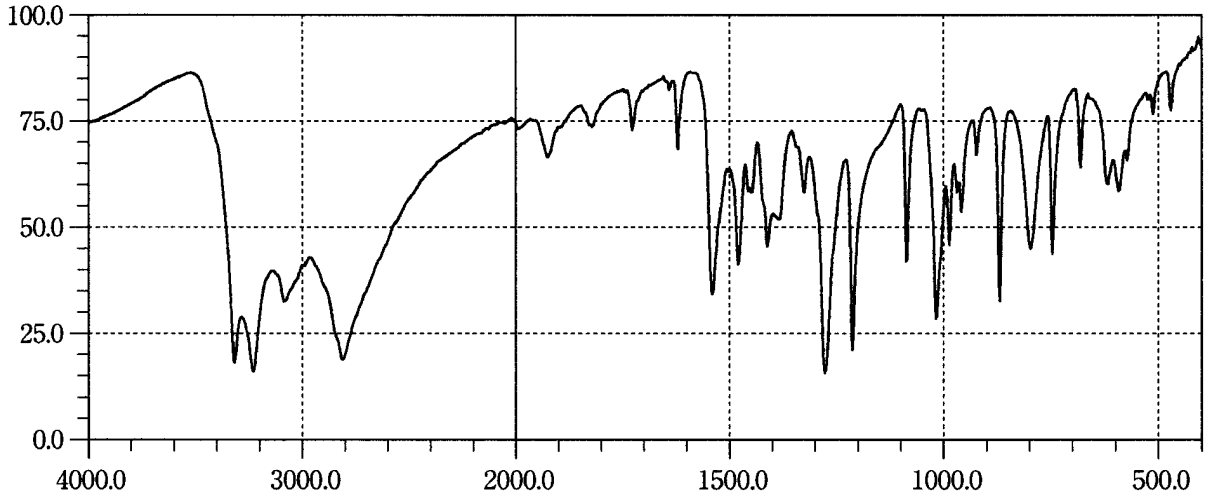
Preparation of sample: Potassium chloride disk method

Piroxicam



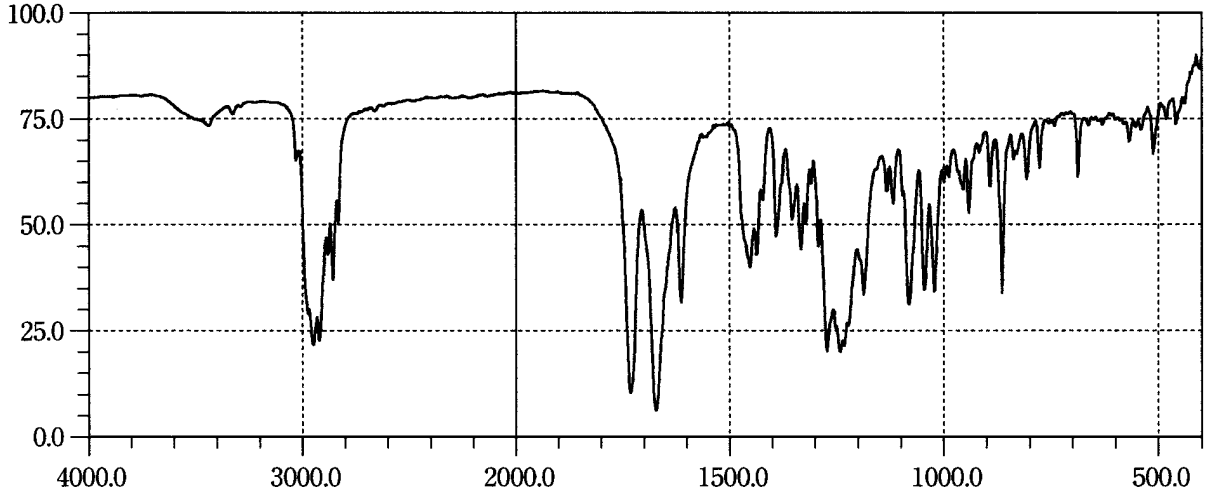
Preparation of sample: Potassium bromide disk method

Pyridoxine Hydrochloride



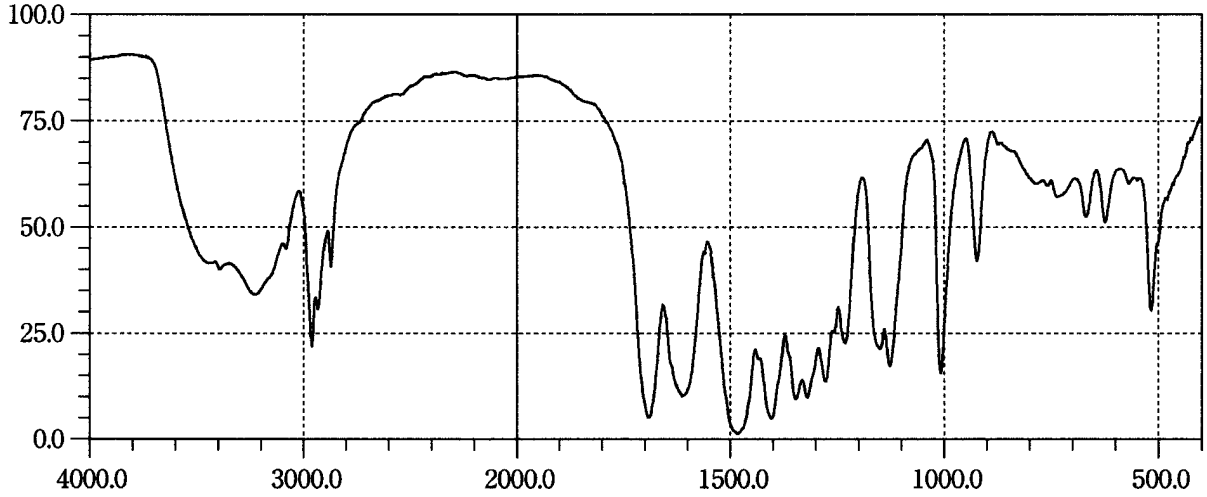
Preparation of sample: Potassium chloride disk method

Testosterone Propionate



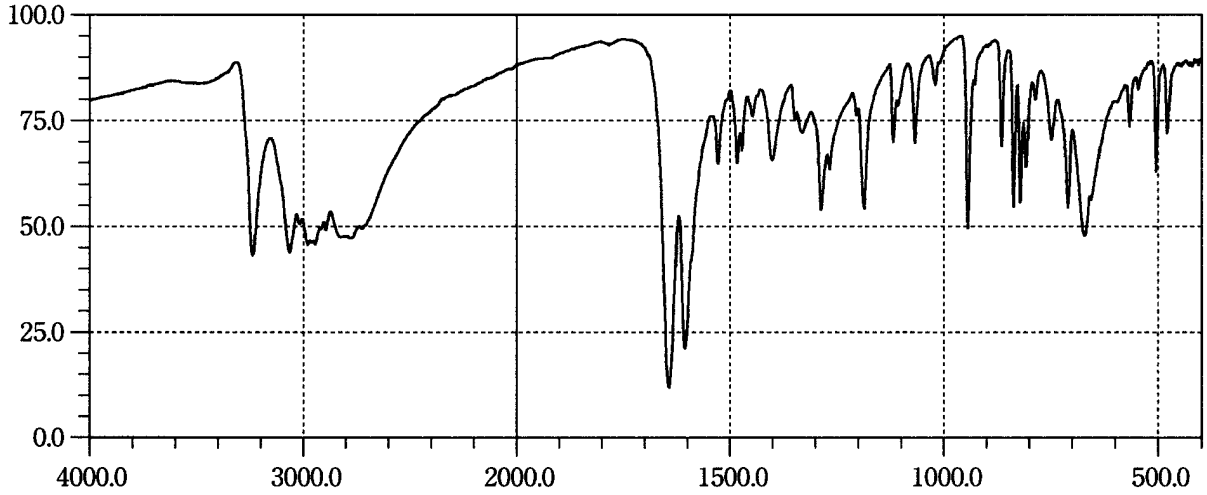
Preparation of sample: Potassium bromide disk method

Thimylal Sodium



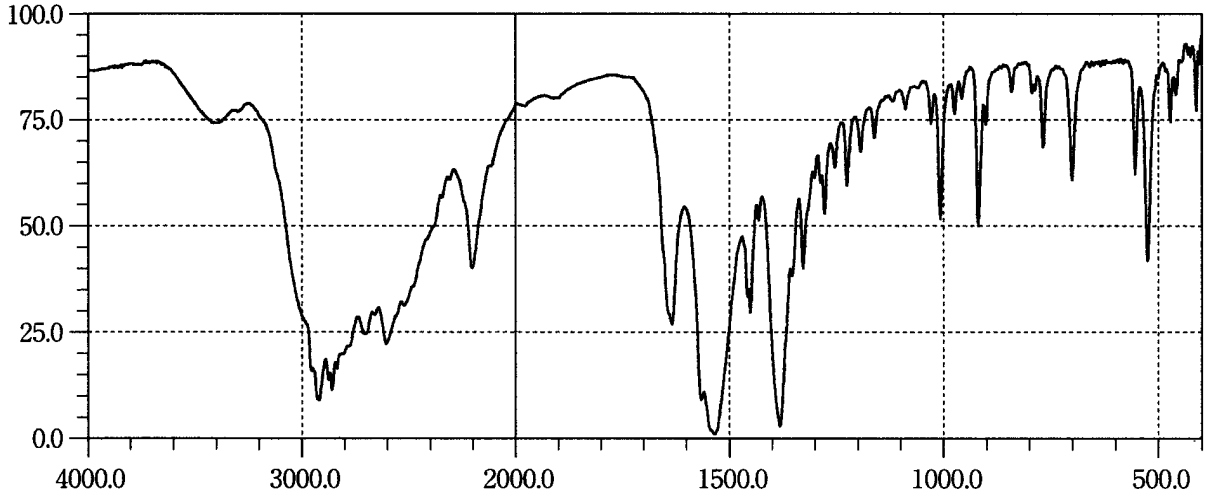
Preparation of sample: Potassium bromide disk method

Tizanidine Hydrochloride



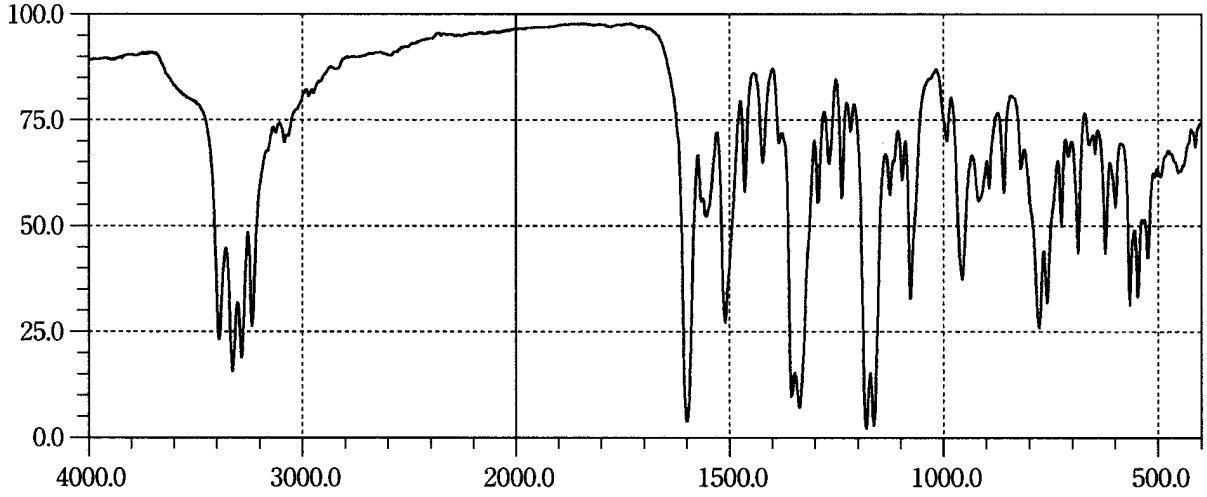
Preparation of sample: Potassium chloride disk method

Tranexamic Acid



Preparation of sample: Potassium bromide disk method

Trichlormethiazide

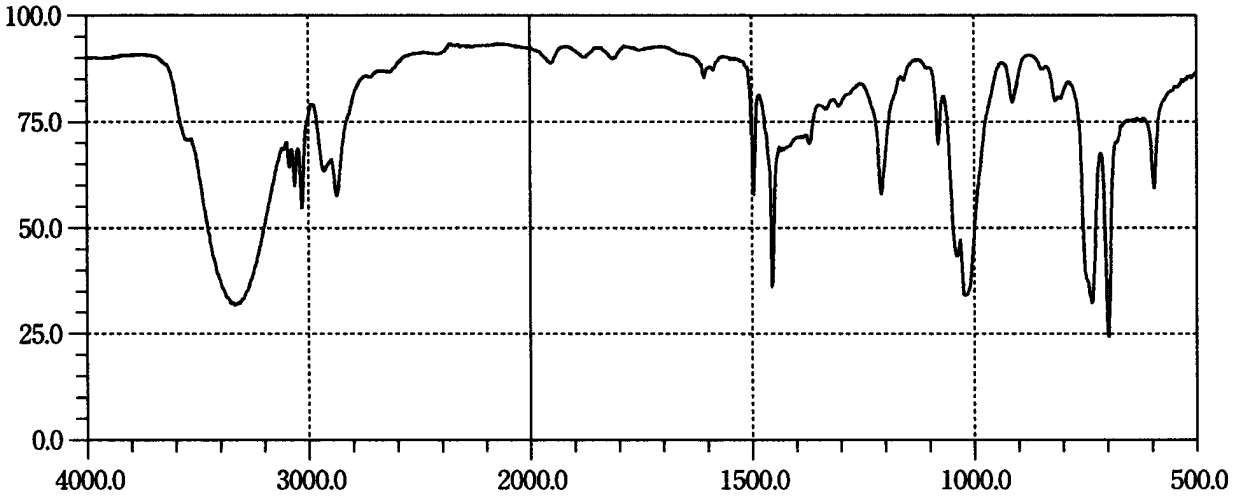


Preparation of sample: Potassium bromide disk method

Add the following:

Part II

Benzyl Alcohol



Preparation of sample: Liquid film method

Delete the following Ultraviolet-visible Reference Spectra:

Part I

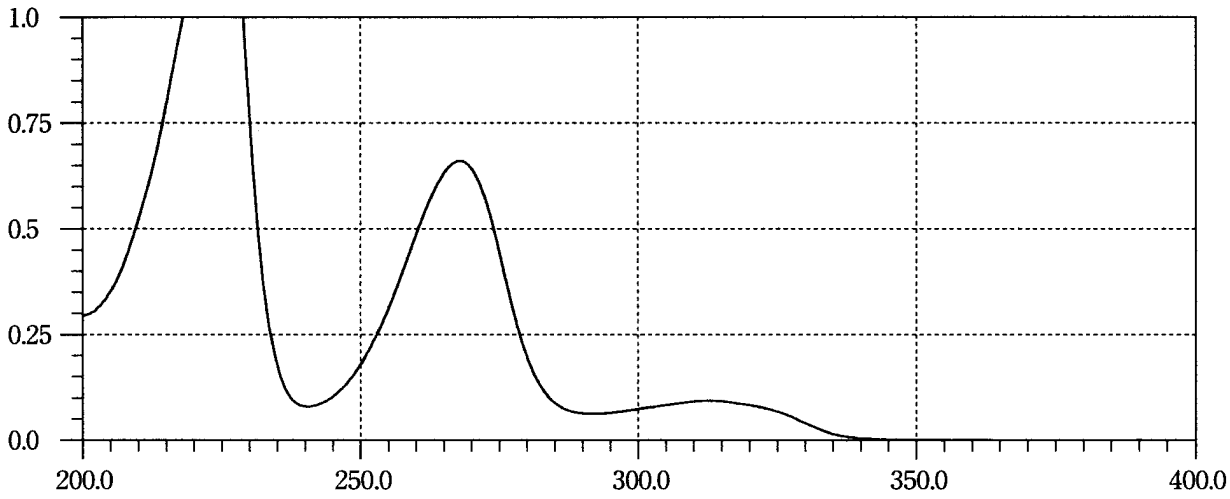
Furosemide 1

Furosemide 2

Change to read:

Part I

Trichlormethiazide

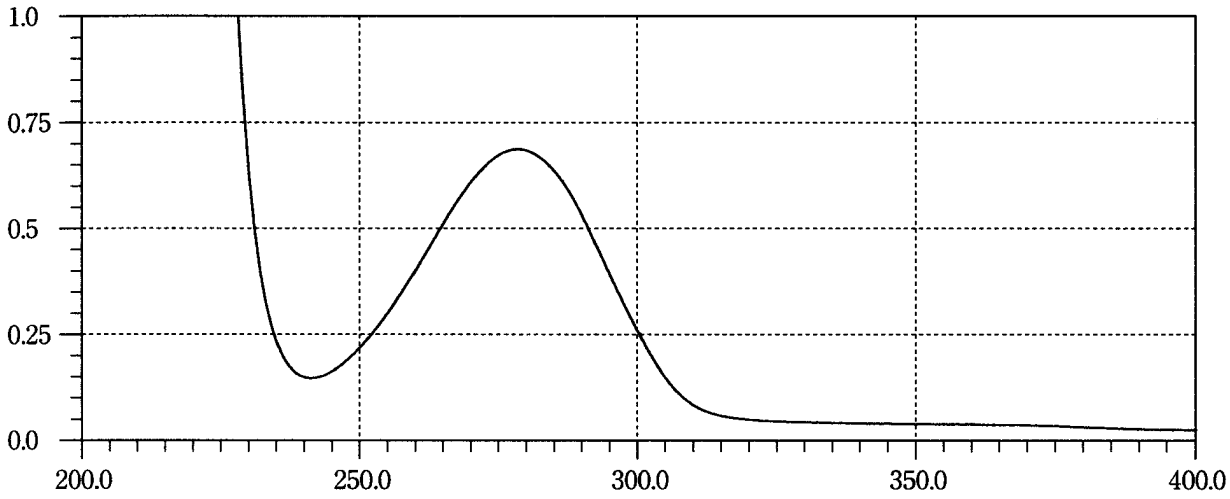


A solution in ethanol (95) (3 in 250,000)

Add the following:

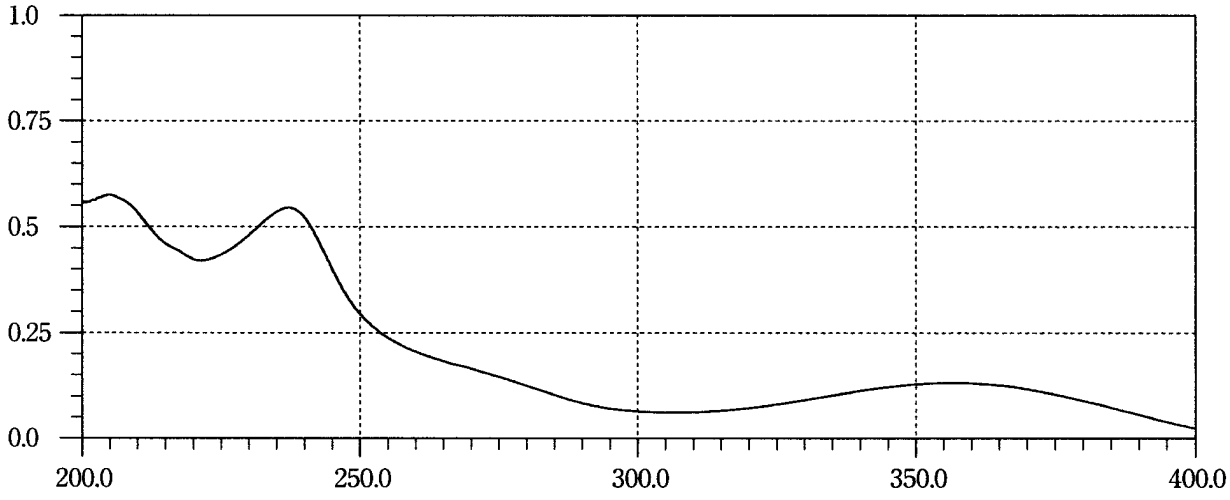
Part I

Alprostadil



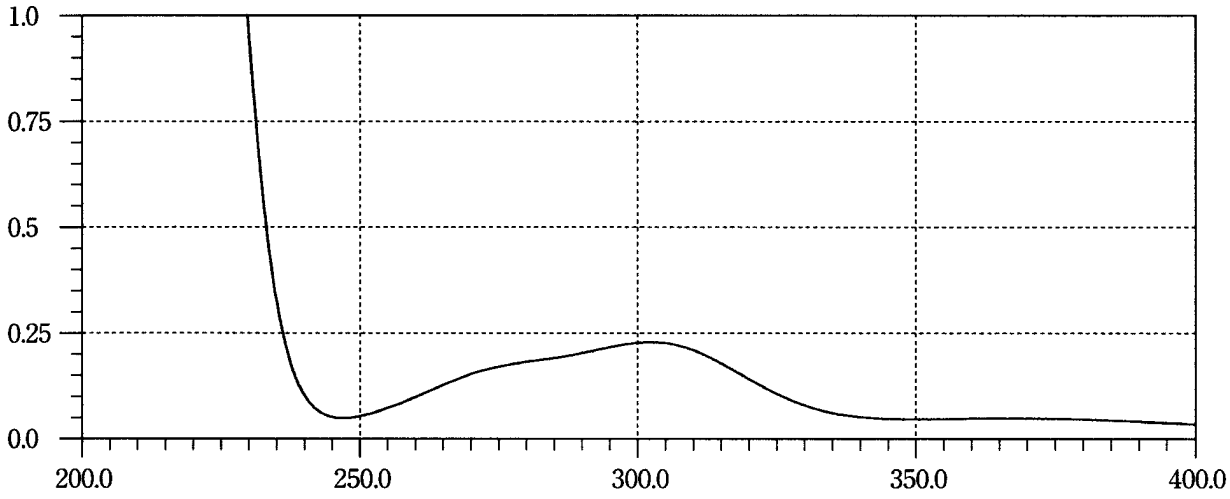
A solution prepared as follows: To 10 mL of a solution in ethanol (99.5) (1 in 100,000) add 1 mL of potassium hydroxide-ethanol TS, and allow to stand for 15 minutes.

Benidipine Hydrochloride



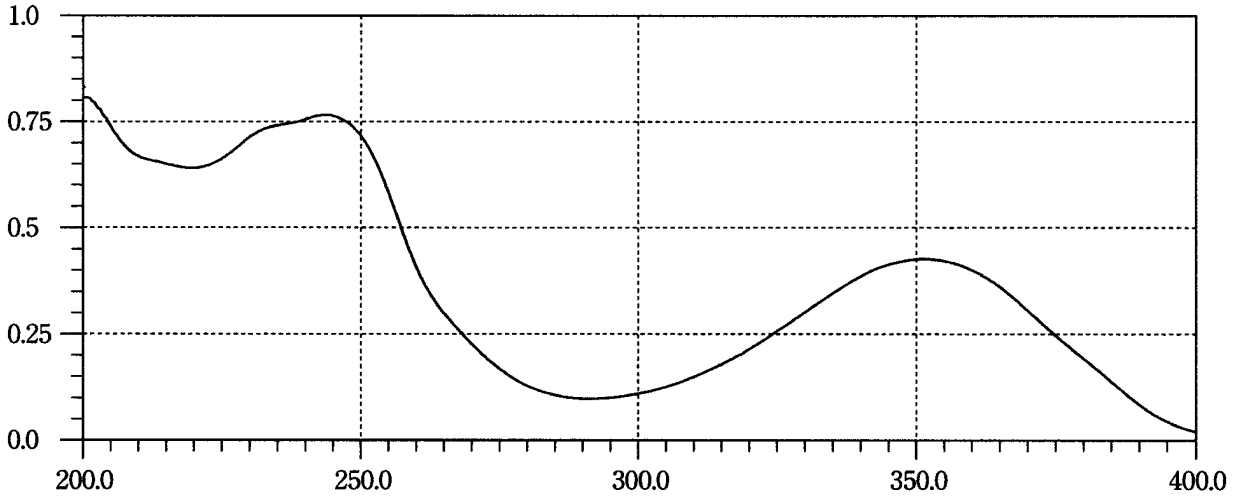
A solution in methanol (1 in 100,000)

Cisplatin



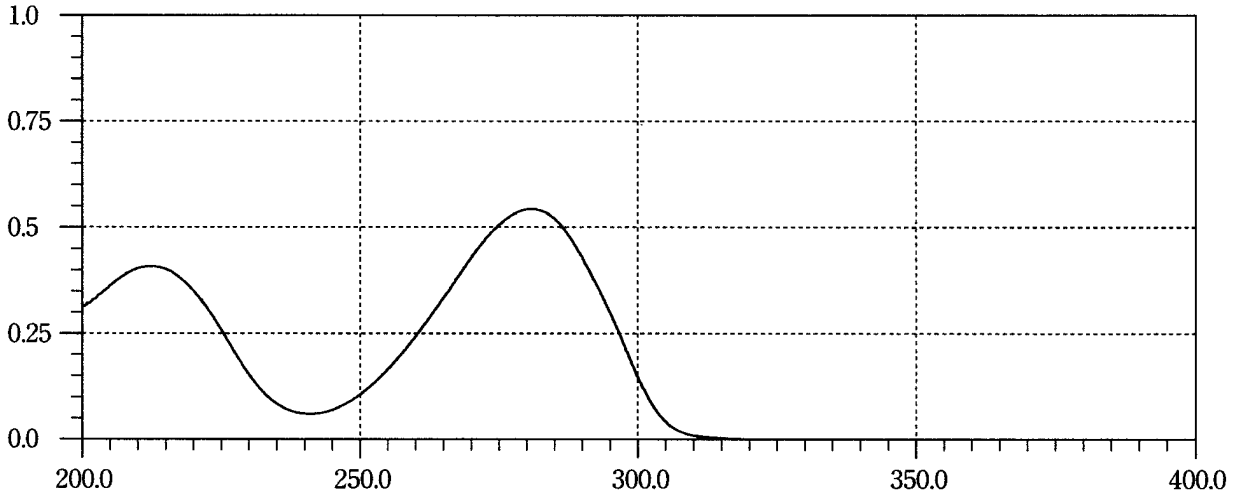
A solution in a solution of sodium chloride in 0.01 mol/L hydrochloric acid TS (9 in 1000) (1 in 2000)

Colchicine



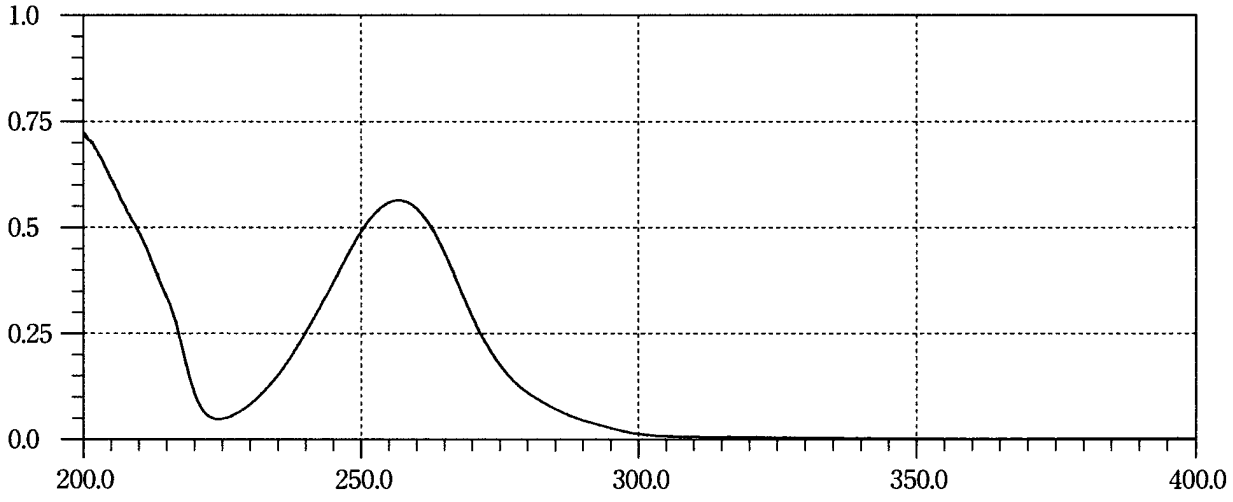
A solution in ethanol (95) (1 in 100,000)

Cytarabine



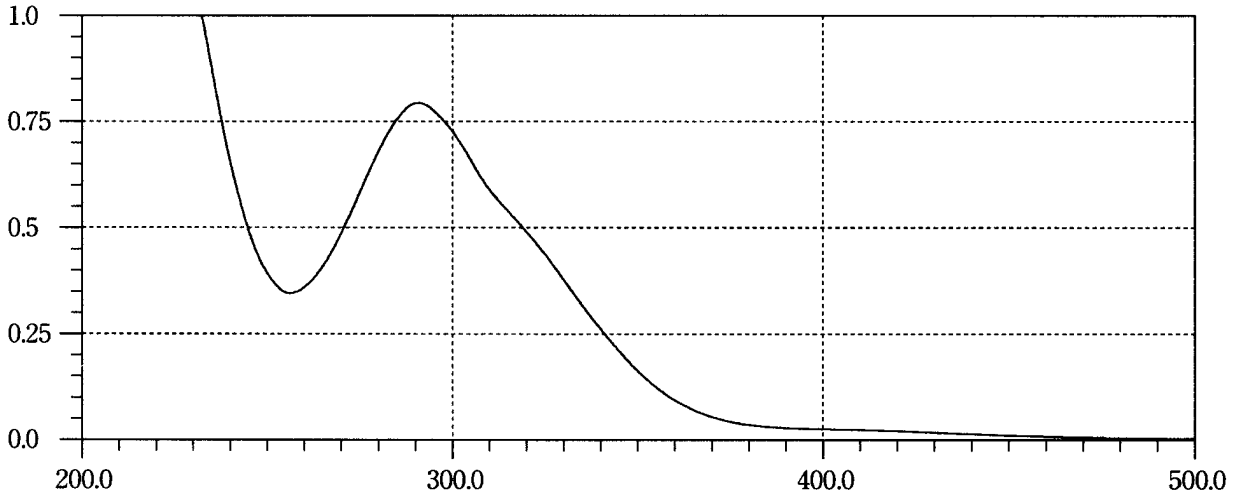
A solution in 0.1 mol/L hydrochloric acid TS (1 in 100,000)

Eperisone Hydrochloride



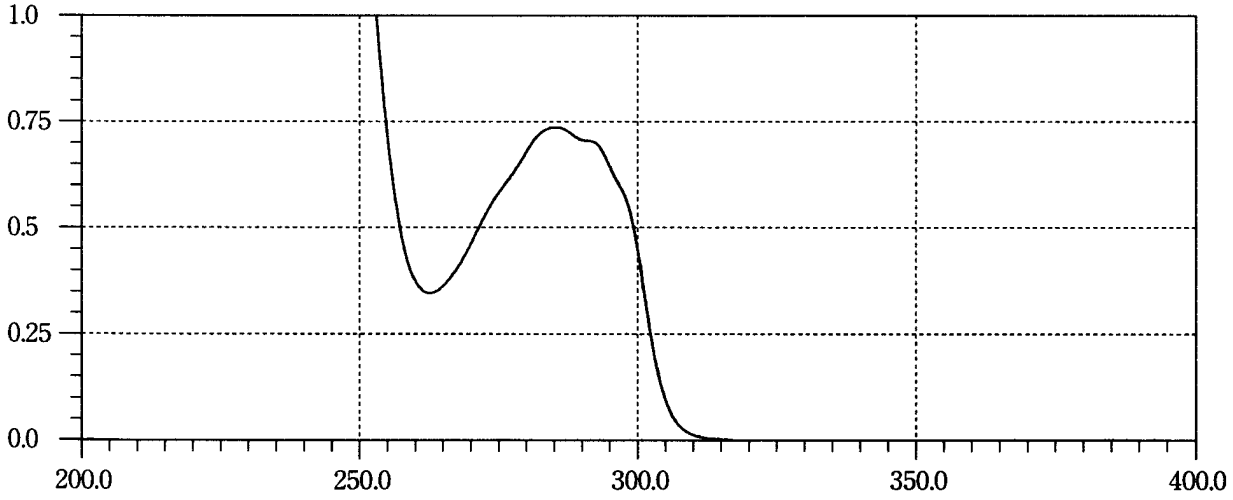
A solution in methanol (1 in 100,000)

Ethionamide



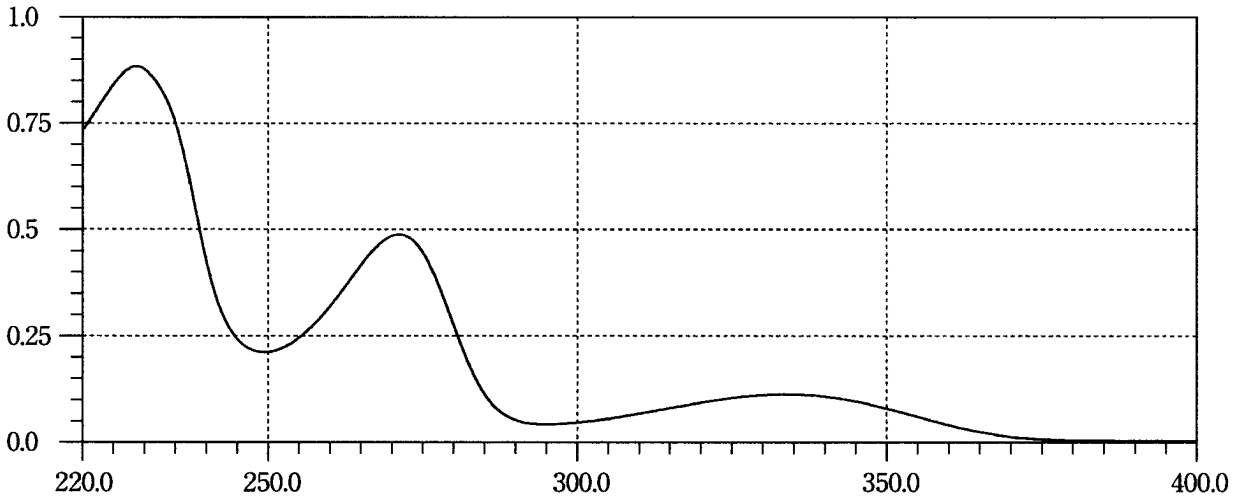
A solution in methanol (3 in 160,000)

Etoposide



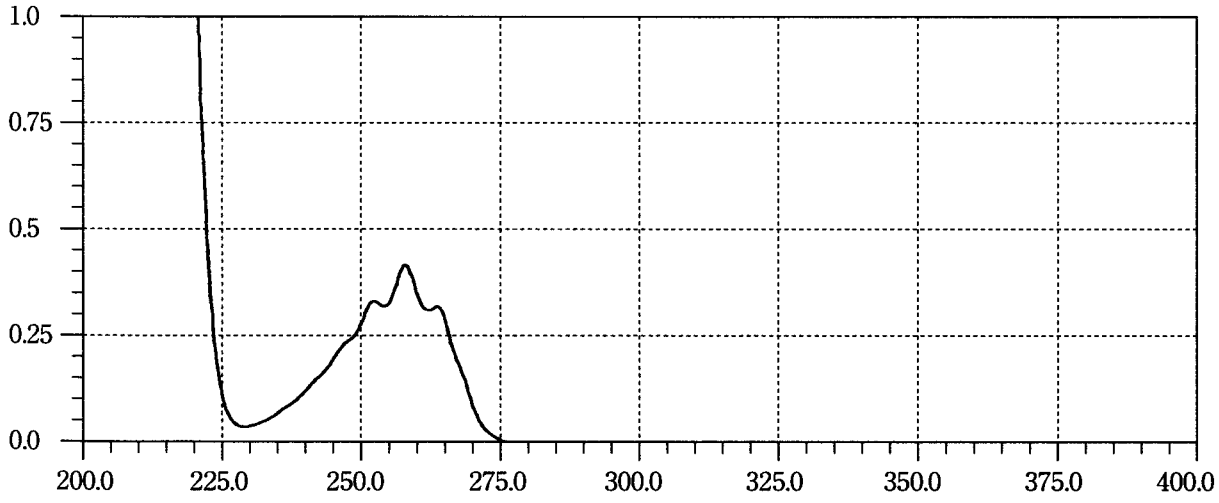
A solution in methanol (1 in 10,000)

Furosemide



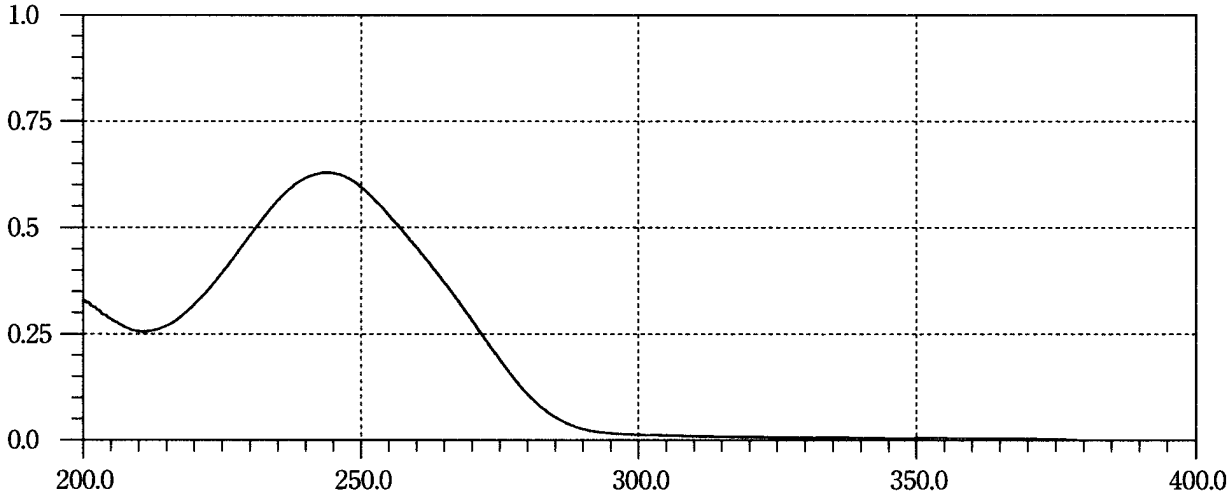
A solution in dilute sodium hydroxide TS (1 in 125,000)

_dl_-Methylephedrine Hydrochloride



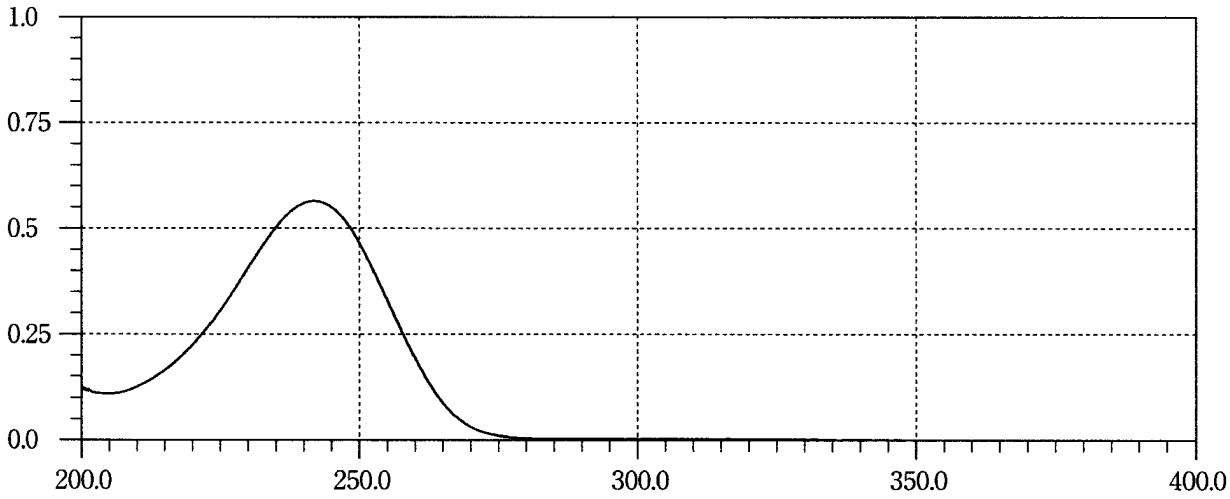
An aqueous solution (1 in 2000)

Methylprednisolone Succinate



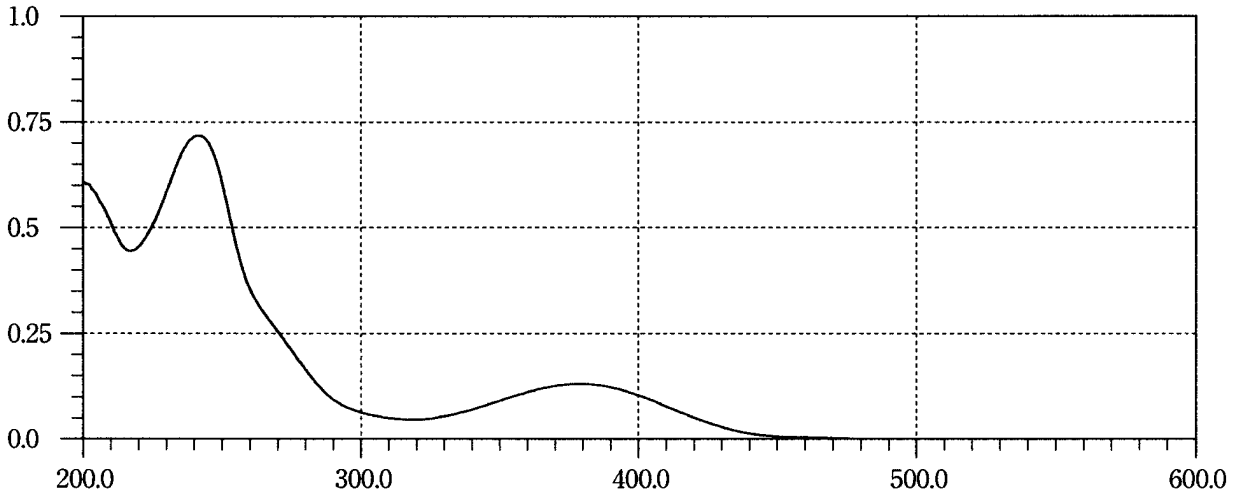
A solution in methanol (1 in 50,000)

Methyltestosterone



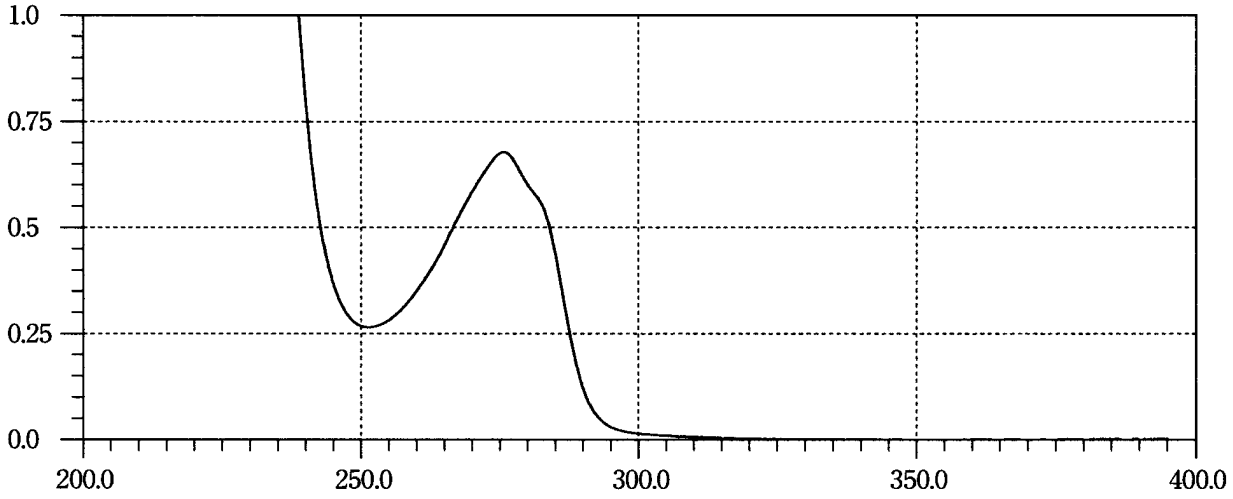
A solution in ethanol (95) (1 in 100,000)

Nilvadipine



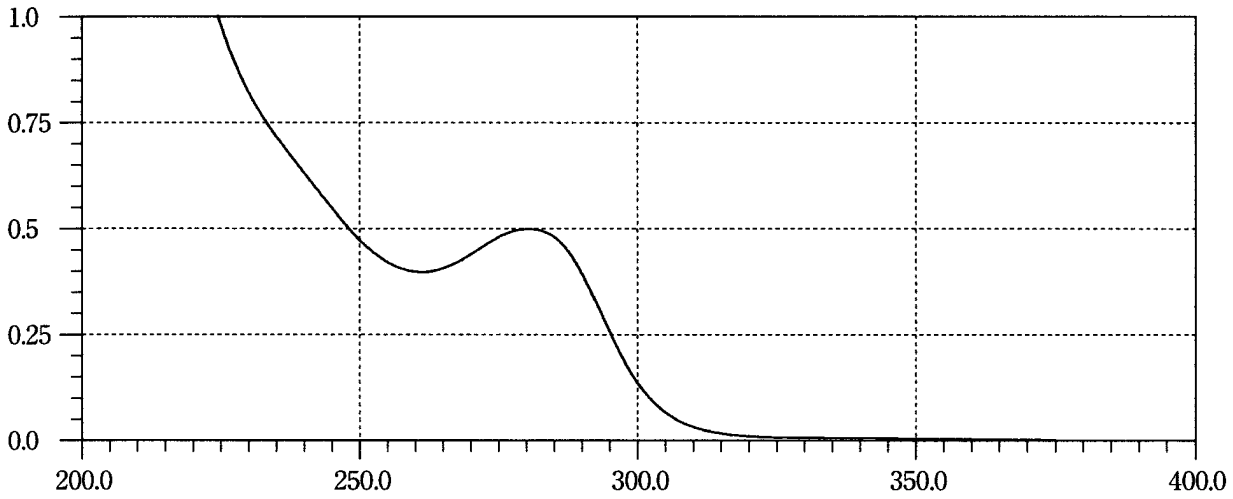
A solution in ethanol (99.5) (1 in 100,000)

Oxytocin



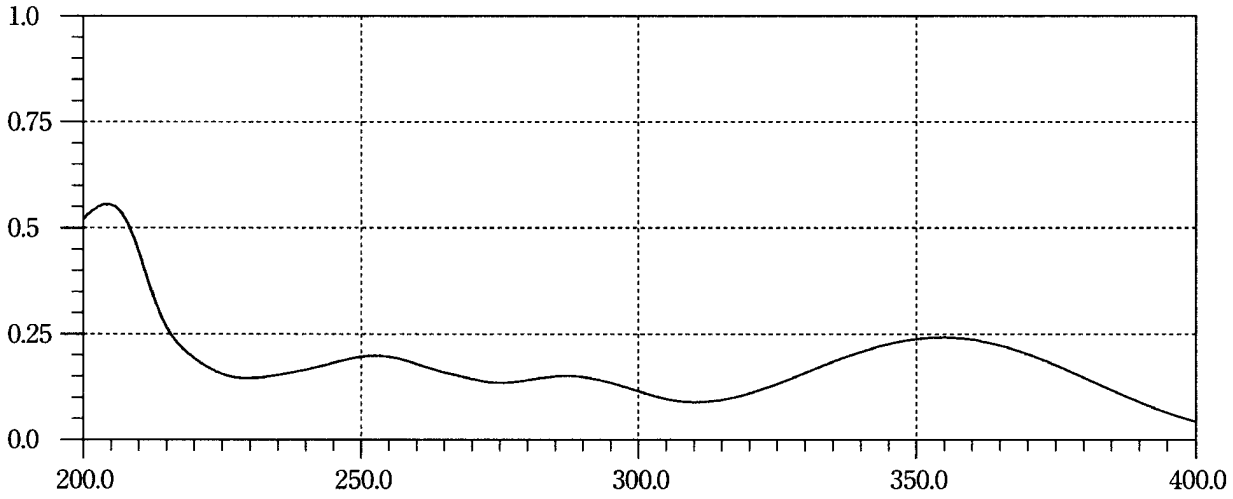
An aqueous solution (1 in 2000)

Pirenzepine Hydrochloride Hydrate



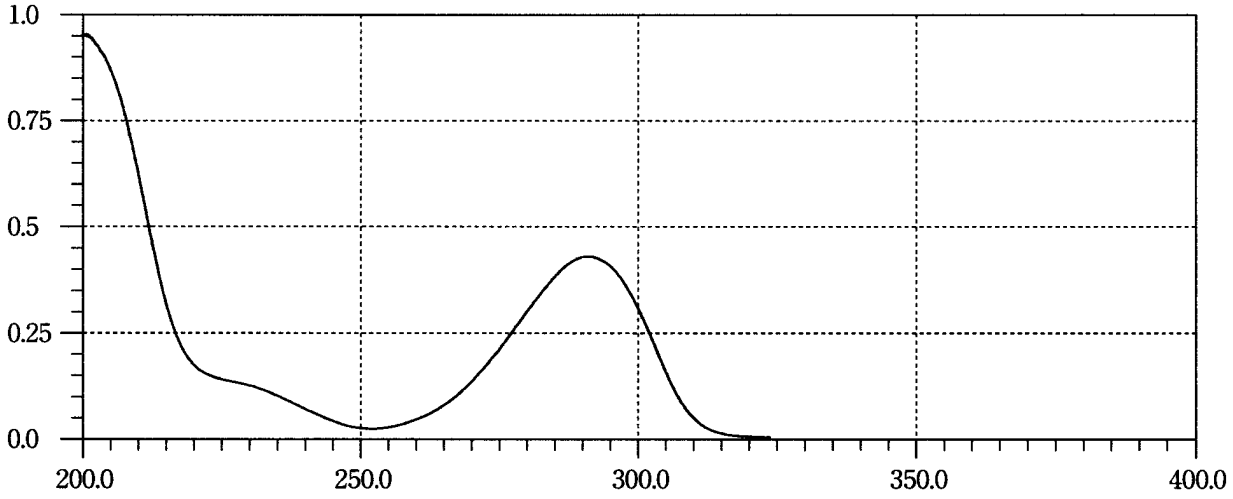
An aqueous solution (1 in 40,000)

Piroxicam



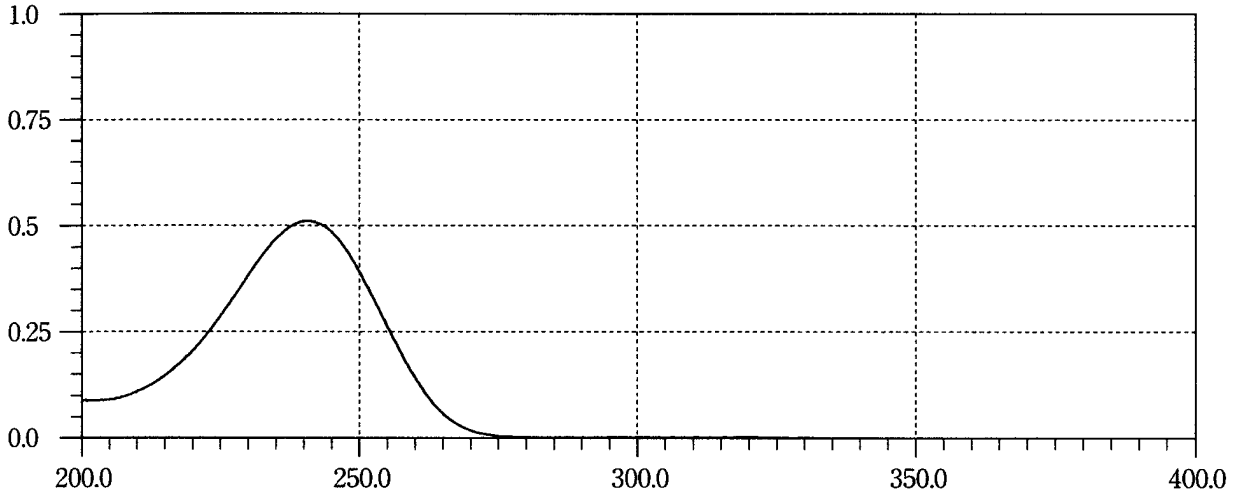
A solution in a mixture of methanol and 0.5 mol/L hydrochloric acid TS (490:1) (1 in 200,000)

Pyridoxine Hydrochloride



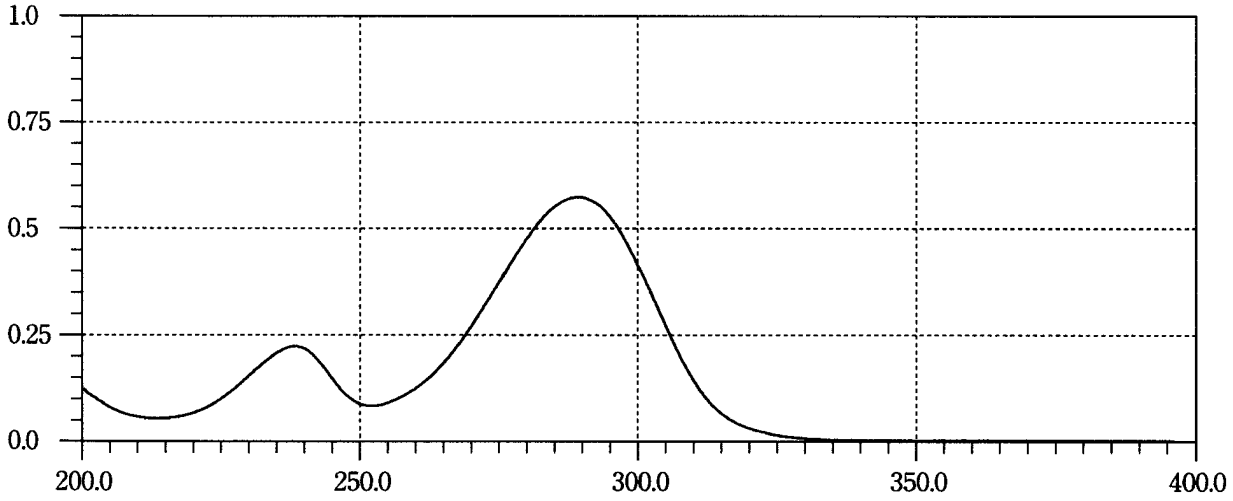
A solution in 0.1 mol/L hydrochloric acid TS (1 in 100,000)

Testosterone Propionate



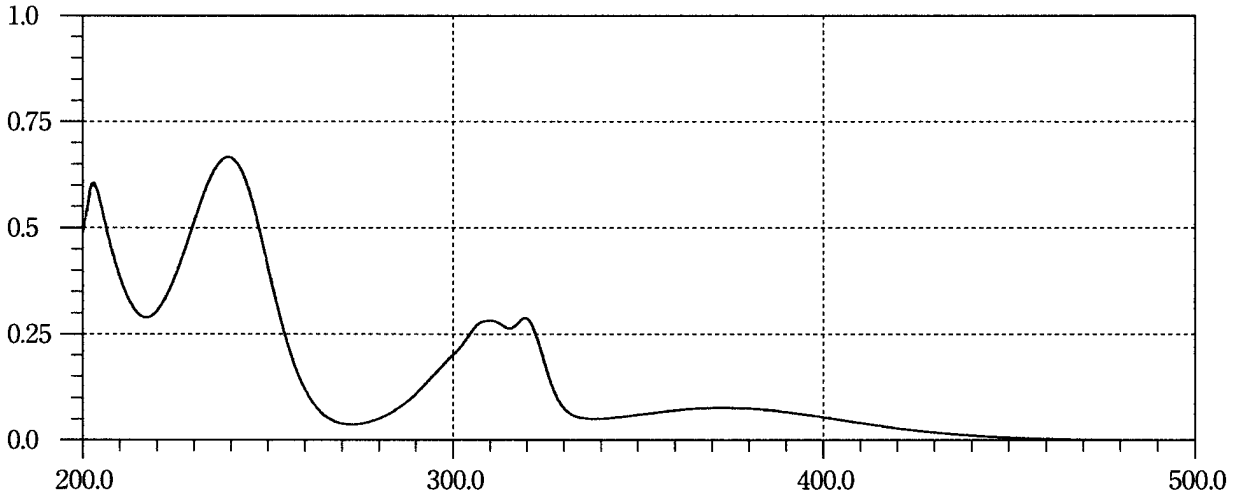
A solution in ethanol (95) (1 in 100,000)

Thiamylal Sodium



A solution in ethanol (95) (7 in 1, 000,000)

Tizanidine Hydrochloride



A solution in diluted 1 mol/L ammonia TS (1 in 10) (1 in 125,000)

General Information

Change to read:

5. International Harmonization Implemented in the Japanese Pharmacopoeia Fourteenth Edition

Items for which harmonization has been agreed among the European Pharmacopoeia, the United States Pharmacopeia and the Japanese Pharmacopoeia are implemented in the Japanese Pharmacopoeia Fourteenth Edition (JP 14). They are shown in the tables below. The column headed Harmonized items shows the harmonized items written in the Pharmacopoeial Harmonization Agreement Document, and the column headed JP 14 shows the items as they appear in JP 14. In the Remarks column, notes on any differences between JP 14 and the agreement are shown as occasion demands.

Harmonized items	JP 14	Remarks
Bacterial Endotoxins Test	**Bacterial Endotoxins Test**	
Apparatus	Apparatus	
Preparation of Standard Endotoxin Stock Solution	Preparation of Standard Endotoxin Stock Solution	
Preparation of Standard Endotoxin Solution	Preparation of Standard Endotoxin Solution	
Preparation of Sample Solutions	Preparation of Sample Solutions	
Determination of Maximum Valid Dilution	Determination of Maximum Valid Dilution	
Gel-clot technique (1) Preparatory testing (2) Limit test (3) Assay	Gel-clot techniques (1) Preparatory testing (2) Limit test (3) Assay	
Photometric techniques (1) Turbidimetric technique (2) Chromogenic technique (3) Preparatory testing (4) Assay	Photometric techniques (1) Turbidimetric technique (2) Chromogenic technique (3) Preparatory testing (4) Assay	
Reagents, Test Solutions	**Reagents, Test Solutions**	
Amebocyte lysate	Lysate reagent	
Lysate TS	Lysate TS	
Water for bacterial endotoxins test (BET)	Water for bacterial endotoxins test (BET)	

Note: The method for decision of the limit for bacterial endotoxins was agreed between the three pharmacopoeias, but in the Decision of Limit for Bacterial Endotoxins under the General Information in JP 14, the maximum adult dose is calculated based on an average body mass of an adult of 60 kg.

Harmonized items	JP 14	Remarks
Residue on Ignition/Sulphated Ash Test	**Residue on Ignition Test**	
(Introduction)	(Introduction)	Explanation of JP's particular expressions in the introduction: The description, for example, ''not more than 0.10% (1 g),'' in a monograph, indicates that the mass of the residue is not more than 1.0 mg per g of the substance to be tested in which about 1 g of the substance is weighed accurately and ignited by the procedure described below, and ''after drying'' indicates that the sample is tested after being dried under the conditions specified in the test for Loss on drying.
Procedure	Procedure	Explanation of JP's particular expressions for taking the sample: When the quantity of the sample to be taken is indicated in a volume, pipet exactly the amount directed in the monograph and transfer to the above crucible. When directed as ''after evaporating,'' heat properly to evaporate the solution.

Harmonized items	JP 14	Remarks
Sterility	**Sterility Test**	
Precautions against microbial contamination		Comment 1
Culture media and incubation temperatures	Media and rinsing fluids	
Fluid thioglycollate medium	Fluid thioglycolate medium	Note 1 – 5
Soya-bean casein digest medium	Soybean-casein digest medium	
Sterility	Sterility of media	
Growth promotion test of aerobes, anaerobes and fungi	Growth promotion test	
Validation test	Validation test	Note 6
Membrane filtration	Membrane filtration	
Direct inoculation	Direct inoculation	
Test for sterility of the product to be examined	Test for sterility of the products to be examined	
Membrane filtration	Membrane filtration	
Aqueous solutions	a) Liquid medicines	Note 7
Soluble solids	b) Solid medicines	Comment 2
Oils and oily solutions	c) Oils and oily solutions	
Ointments and creams	d) Ointments and creams	
Direct inoculation of the culture medium	Direct inoculation of the culture medium	
Oily liquids	a) Oily liquids	
Ointments and creams	b) Ointments and creams	

Catgut and other surgical sutures for veterinary use		Comment 3
Observation and interpretation of results	Cultivation and observation, Observation and interpretation of results	Note 8 – 9
Application of the test to parenteral preparations, ophthalmic and other non-injectable preparations required to comply with the test for sterility		Comment 4
Table 2.6.1.-1. Strains of the test micro-organisms suitable for use in the Growth Promotion Test and the Validation Test	Table 1. Microorganisms for growth promotion test and the validation test	
Table 2.6.1.-2. Minimum quantity to be used for each medium	Table 3. Minimum quantity to be used for each medium	
Table 2.6.1.-3. Minimum number of items to be tested	Table 2. Number of items to be taken from the lot	Note 10 – 11

Note: 1) Non-pharmaceutical media: Not to be used.
2) Water content of agar: Not being specified.
3) Effective period of media: Unnecessary to be validated.
4) Effective period of media stored in hermetic containers: Usable for maximum one year.
5) Medium for sterility test of the products containing a mercurial preservative: For products that cannot be tested by the membrane-filtration method, use fluid thioglycolate medium incubated at 20 – 25°C instead of soybean-casein digest medium.
6) Periodic growth promotion test for a ready-made powder medium: Not need to test with respect to each batch, provided it is prepared under well-controlled conditions.
7) Amount of the rinsing fluid each time in the membrane-filtration method: 100 mL per filter.
8) Transferring amount from turbid medium to fresh medium: A suitable amount.
9) Requirements for the retesting in the case when the evidence of microbial growth is found: If the microbial growth is found the product examined does not comply with the test for sterility. However, provided that various factors and/or properties of the contaminant suggest that the test itself was inadequate, the test may be repeated. If no evidence of microbial growth is found in the repeat test the product complies with the Sterility Test. If microbial growth is found in the repeat test the product does not comply with the Sterility Test.
10) The table of 'Number of items to be taken from the lot': Specified as a part of the General Test.
11) Number of large-volume products (more than 100 mL) to be taken from the lot: Maximum 10 containers.

Comment 1: The same statement appears in the introduction.
Comment 2: There is a statement in 11. Injections (9) under the General Rules for Preparations that ''Unless otherwise specified, Injections meet the requirements of the Sterility Test. In the case of drugs to be dissolved before use, carry out the test with the solution obtained by dissolving the contents in the attached solvent.''
Comment 3: The items not included in JP.
Comment 4: A part of this is directed in the General Test.

Harmonized items	JP 14	Remarks
Sodium Chloride	**Sodium Chloride**	
Definition	limits of the content	
Identification A	Identification (1)	
Identification B	Identification (2)	
Acidity or alkalinity	Purity (2) Acidity or alkalinity	
Bromides	Purity (5) Bromides	
Ferrocyanides	Purity (7) Ferrocyanides	
Iodides	Purity (6) Iodides	
Nitrites	not specified	
Phosphates	Purity (4) Phosphates	
Sulphates	Purity (3) Sulfates	
Aluminium	not specified	
Barium	Purity (10) Barium	
Iron	Purity (9) Iron	
Magnesium and alkaline-earth metals	Purity (11) Magnesium and alkaline-earth materials	
Potassium	not specified	
Loss on drying	Loss on drying	
Bacterial endotoxins	not specified	
Assay	Assay	

Harmonized items	JP 14	Remarks
Citric Acid Monohydrate	**Citric Acid**	
Definition	limits of the content	
Identification	Identification	
Appearance of solution	Purity (1) Clarity and color of solution	
Readily carbonisable substances	Purity (5) Readily carbonizable substances	
Oxalic acid	Purity (3) Oxalate	
Sulphates	Purity (2) Sulfates	
Aluminium	not specified	
Water	Water	
Sulphated ash	Residue on ignition	
Bacterial endotoxins	not specified	
Assay	Assay	
Storage	Containers and storage	

Harmonized items	JP 14	Remarks
Citric Acid, Anhydrous	**Anhydrous Citric Acid**	
Definition	limits of the content	
Identification	Identification	
Appearance of solution	Purity (1) Clarity and color of solution	
Readily carbonisable substances	Purity (5) Readily carbonizable substances	
Oxalic acid	Purity (3) Oxalate	
Sulphates	Purity (2) Sulfates	
Aluminium	not specified	
Water	Water	
Sulphated ash	Residue on ignition	
Bacterial endotoxins	not specified	
Assay	Assay	
Storage	Containers and storage	

Harmonized items	JP 14 General Infromation	Remarks
Carboxymethylcellulose Calcium	**Carmellose Calcium**	
Definition	origin	
Identification A	Identification (1)	
Identification B	Identification (2)	
Identification C	Identification (3)	
Identification D	Identification (4)	
Alkalinity	Purity (1) Alkali	
Loss on drying	Loss on drying	
Residue on ignition	Residue on ignition	
Limit of chloride	Purity (2) Chloride	
Limit of sulfate	Purity (3) Sulfate	

Harmonized items	JP 14 General Infromation	Remarks
Wheat Starch	**Wheat Starch**	
Definition	origin	
Identification A	Identification (1)	
Identification B	Identification (2)	
Identification C	Identification (3)	
pH	pH	
Iron	Purity (1) Iron	
Total protein	not specified	
Oxidising substances	Purity (2) Oxidizing substances	
Sulphur dioxide	Purity (3) Sulfur dioxide	
Loss on drying	Loss on drying	
Sulphated ash	Residue on ignition	

Harmonized items	JP 14	Remarks
Cellacefate	**Cellulose Acetate Phthalate**	
Definition	limits of the content	
Identification	Identification	
Viscosity	Viscosity	
Limit of free acid	Purity (2) Free acids	
Water	Water	
Residue on ignition	Residue on ignition	
Phthalyl content	Assay (1) Carboxybenzoyl	
Content of acetyl	Assay (2) Acetyl group	
Packaging and storage	Containers and storage	

Harmonized items	JP 14 General Infromation	Remarks
Corn Starch	**Corn Starch**	
Definition	origin	
Identification A	Identification (1)	
Identification B	Identification (2)	
Identification C	Identification (3)	
pH	pH	
Loss on drying	Loss on drying	
Residue on ignition	Residue on ignition	
Limit of iron	Purity (1) Iron	
Limit of oxidizing substances	Purity (2) Oxidizing substances	
Sulfur dioxide determination	Purity (3) Sulfur dioxide	

Harmonized items	JP 14 General Infromation	Remarks
Potato Starch	**Potato Starch**	
Definition	origin	
Identification A	Identification (1)	
Identification B	Identification (2)	
Identification C	Identification (3)	
pH	pH	
Iron	Purity (1) Iron	
Oxidising substances	Purity (2) Oxidizing substances	
Sulphur dioxide	Purity (3) Sulfur dioxide	
Loss on drying	Loss on drying	
Sulphated ash	Residue on ignition	

Harmonized items	JP 14	Remarks
	General Information	
Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)	**SDS-Polyacrylamide Gel Electrophoresis**	
Characteristics of Polyacrylamide Gels	1. Characteristics of Polyacrylamide Gels	
Denaturing Polyacrylamide Gel Electrophoresis	2. Polyacrylamide Gel Electrophoresis under Denaturing Conditions	
Reducing conditions	1) Reducing conditions	
Non-reducing conditions	2) Non-reducing conditions	
Characteristics of Discontinuous Buffer System Gel Electrophoresis	3. Characteristics of Discontinuous Buffer System Gel Electrophoresis	
Preparing Vertical Discontinuous Buffer SDS Polyacrylamide Gels	4. Preparing Vertical Discontinuous Buffer SDS-Polyacrylamide Gels	
Assembling of the gel moulding cassette	1) Assembling of the gel moulding cassette	
Preparation of the gel	2) Preparation of the gel	
Mounting the gel in the electrophoresis apparatus and electrophoretic separation	3) Mounting the gel in the electrophoresis apparatus and electrophoretic separation	
Detection of Protein in Gels	5. Detection of Proteins in Gels	
Coomassie staining	1) Coomassie staining	
Silver staining	2) Silver staining	
Drying of Stained SDS Polyacrylamide Gels	6. Drying of Stained SDS-Polyacrylamide Gels	
Molecular-Mass Determination	7. Molecular-Mass Determination	
Validation of the Test	8. Suitability of the Test	
Quantification of Impurities	9. Quantification of Impurities	
Reagents, Test Solutions	Test Solutions	
Blocking solution	Blocking TS	

Coomassie staining solution	Coomassie staining TS	
Destaining solution	Destaining TS	
Developer solution	Developer TS	
Fixing solution	Fixing TS	
Silver nitrate reagent	Silver nitrate TS for silver staining	
Trichloroacetic acid reagent	Trichloroacetic acid TS for fixing	
Table 1 − Preparation of resolving gel	Table 1. Preparation of resolving gel	
Table 2 − Preparation of stacking gel	Table 2. Preparation of stacking gel	

Harmonized items	JP 14 General Infromation	Remarks
Benzyl Alcohol	**Benzyl Alcohol**	
Definition	limits of the content	
Identification	Identification	
Appearance of solution	Purity (1) Clarity and color of solution	
Refractive index	Refractive index	
Acidity	Purity (2) Acid	
Benzaldehyde and other related substances	Purity (3) Benzaldehyde and other related substances	
Peroxide value	Purity (4) Peroxide value	
Residue on evaporation	Purity (5) Residue on evaporation	
Assay	Assay	

Harmonized items	JP 14	Remarks
	General Information	
Amino Acid Analysis	**Amino Acid Analysis**	
Apparatus	Apparatus	
General Precautions	General Precautions	
Reference Standard Material	Reference Standard Material	
Calibration of Instrumentation	Calibration of Instrumentation	
Repeatability	Repeatability	
Sample Preparation	Sample Preparation	
Internal Standards	Internal Standards	
Protein Hydrolysis	Protein Hydrolysis	
Method 1	Method 1	
Hydrolysis Solution	Hydrolysis Solution	
Procedure	Procedure	
Method 2	Method 2	
Hydrolysis Solution	Hydrolysis Solution	
Vapor Phase Hydrolysis	Vapor Phase Hydrolysis	
Method 3	Method 3	

Hydrolysis Solution
Vapor Phase Hydrolysis
Method 4
Oxidation Solution
Procedure
Method 5
Hydrolysis Solution
Liquid Phase Hydrolysis
Method 6
Hydrolysis Solution
Vapor Phase Hydrolysis
Method 7
Reducing Solution
Procedure
Method 8
Stock Solutions
Reducing Solution
Procedure
Method 9
Stock Solutions
Carboxymethylation Solution
Buffer Solution
Procedure
Method 10
Reducing Solution
Procedure
Method 11
Reducing Solutions
Procedure
Methodologies of Amino Acid Analysis General Principles
Method 1-Postcolumn Ninhydrin Detection General Principle
Method 2-Postcolumn OPA Fluorometric Detection General Principle
Method 3-Precolumn PITC Derivatization General Principle
Method 4-Precolumn AQC Derivatization General Principle
Method 5-Precolumn OPA Derivatization General Principle
Method 6-Precolumn DABS-Cl Derivatization General Principle
Method 7-Precolumn FMOC-Cl Derivatization General Principle

Method 8-Precolumn NBD-F Derivatization General Principle	Method 8-Precolumn NBD-F Derivatization General Principle	
Data Calculation and Analysis	Data Calculation and Analysis	
Calculations	Calculations	
Amino Acid Mole Percent	Amino Acid Mole Percent	
Unknown Protein Samples	Unknown Protein Samples	
Known Protein Samples	Known Protein Samples	

Harmonized items	JP 14	Remarks
	General Information	
Capillary Electrophoresis	**Capillary Electrophoresis**	
Apparatus	Apparatus	
Capillary Zone Electrophoresis	Capillary Zone Electrophoresis	
Optimisation	Optimization	
Instrumental parameters	Instrumental parameters	
Voltage	Voltage	
Polarity	Polarity	
Temperature	Temperature	
Capillary	Capillary	
Electrolytic solution parameters	Electrolytic solution parameters	
Buffer type and concentration	Buffer type and concentration	
Buffer pH	Buffer pH	
Organic solvents	Organic solvents	
Additives for chiral separations	Additives for chiral separations	
Capillary Gel Electrophoresis	Capillary Gel Electrophoresis	
Characteristics of Gels	Characteristics of Gels	
Capillary Isoelectric Focusing	Capillary Isoelectric Focusing	
Loading step	Loading step	
loading in one step	loading in one step	
sequential loading	sequential loading	
Focusing step	Focusing step	
Mobilisation step	Mobilization step	
Optimisation	Optimization	
Voltage	Voltage	
Capillary	Capillary	
Solutions	Solutions	
Micellar Electrokinetic Chromatography (MEKC)	Micellar Electrokinetic Chromatography (MEKC)	
Optimisation	Optimization	
Instrumental parameters	Instrumental parameters	
Voltage	Voltage	
Temperature	Temperature	
Capillary	Capillary	
Electrolytic solution parameters	Electrolytic solution parameters	

Surfactant type and concentration	Surfactant type and concentration	
Buffer pH	Buffer pH	
Organic solvents	Organic solvents	
Additives for chiral separations	Additives for chiral separations	
Other additives	Other additives	
Quantification	Quantification	
Calculations	Calculations	
System Suitability	System Suitability	
Apparent Number of Theoretical Plates	Apparent Number of Theoretical Plates	
Resolution	Resolution	
Symmetry Factor	Symmetry Factor	
Signal-to-noise Ratio	Signal-to-noise Ratio	

Harmonized items	JP 14	Remarks
	General Information	
Total Protein Assay	**Total Protein Assay**	
Method 1	Method 1	
Standard Solution	Standard Solution	
Test Solution	Test Solution	
Procedure	Procedure	
Light-Scattering	Light-Scattering	
Calculations	Calculations	
Method 2	Method 2	Explanatory footnote ''Example: the Minimum Requirements for Biological Products and individual monograph of JP'' is added.
Standard Solutions	Standard Solutions	
Test Solution	Test Solution	
Blank	Blank	
Reagents and Solutions	Reagents and Solutions	
Copper Sulfate Reagent	Copper Sulfate Reagent	
SDS Solution	5% SDS TS	
Sodium Hydroxide Solution	Sodium Hydroxide Solution	
Alkaline Copper Reagent	Alkaline Copper Reagent	
Diluted Folin-Ciocalteu's Phenol Ragent	Diluted Folin's TS	
Procedure	Procedure	
Calculations	Calculations	
Interfering Substances	Interfering Substances	
Sodium Deoxycholate Reagent	Sodium Deoxycholate Reagent	
Trichloroacetic Acid Reagent	Trichloroacetic Acid Reagent	
Procedure	Procedure	
Method 3	Method 3	
Standard Solutions	Standard Solutions	
Test Solution	Test Solution	
Blank	Blank	
Coomassie Reagent	Coomassie Reagent	

Procedure	Procedure	
Calculations	Calculations	
Method 4	Method 4	
Standard Solutions	Standard Solutions	
Test Solution	Test Solution	
Blank	Blank	
Reagents	Reagents and Solutions	
BCA Reagent	BCA Reagent	
Copper Sulfate Reagent	Copper Sulfate Reagent	
Copper-BCA Reagent	Copper-BCA Reagent	
Procedure	Procedure	
Calculations	Calculations	
Method 5	Method 5	For the preparation of biuret reagent, dissolving copper (II) sulfate and trisodium citrate dihydrate in water, not in hot water, and warming if necessary. Addition of the handling of standard solutions to the Procedure.
Standard Solutions	Standard Solutions	
Test Solution	Test Solution	
Blank	Blank	
Biuret Reagent	Biuret Reagent	
Procedure	Procedure	
Calculations	Calculations	
Interfering Substances	Interfering Substances	
Comments	Comments	
Method 6	Method 6	
Standard Solutions	Standard Solutions	
Test Solution	Test Solution	
Blank	Blank	
Reagents	Reagents and Solutions	
Borate Buffer	Borate Buffer	
Stock OPA Reagent	Stock OPA Reagent	
OPA Reagent	OPA Reagent	
Procedure	Procedure	
Calculations	Calculations	
Method 7	Method 7	
Procedure A	Procedure A	
Procedure B	Procedure B	
Calculations	Calculations	

Harmonized items	JP 14	Remarks
	General Information	
Isoelectric Focusing	**Isoelectric Focusing**	
Theoretical Aspects	Theoretical Aspects	
Practical Aspects	Practical Aspects	
Apparatus	Apparatus	
Isoelectric Focusing in Polyacrylamide Gels: Detailed Procedure	Isoelectric Focusing in Polyacrylamide Gels: Detailed Procedure	
Preparation of the Gels	Preparation of the Gels	
1) 7.5 per cent polyacrylamide gel	7.5% Polyacrylamide gel	
2) Preparation of the mould	Preparation of the mould	
Method	Method	
Variations to the Detailed Procedure (subject to validation)	Variations to the Detailed Procedure (Subject to Validation)	
Validation of Iso-electric Focusing Procedures	Validation of Iso-Electric Focusing Procedures	
Specified Variation to the General Method	Specified Variations to the General Method	
Point to Consider	Points to Consider	
Figure-Mould	Figure. Mould	
Reagents	Reagents and Solutions	Coomassie staining solution and destaining solution are specified.
Fixing solution for isoelectric focusing in polyacrylamide gel	Fixing solution for isoelectric focusing in polyacrylamide gel	
	Coomassie staining TS	
	Destaining solution	

Harmonized items	JP 14	Remarks
	General Information	
Peptide Mapping	**Peptide Mapping**	
Purpose and Scope	Purpose and Scope	
The Peptide Map	The Peptide Map	
Isolation and Purification	Isolation and Purification	
Selective Cleavage of Peptide Bonds	Selective Cleavage of Peptide Bonds	
Pretreatment of Sample	Pretreatment of Sample	
Pretreatment of the Cleavage Agent	Pretreatment of the Cleavage Agent	
Pretreatment of the Protein	Pretreatment of the Protein	
Establishment of Optimal Digestion Conditions	Establishment of Optimal Digestion Conditions	
pH	pH	
Temperature	Temperature	
Time	Time	
Amount of Cleavage Agent	Amount of Cleavage Agent	
Chromatographic Separation	Chromatographic Separation	
Chromatographic Column	Chromatographic Column	
Solvent	Solvent	

Mobile Phase	Mobile Phase	
Gradient Selection	Gradient Selection	
Isocratic Selection	Isocratic Selection	
Other Parameters	Other Parameters	
Validation	Validation	
Analysis and Identification of Peptides	Analysis and Identification of Peptides	
Table 1. Examples of Cleavage Agents.	Table 1. Examples of Cleavage Agents	
Table 2. Techniques Used for the Separation of Peptides.	Table 2. Techniques Used for the Separation of Peptides	

Add the following:

20. Amino Acid Analysis

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 organized 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 protein 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 hydrolyze 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 derivatized 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 postcolumn derivatization capability, unless the sample is analyzed using precolumn derivatization. The detector is usually an ultraviolet-visible or fluorescence detector depending on the derivatization method used. A recording device (e.g., integrator) is used for transforming the analog 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 Standard 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 analyzed 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 analyzing 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 analyzed. 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.

Four to six amino acid standard levels are analyzed to determine a response factor for each amino acid. The response factor is calculated as the average peak area or peak height per nmol 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 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 nmol 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 analyzing many replicates (i.e., six 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. Often the amino acid composition of a standard protein (e.g., bovine serum albumin) is analyzed 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 utilize postcolumn derivatization of the amino acids are generally not affected by buffer components to the extent seen with precolumn derivatization methods. It is desirable to limit the number of sample manipulations to reduce potential background contamination, to improve analyte recovery, and to reduce labor. 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, eluting 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); and (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 because their 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 under 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 hour in 1 mol/L hydrochloric acid or soak tubes in concentrated nitric acid or in a mixture of concentrated hydrochloric acid and concentrated nitric acid (1:1). 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 hours 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 hydrolyzing 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, 48, and 72 hours) is often employed to analyze the starting concentration of amino acids that are partially destroyed or slow to cleave. By plotting the observed concentration of labile amino acids (i.e., 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.

Note: 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 mol/L hydrochloric acid containing 0.1% to 1.0% of phenol.

Procedure—

Liquid Phase Hydrolysis Place the protein or peptide sample in a hydrolysis tube, and dry. [Note: 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 lyophilized protein. Freeze the sample tube in a dry ice-acetone bath, and flame seal in vacuum. Samples are typically hydrolyzed at 110°C for 24 hours in vacuum or inert atmosphere to prevent oxidation. Longer hydrolysis times (e.g., 48 and 72 hours) are investigated if there is a concern that the protein is not completely hydrolyzed.

Vapor 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 minimized by using vapor 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-hour hydrolysis time. Acid vapor hydrolyzes the dried sample. Any condensation of the acid in the sample vials is minimized. After hydrolysis, dry the test sample in vacuum to remove any residual acid.

Method 2

Tryptophan oxidation during hydrolysis is decreased by using mercaptoethanesulfonic acid (MESA) as the reducing acid.

Hydrolysis Solution 2.5 mol/L MESA solution.

Vapor Phase Hydrolysis About 1 to 100 μg of the protein/peptide under test is dried in a hydrolysis tube. The hydrolysis tube is placed in a larger tube with about 200 μL

of the *Hydrolysis Solution*. The larger tube is sealed in vacuum (about 50 μm of mercury or 6.7 Pa) to vaporize the *Hydrolysis Solution*. The hydrolysis tube is heated to 170°C to 185°C for about 12.5 minutes. After hydrolysis, the hydrolysis tube is dried in vacuum for 15 minutes to remove the residual acid.

Method 3

Tryptophan oxidation during hydrolysis is prevented by using thioglycolic acid (TGA) as the reducing acid.

Hydrolysis Solution A solution containing 7 mol/L hydrochloric acid, 10% of trifluoroacetic acid, 20% of thioglycolic acid, and 1% of phenol.

Vapor Phase Hydrolysis About 10 to 50 μg of the protein/peptide under test is dried in a sample tube. The sample tube is placed in a larger tube with about 200 μL of the *Hydrolysis Solution*. The larger tube is sealed in vacuum (about 50 μm of mercury or 6.7 Pa) to vaporize the TGA. The sample tube is heated to 166°C for about 15 to 30 minutes. After hydrolysis, the sample tube is dried in vacuum for 5 minutes 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 The performic acid is prepared fresh by mixing formic acid and 30 percent hydrogen peroxide (9:1), and incubated at room temperature for 1 hour.

Procedure The protein/peptide sample is dissolved in 20 μL of formic acid, and heated at 50°C for 5 minutes; then 100 μL of the *Oxidation Solution* is added. The oxidation is allowed to proceed for 10 to 30 minutes. In this reaction, cysteine is converted to cysteic acid and methionine is converted to methionine sulfone. The excess reagent is removed from the sample in a vacuum centrifuge. This technique may cause modifications to tyrosine residues in the presence of halides. The oxidized protein can then be acid hydrolyzed using *Method 1* or *Method 2*.

Method 5

Cysteine-cystine oxidation is accomplished during the liquid phase hydrolysis with sodium azide.

Hydrolysis Solution 6 mol/L hydrochloric acid containing 0.2% of phenol, to which is added sodium azide to obtain a final concentration of 0.2% (w/v). The added phenol prevents halogenation of tyrosine.

Liquid Phase Hydrolysis The protein/peptide hydrolysis is conducted at about 110°C for 24 hours. 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 two oxidative products, methionine sulfoxide and methionine sulfone.

Method 6

Cysteine-cystine oxidation is accomplished with dimethyl sulfoxide (DMSO).

Hydrolysis Solution 6 mol/L hydrochloric acid containing 0.1% to 1.0% of phenol, to which DMSO is added to obtain a final concentration of 2% (v/v).

Vapor Phase Hydrolysis The protein/peptide hydrolysis is conducted at about 110°C for 24 hours. 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 hydrolyses of standard proteins containing 1 to 8 mol of cysteine. The response factors from protein/peptide hydrolysates are typically about 30% lower than those for nonhydrolyzed 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 vapor 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 vacuum (about 50 μm of mercury or 6.7 Pa), and incubate at about 100°C for 5 minutes. Then remove the inner hydrolysis tube, and dry it in a vacuum desiccator for 15 minutes to remove residual reagents. The pyridylethylated protein/peptide can then be acid hydrolyzed using previously described procedures. The pyridylethylation reaction is performed simultaneously with a protein standard sample containing 1 to 8 mol of cysteine to improve accuracy in the pyridylethylcysteine 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 three solutions: 1 mol/L Tris hydrochloride (pH 8.5) containing 4 mmol/L edetate disodium (*Stock Solution A*), 8 mol/L guanidine hydrochloride (*Stock Solution B*), and 10% of 2-mercaptoethanol in water (*Stock Solution C*).

Reducing Solution Prepare a mixture of *Stock Solution B* and *Stock Solution A* (3:1) to obtain a buffered solution of 6 mol/L guanidine hydrochloride in 0.25 mol/L 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 hours 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 hours at room temperature in the dark. The protein/peptide is

desalted 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 solution containing 100 mg of iodoacetamide per mL of ethanol (95).

Buffer Solution Use the *Reducing Solution*, prepared as directed 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 hours 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 minutes at room temperature in the dark. [Note: If the thiol content of the protein is unknown, then add 5 µL of 100 mmol/L iodoacetamide for every 20 nmol of protein present.] The reaction is stopped by adding excess of 2-mercaptoethanol. The protein/peptide is desalted 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-carboxymethylcysteine during acid hydrolysis.

Method 10

Cysteine-cystine is reacted with dithiodiglycolic acid or dithiodipropionic acid to produce a mixed disulfide. [Note: The choice of dithiodiglycolic acid or dithiodipropionic acid depends on the required resolution of the amino acid analysis method.]

Reducing Solution A solution containing 10 mg of dithiodiglycolic acid (or dithiodipropionic acid) per mL of 0.2 mol/L 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 hydrolyzed using *Method 1*. This method has the advantage that other amino acid residues are not derivatized by side reactions, and 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 three solutions: a solution of 10 mmol/L trifluoroacetic acid (*Solution A*), a solution of 5 mol/L guanidine hydrochloride and 10 mmol/L trifluoroacetic acid (*Solution B*), and a freshly prepared solution of *N*,*N*-dimethylformamide containing 36 mg of BTI per mL (*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 vacuum. Heat the sample at 60°C for 4 hours in the dark. The sample is then dialyzed with water to remove the excess reagents. Extract the dialyzed sample three times with equal volumes of *n*-butyl acetate, and then lyophilize. The protein can then be acid hydrolyzed 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 underivatized and BTI-derivatized acid hydrolysis. [Note: The threonine, methionine, cysteine, tyrosine, and histidine assayed content can be altered by BTI derivatization; 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 postcolumn derivatization (e.g., with ninhydrin or *o*-phthalaldehyde). Postcolumn detection techniques can be used with samples that contain small amounts of buffer components, such as salts and urea, and generally require between 5 and 10 µg of protein sample per analysis. The remaining amino acid techniques typically involve precolumn derivatization of the free amino acids (e.g., phenyl isothiocyanate; 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate or *o*-phthalaldehyde; (dimethylamino)azobenzenesulfonyl chloride; 9-fluorenylmethylchloroformate; and, 7-fluoro-4-nitrobenzo-2-oxa-1,3-diazole) followed by reversed-phase HPLC. Precolumn derivatization techniques are very sensitive and usually require between 0.5 and 1.0 µg of protein sample per analysis but may be influenced by buffer salts in the samples. Precolumn derivatization techniques may also result in multiple derivatives of a given amino acid, which complicates the result interpretation. Postcolumn derivatization techniques are generally influenced less by performance variation of the assay than precolumn derivatization 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 utilize more than one amino acid analysis technique to exploit the advantages offered by each. In each of these *Methods*, the analog signal is visualized by means of a data acquisition system, and the peak areas are integrated for quantification purposes.

Method 1—Postcolumn Ninhydrin Detection General Principle

Ion-exchange chromatography with postcolumn ninhydrin detection is one of the most common methods employed for quantitative amino acid analysis. As a rule, a Li-based cation-exchange system is employed for the analysis of the more complex physiological samples, and the faster Na-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 characteristic purple or yellow color. Amino acids, except imino acid, give a purple color, and show the maximum absorption at 570 nm. The imino acids such as proline give a yellow color, and show the maximum absorption at 440 nm. The postcolumn reaction between ninhydrin and amino acid eluted from column is monitored at 440 and 570 nm, and the chromatogram obtained is used for the determination of amino acid composition.

Detection limit is considered to be 10 pmol for most of the amino acid derivatives, but 50 pmol for proline. Response linearity is obtained in the range of 20 to 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—Postcolumn OPA Fluorometric Detection General Principle

o-Phthalaldehyde (OPA) reacts with primary amines in the presence of thiol compound, to form highly fluorescent isoindole products. This reaction is utilized for the postcolumn derivatization in analysis of amino acids by ion-exchange chromatography. The rule of the separation is the same as *Method 1*. Instruments and reagents for this form of amino acid analysis are available commercially. Many modifications of this methodology exist.

Although OPA does not react with secondary amines (imino acids such as proline) to form fluorescent substances, the oxidation with sodium hypochlorite allows secondary amines to react with OPA. The procedure employs a strongly acidic cation-exchange column for separation of free amino acids followed by postcolumn oxidation with sodium hypochlorite and postcolumn derivatization using OPA and thiol compound such as *N*-acetyl-L-cysteine and 2-mercaptoethanol. The derivatization of primary amino acids are not noticeably affected by the continuous supply of sodium hypochlorite.

Separation of the amino acids on an ion-exchange column is accomplished through a combination of changes in pH and cation strength. After postcolumn derivatization of eluted amino acids with OPA, the reactant passes through the fluorometric detector. Fluorescence intensity of OPA-derivatized amino acids are monitored with an excitation wavelength of 348 nm and an emission wavelength of 450 nm.

Detection limit is considered to be a few tens of picomole level for most of the amino acid derivatives. Response linearity is obtained in the range of a few picomole level to a few tens of nanomole level. To obtain good compositional data, the starting with greater than 500 ng of sample before hydrolysis is best suited for the amino acid analysis of protein/peptide.

Method 3—Precolumn PITC Derivatization General Principle

Phenylisothiocyanate (PITC) reacts with amino acids to form phenylthiocarbamyl (PTC) derivatives which can be detected with high sensitivity at 245 nm. Therefore, precolumn derivatization of amino acids with PITC followed by a reversed-phase HPLC separation with UV detection is used to analyze the amino acid composition.

After the reagent is removed under vacuum, the derivatized 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 three days.

Separation of the PTC-amino acids on a reversed-phase HPLC with ODS column is accomplished through a combination of changes in concentrations of acetonitrile and buffer ionic strength. PTC-amino acids eluted from column are monitored at 254 nm.

Detection limit is considered to be 1 pmol for most of the amino acid derivatives. Response linearity is obtained in the range of 20 to 500 pmol with correlation coefficients exceeding 0.999. To obtain good compositional data, samples larger than 500 ng of protein/peptide before hydrolysis is best suited for this amino acid analysis of proteins/peptides.

Method 4—Precolumn AQC Derivatization General Principle

Precolumn derivatization of amino acids with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) followed by reversed-phase HPLC separation with fluorometric detection is used.

6-Aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (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, precolumn derivatization of amino acids with AQC followed by reversed-phase HPLC separation is used to analyze the amino acid composition.

Separation of the AQC-amino acids on ODS column is accomplished through a combination of changes in concen-

trations of acetonitrile and salt. Selective fluorescence detection of the derivatives with excitation wavelength at 250 nm and 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 hydrolyzed ($t_{1/2}$ <15 seconds) to yield 6-aminoquinoline, *N*-hydroxysuccinimide and carbon dioxide, and after 1 minute no further derivatization can take place.

Peak areas for AQC-amino acids are essentially unchanged for at least 1 week at room temperature, and the derivatives have more than sufficient stability to allow for overnight automated chromatographic analysis.

Detection limit is considered to be ranging from ca. 40 to 320 fmol for each amino acid, except for Cys. Detection limit for Cys is approximately 800 fmol. Response linearity is obtained in the range of 2.5 to 200 μmol/L with correlation coefficients exceeding 0.999. Good compositional data could be obtained from the analysis of derivatized protein hydrolysates containing as little as 30 ng of protein/peptide.

Method 5—Precolumn OPA Derivatization General Principle

Precolumn derivatization 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).

o-Phthalaldehyde (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 derivatization and analysis using an autosampler to mix the sample with the reagent. However, lack of reactivity with secondary amino acids has been 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*.

Precolumn derivatization 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 derivatization. The liquid chromatograph is equipped with a fluorometric detector for the detection of derivatized amino acids. Fluorescence intensity of OPA-derivatized 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—Precolumn DABS-Cl Derivatization General Principle

Precolumn derivatization of amino acids with (dimethylamino)azobenzenesulfonyl chloride (DABS-Cl) followed by reversed-phase HPLC separation with visible light detection is used.

(Dimethylamino)azobenzenesulfonyl chloride (DABS-Cl) is a chromophoric reagent employed for the labeling of amino acids. Amino acids labeled with DABS-Cl (DABS-amino acids) are highly stable and show the maximum absorption at 436 nm.

DABS-amino acids, all 19 naturally occurring amino acids 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 column are detected at 436 nm in the visible region.

This *Method* can analyze the imino acids such as proline together with the amino acids at the same degree of sensitivity, DABS-Cl derivatization 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 under *Method 2* in ''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 under *Method 11* in ''Protein Hydrolysis''.

The non-proteinogenic amino acid, norleucine cannot be used as 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.

Detection limit of DABS-amino acid is about 1 pmol. As little as 2 to 5 pmol of an individual DABS-amino acid can be quantitatively analysed with reliability, and only 10 to 30 ng of the dabsylated protein hydrolysate is required for each analysis.

Method 7—Precolumn FMOC-Cl Derivatization General Principle

Precolumn derivatization of amino acids with 9-fluorenylmethyl chloroformate (FMOC-Cl) followed by reversed-phase HPLC separation with fluorometric detection is used.

9-Fluorenylmethyl chloroformate (FMOC-Cl) reacts with both primary and secondary amino acids to form highly fluorescent products. The reaction of FMOC-Cl with amino acid proceeds under mild conditions in aqueous solution and is completed in 30 seconds. 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 ODS column. The separation is carried out by gradient elution varied linearly from a mixture of acetonitrile methanol and acetic acid buffer (10:40:50) to a mixture of acetonitrile and acetic acid buffer (50:50), and 20 amino acid derivatives are separated in 20 minutes. Each derivative

eluted from 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 fmol range. A linearity range of 0.1 to 50 μmol/L is obtained for most of the amino acids.

Method 8—Precolumn NBD-F Derivatization General Principle

Precolumn derivatization 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.

7-fluoro-4-nitrobenzo-2-oxa-1.3-diazole (NBD-F) reacts with both primary and secondary amino acids to form highly fluorescent products. Amino acids are derivatized with NBD-F by heating to 60°C for 5 minutes.

NBD-amino acid derivatives are separated on an ODS column of a reversed-phase HPLC by employing gradient elution system consisting of acetonitrile and aqueous buffer mixture, and 17 amino acid derivatives are separated in 35 minutes. ε-Aminocaproic acid can be used as an internal standard, because it is eluted in a clean chromatographic region. Each derivative eluted from 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 precolumn OPA derivatization 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 was achieved for about 1.5 μg of protein hydrolysates in the final precolumn labeling reaction mixture for HPLC.

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 (≦not less than 200 μm of mercury or 26.7 Pa) or introduction of inert gas (argon) in the headspace of the reaction vessel during vapor 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.

Calculations

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 weight 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 by the formula:

$$100r_U/r,$$

in which r_U is the peak response, in nmol, of the amino acid under test; and r is the sum of peak responses, in nmol, 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 μg, of each recovered amino acid by the formula:

$$mM_W/1000,$$

in which m is the recovered quantity, in nmol, of the amino acid under test; and M_W is the average molecular weight for that amino acid, corrected for the weight 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 analyzed after appropriate correction for partially and completely destroyed amino acids. If the molecular weight 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 by the formula:

$$m/(1000M/M_{WT}),$$

in which m is the recovered quantity, in nmol, of the amino acid under test; M is the total mass, in μg, of the protein; and M_{WT} is the molecular weight 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 weight and amino acid composition using the amino acid analysis data. When the composition of the protein being analyzed 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 nmol, 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% 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, by the formula:

$$100m/m_S,$$

in which m is the experimentally determined quantity, in nmol 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.

Add the following:

21. Capillary Electrophoresis

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:

$$v_{ep} = \mu_{ep}E = \left(\frac{q}{6\pi\eta r}\right)\left(\frac{V}{L}\right)$$

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:

$$v_{eo} = \mu_{eo}E = \left(\frac{\varepsilon\zeta}{\eta}\right)\left(\frac{V}{L}\right)$$

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:

$$t = \frac{l}{v_{ep} + v_{eo}} = \frac{l \times L}{(\mu_{ep} + \mu_{eo})V}$$

In general, uncoated fused-silica capillaries above pH 3 have negative charge due to ionized 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 two 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:

$$R_S = \frac{\sqrt{N}(\mu_{epb} - \mu_{epa})}{4(\bar{\mu}_{ep} + \mu_{eo})}$$

μ_{epa} and μ_{epb}: electrophoretic mobilities of the two analytes separated,

$\bar{\mu}_{ep}$: mean electrophoretic mobility of the two analytes

$$\bar{\mu}_{ep} = \frac{1}{2}(\mu_{epb} + \mu epa).$$

Apparatus

An apparatus for capillary electrophoresis is composed of:

—a high-voltage, controllable direct-current power supply,

—two buffer reservoirs, held at the same level, containing the prescribed anodic and cathodic solutions,

—two 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 fluorometry, 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.

1. 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 free solution capillary 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.

Optimization

Optimization 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 optimizing the applied voltage and column 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 electro-osmotic 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 minimize current generation.

Matching buffer-ion mobility to solute mobility, whenever possible, is important for minimizing 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 ionization 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 optical isomers, 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 ionizable (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.

2. 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

Two 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 polymerization 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 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 optimized by modifying the separation buffer (as indicated in the free solution capillary 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.

3. 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 mobilization.

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.

Mobilization step: If mobilization is required for detection, use one of the following methods. Three methods are available:

—in the first method, mobilization 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, mobilization is accomplished by applying positive pressure after the focusing step;

—in the third method, mobilization is achieved after the focusing step by adding salts to the cathode reservoir or the anode reservoir (depending on the direction chosen for mobilization) in order to alter the pH in the capillary when the voltage is applied. As the pH is changed, the proteins and ampholytes are mobilized 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 ($\mathrm{d}pH/\mathrm{d}x$), 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 ($-\mathrm{d}\mu/\mathrm{d}pH$):

$$\Delta PI = 3\sqrt{\frac{D(\mathrm{d}pH/\mathrm{d}x)}{E(-\mathrm{d}\mu/\mathrm{d}pH)}}$$

Optimization

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 mobilization 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.

4. 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

ionized solutes is essentially chromatographic, migration of the solute and resolution can be rationalized 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:

$$k' = \frac{t_R - t_0}{t_0\left(1 - \frac{t_R}{t_{mc}}\right)} = K\frac{V_S}{V_M}$$

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 two closely-migrating solutes (R_S) is given by:

$$R_S = \frac{\sqrt{N}}{4} \times \frac{\alpha - 1}{\alpha} \times \frac{k'_b}{k'_b + 1} \times \frac{1 - \left(\frac{t_0}{t_{mc}}\right)}{1 + \left(\frac{t_0}{t_{mc}}\right)k'_a}$$

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.

Optimization

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 free solution capillary 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 $\sqrt{t_m/t_0}$, 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-ionized 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 micellization 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 micellized 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 (normalization procedure). The use of an automatic integration system (integrator or data acquisition and processing system) is recommended.

System Suitability

In order to check the behavior 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:

$$N = 5.54\left(\frac{t_R}{w_h}\right)^2$$

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 (RS) between peaks of similar height of two components may be calculated using the expression:

$$R_S = \left(\frac{1.18(t_{R2} - t_{R1})}{w_{h1} + w_{h2}}\right)$$

$t_{R2} > t_{R1}$

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 two partly resolved peaks in a standard preparation and the height of the smaller peak (H_p) and calculating the peak-to-valley ratio:

$$p/v = \frac{H_p}{H_v}$$

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:

$$S/N = \frac{2H}{h}$$

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.

Add the following:

22. Isoelectric Focusing

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 immobilized 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), their charge is neutralized 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 neighboring bands:

$$\Delta pI = 3\sqrt{\frac{D(dpH/dx)}{E(-d\mu/dpH)}}$$

D: Diffusion coefficient of the protein
dpH/dx: pH gradient
E: Intensity of the electric field, in volts per centimeter
$-d\mu/dpH$: Variation of the solute mobility with the pH in the region close to the pI

Since D and $-d\mu/dpH$ 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 immobilized pH gradients where the buffering species, which are analogous to carrier ampholytes, are copolymerized 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 immobilized 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 deionized 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 colored protein (e.g. hemoglobin) 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. 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) 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.

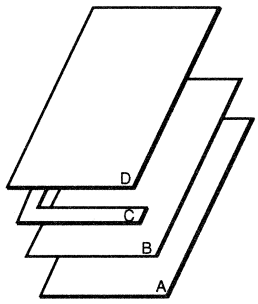


Figure. Mould

7.5% Polyacrylamide gel Dissolve 29.1 g of acrylamide and 0.9 g of *N*,*N*′-methylenebisacrylamide in 100 mL of water. To 2.5 volumes of this solution, add the mixture of ampholytes specified in the monograph and dilute to 10 volumes with water. 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. Place 7.5% polyacrylamide gel prepared before use on a magnetic stirrer, and add 0.25 volumes of a solution of ammonium persulfate (1 in 10) and 0.25 volumes of *N*,*N*,*N*′,*N*′-tetramethylethylenediamine. 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 stabilized. Using forceps, remove the sample application strips and the 2 electrode wicks. Immerse the gel in fixing solution for isoelectric focusing in polyacrylamide gel. Incubate with gentle shaking at room temperature for 30 minutes. Drain off the solution and add 200 mL of destaining solution. Incubate with shaking for 1 hour. Drain the gel, add coomassie staining TS. Incubate for 30 minutes. Destain the gel by passive diffusion with destaining solution until the bands are well visualized 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 immobilized 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 colored 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 mol/L concentration is often satisfactory to keep protein in solution but up to 8 mol/L 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. Immobilized 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.

Reagents and Solutions—

Fixing solution for isoelectric focusing in polyacrylamide gel Dissolve 35 g of 5-sulfosalicylic acid dihydrate and 100 g of trichloroacetic acid in water to make 1000 mL.

Coomassie staining TS Dissolve 125 mg of coomassie brilliant blue R-250 in 100 mL of a mixture of water, methanol and acetic acid (100) (5:4:1), and filter.

Destaining solution A mixture of water, methanol and acetic acid (100) (5:4:1).

Add the following:

23. Peptide Mapping

Purpose and Scope

Peptide mapping is an identity test for proteins, especially those obtained by r-DNA technology. It involves the chemical or enzymatic treatment of a protein resulting in the formation of peptide fragments followed by separation and identification of the fragments in a reproducible manner. It is a powerful test that is capable of identifying 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 standard or reference material 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 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 characterize the desired protein product, 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 product, or to detect the presence of protein variant.

The Peptide Map

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 analyzed. Four major 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 standard or a reference material. 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 should 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 should 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 1. This list is not all-inclusive and will be expanded as other cleavage agents are identified.

Table 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 & 2)	Nonspecific 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) (EC 3.24.33)	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

Pretreatment of Sample Depending on the size or the configuration of the protein, different approaches in the pretreatment of samples can be used. For monoclonal antibodies the heavy and light chains will need to be separated before mapping. 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, 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 HPLC or immobilization 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 added substances and stabilizers 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 tryptic map due to side reactions occurring during the digestion reaction, such as nonspecific cleavage, deamidation, disulfide isomerization, 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: The pH of the digestion mixture is empirically determined to ensure the optimization 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 should not alter the chemical integrity of the protein during the digestion and should 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 minimize 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 to 30 hours. The reaction is stopped by the addition of an acid which does not interfere in the tryptic map or by freezing.

Amount of Cleavage Agent: Although excessive amounts of cleavage agent are used to accomplish a reasonably rapid digestion time (i.e., 6 to 20 hours), the amount of cleavage agent is minimized 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 can be added in two or more stages to optimize 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 be interfering 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. In this section, a most widely used reverse-phase High Performance Liquid Chromatographic (RP-HPLC) method is described as one of the procedures of chromatographic separation.

Table 2. Techniques Used for the Separation of Peptides

Reverse-Phase High Performance Liquid Chromatography (RP-HPLC)
Ion-Exchange Chromatography (IEC)
Hydrophobic Interaction Chromatography (HIC)
Polyacrylamide Gel Electrophoresis (PAGE), nondenaturating
Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)
Capillary Electrophoresis (CE)
Paper Chromatography-High Voltage (PCHV)
High-Voltage Paper Electrophoresis (HVPE)

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 RP-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.

Chromatographic Column The selection of a chromatographic column is empirically determined for each protein. Columns with 100 Å or 300 Å pore size with silica support can give optimal separation. For smaller peptides, octylsilane chemically bonded to totally porous silica articles, 3 to 10 μm in diameter (L7) and octadecylsilane chemically bonded to porous silica or ceramic micro-particles, 3 to 10 μm in diameter (L1) column packings are more efficient than the butyl silane chemically bonded to totally porous silica particles, 5 to 10 μm in diameter (L26) packing.

Solvent The most commonly used solvent is water with acetonitrile as the organic modifier to which less than 0.1% trifluoroacetic acid is added. If necessary, add isopropyl alcohol or n-propyl alcohol to solubilize 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 to 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, and 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 Selection Gradients can be linear, nonlinear, or include step functions. A shallow gradient is recommended in order to separate complex mixtures. Gradients are optimized to provide clear resolution of one or two peaks that will become ''marker'' peaks for the test.

Isocratic Selection 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 should 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 to 2.0 mL per minute, and the detection of peptides is performed with a UV detector at 200 to 230 nm. Other methods of detection have been used (e.g., postcolumn derivatization), 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 was achieved is by the comparison with a Reference Standard, which is treated exactly as the article under test. The use of a reference standard or reference material in parallel with the protein under test is critical in the development and establishment of system suitability limits. In addition a specimen chromatogram should be included with the Reference Standard or Reference Material 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 covers 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 standard/reference material for the specified protein. The use of a digested reference standard or reference material for a given protein in the determination of peptide resolution is the method of choice. For an analysis of a variant protein, a characterized mixture of a variant and a reference standard or reference material 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 standard or reference material 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 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 retention times, 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 standard or reference material digest. If all peaks in the sample digest and in the reference standard or reference material digest have the same relative retention times and peaks response ratios, then the identity of the sample under test is confirmed.

If peaks that initially eluted with significantly different

relative retention times 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 standard or reference material 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 standard or reference material and sample fragments.

The numerical comparison of the 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 one 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 standard/reference material. 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 characterization of the individual peptide peaks. However, validation of peptide mapping in support of regulatory applications requires rigorous characterization of each of the individual peaks in the peptide map. Methods to characterize peaks range from N-terminal sequencing of each peak followed by amino acid analysis to the use of mass spectroscopy (MS).

For characterization 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 MALDITOF-MS can also be used for characterization purposes.

The use of MS for characterization 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 matrix-assisted laser desorption ionization coupled to time-of-flight analyzer (MALDITOF) 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 sulfhydryl-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 characterization of a protein through peptide mapping is to reconcile and account for at least 95% of the theoretical composition of the protein structure.

Add the following:

24. Rapid Identification of Microorganisms Based on Molecular Biological Method

This chapter describes the methods for the identification or estimation of microorganisms (bacteria and fungi), found in in-process control tests or lot release tests of pharmaceutical products, at the species or genus level based on their DNA sequence homology. The identification of isolates found in the sterility test or aseptic processing can be helpful for investigating the causes of contamination. Furthermore, information on microorganisms found in raw materials used for pharmaceutical products, processing areas of pharmaceutical products, and so on is useful in designing measures to control the microbiological quality of drugs. For the identification of microorganisms, phenotypic analysis is widely used, based on morphological, physiological, and biochemical features and analysis of components. Commercial kits based on differences in phenotype patterns have been used for the identification of microorganisms, but are not always applicable to microorganisms found in raw materials used for pharmaceutical products and in processing areas of pharmaceutical products. In general, the

identification of microorganisms based on phenotypic analysis needs special knowledge and judgment is often subjective. It is considered that the evolutionary history of microorganisms (bacteria and fungi) is memorized in their ribosomal RNAs (rRNAs), so that systematic classification and identification of microorganisms in recent years have been based on the analysis of these sequences. This chapter presents a rapid method to identify or estimate microorganisms based on partial sequences of divergent regions of the 16S rRNA gene for bacteria and of the internal transcribed spacer 1 (ITS1) region located between 18S rRNA and 5.8S rRNA for fungi, followed by comparison of the sequences with those in the database. Methods described in this chapter do not take the place of usual other methods for the identification, and can be modified based on the examiner's experience, and on the available equipment or materials. Other gene regions besides those mentioned in this chapter can be used if appropriate.

Apparatuses

(1) DNA sequencer

Various types of sequencers used a gel board or capillary can be used.

(2) DNA amplifier

To amplify target DNA and label amplified (PCR) products with sequencing reagents.

Procedures

The following procedures are described as an example.

1. Preparation of template DNA

It is important to use a pure cultivated bacterium or fungus for identification. In the case of colony samples, colonies are picked up with a sterilized toothpick (in the case of fungi, a small fragment of colony sample is picked up), and suspended in 0.3 mL of DNA releasing solution in a 1.5 mL centrifuge tube. In the case of culture fluid, a 0.5 mL portion of fluid is put in a 1.5 mL centrifuge tube and centrifuged at 10,000 rpm for 10 min. After removal of the supernatant, the pellet is suspended in 0.3 mL of DNA releasing solution, and then heated at 100°C for 10 min. In general, PCR can be run for bacteria and yeasts heated in DNA releasing solution. For fungi, DNA extraction after treatment with a mixer or ultrasonic generator may be necessary before PCR.

2. PCR

Add 2 μL of template DNA in PCR reaction solution. Use 10F/800R primers for bacteria and ITS1F/ITS1R primers for fungi, and then perform 30 amplification cycles at 94°C for 30 sec, 55°C for 60 sec, and 72°C for 60 sec. DNA fragments are amplified about 800 bp in the case of bacteria and about 150 – 470 bp depending on the strain in the case of fungi. Include a negative control (water instead of the test solution) in the PCR.

3. Confirmation of PCR products

Mix 5 μL of PCR product with 1 μL of loading buffer solution, place it in a 1.5 w/v% agarose gel well, and carry out electrophoresis with TAE buffer solution (1-fold concentration). Carry out the electrophoresis together with appropriate DNA size markers. After the electrophoresis, observe PCR products on a trans-illuminator (312 nm) and confirm the presence of a single band of the targeted size. If multiple bands are observed, cut the targeted band out of the gel, and extract DNA by using appropriate commercial DNA extraction kit.

4. Purification of PCR products

Remove unincorporated PCR primers and deoxynucleoside triphosphates (dNTP) from PCR products by using appropriate purification methods.

5. Quantification of purified DNA

When purified DNA is measured by spectrophotometer, calculate 1 $OD_{260\ nm}$ as 50 μg/mL.

6. Labeling of PCR products with sequencing reagents

Use an appropriate fluorescence-labeled sequencing reagent suitable for the available DNA sequencer or its program and label the PCR products according to the instructions provided with the reagent.

7. Purification of sequencing reagent-labeled PCR products

Transfer the product in 75 μL of diluted ethanol (7 in 10) into a 1.5 mL centrifuge tube, keep in an ice bath for 20 min, and centrifuge at 15,000 rpm for 20 min. After removal of supernatant, add 250 μL of diluted ethanol (7 in 10) to the precipitate and centrifuge at 15,000 rpm for 5 min. Remove the supernatant and dry the precipitate.

8. DNA homology analysis

Place sequencing reagent-labeled PCR products in the DNA sequencer and read the nucleotide sequences of the PCR products. Compare the partial nucleotide sequence with those in the BLAST database.

Judgment

If sequencing data show over 90% identity with a sequence in the database, in general, judgment may be made as follows.

1. In the case of bacteria, compare about 300 nucleotides between positions 50 to 350 in the product obtained with the 10F primer, with the BLAST database. Higher ranked species are judged as identified species or closely related species.
2. In the case of fungi, compare sequencing data for the product obtained with the ITS1F primer, with the BLAST database. Higher ranked species are judged as identified species or closely related species.

Reagents, Test Solutions

(1) 0.5 mol/L Disodium dihydrogen ethylenediamine tetraacetate TS

Dissolve 18.6 g of disodium dihydrogen ethylenediamine tetraacetate dihydrate in water to make 100 mL.

(2) 1 mol/L Tris buffer solution, pH 8.0

Dissolve 24.2 g of 2-amino-2-hydroxymethyl-1,3-propanediol in a suitable amount of water, adjust the pH to 8.0 with 0.2 mol/L hydrochloric acid TS, and add water to make 200 mL.

(3) TE buffer solution

Mix 1.0 mL of 1 mol/L tris buffer solution, pH 8.0 and 0.2 mL of 0.5 mol/L disodium dihydrogen ethylenediamine tetraacetate TS, and add water to make 100 mL.

(4) DNA releasing solution

Divide TE buffer solution containing Triton X-100 (1 in 100) into small amounts and store frozen until use.

(5) PCR reaction solution

10-fold buffer solution*	5 μL
dNTP mixture**	4 μL
10 μmol/L Sense primer	1 μL
10 μmol/L Anti-sense primer	1 μL
Heat-resistant DNA polymerase (1 U/μL)	1 μL
Water	36 μL

* Being composed of 100 mmol/L 2-amino-2-hydroxymethyl-1,3-propanediol hydrochloride, pH 8.4, 500 mmol/L potassium chloride, 20 mmol/L magnesium chloride and 0.1 g/L gelatin.

** A solution containing 2.5 mmol/L each of dGTP (sodium 2′-deoxyguanosine 5′-triphosphate), dATP (sodium 2′-deoxyadenosine 5′-triphosphate), dCTP (sodium 2′-deoxycytidine 5′-triphosphate) and dTTP (sodium 2′-deoxythymidine 5′-triphosphate). Adequate products containing these components as described above may be used.

(6) Sequencing reagent

There are many kinds of sequencing methods, such as the dye-primer method for labeling of primer, the dye-terminator method for labeling of dNTP terminator and so on. Use an appropriate sequencing reagent kit for the apparatus and program to be used.

(7) 50-Fold concentrated TAE buffer solution

Dissolve 242 g of 2-amino-2-hydroxymethyl-1,3-propanediol in 57.1 mL of acetic acid (100) and 100 mL of 0.5 mol/L disodium dihydrogen ethylenediamine tetraacetate TS, and add water to make 1000 mL.

(8) 1-Fold concentrated TAE buffer solution

Diluted 50-fold concentrated TAE buffer solution (1 in 50) prepared before use is referred to as 1-fold concentrated TAE buffer solution.

(9) Agarose gel

Mix 1.5 g of agarose, 2.0 mL of 50-fold concentrated TAE buffer solution, 10 μL of a solution of ethidium bromide (3,8-diamino-5-ethyl-6-phenylphenanthridinium bromide) (1 in 100) and 100 mL of water. After dissolving the materials by heating, cool the solution to about 60°C, and prepare gels.

(10) Loading buffer solution (6-fold concentrated)

Dissolve 0.25 g of bromophenol blue, 0.25 g of xylene cyanol FF and 1.63 g of disodium dihydrogen ethylenediamine tetraacetate dihydrate in 50 mL of water, and add 30 mL of glycerol and water to make 100 mL.

(11) PCR primers

For	Primer	
Bacteria	10F	5′-GTTTGATCCTGGCTCA-3′
	800R	5′-TACCAGGGTATCTAATCC-3′
Fungi	ITS1F	5′-GTAACAAGGT(T/C)TCCGT-3′
	ITS1R	5′-CGTTCTTCATCGATG-3′

Add the following:

25. Solid and Particle Densities

Density of a solid or a powder as a state of aggregation has different definitions depending on the way of including of the interparticulate and intraparticulate voids that exist between the particles or inside the powder. Different figures are obtained in each case, and there are different practical meanings. Generally, there are three levels of definitions of the solid or powder density.

(1) Crystal density: It is assumed that the system is homogeneous with no intraparticulate void. Crystal density is also called true density.

(2) Particle density: The sealed pores or the experimentally non-accessible open pores is also included as a part of the volumes of the solid or the powder.

(3) Bulk density: The interparticulate void formed in the powder bed is also included as a part of the volumes of the solid or the powder. Bulk density is also called apparent density. Generally, the powder densities at loose packing and at tapping are defined as the bulk density and the tapped density, respectively.

Generally, the densities of liquid or gas are affected only by temperature and pressure, but the solid or powder density is affected by the state of aggregation of the molecules or the particles. Therefore, the solid or powder densities naturally vary depending on crystal structure or crystallinity of the substance concerned, and also varies depending on the method of preparation or handling if the sample is amorphous form or partially amorphous. Consequently, even in a case that two solids or powders are chemically identical, it may be possible that the different figures of density are obtained if their crystal structures are different. As the solid or powder particle densities are important physical properties for the powdered pharmaceutical drugs or the powdered raw materials of drugs, Japanese Pharmacopoeia XIV specifies each density determination as ''Powder Particle Density Determination'' for the particle density and as ''Determination of Bulk and Tapped Densities'' for the bulk density.

The solid or powder densities are expressed in mass per unit volume (kg/m^3), and generally expressed in g/cm^3 ($1\ g/cm^3 = 1000\ kg/m^3$).

Crystal Density

The crystal density of a substance is the average mass per unit volume, exclusive of all voids that are not a fundamental part of the molecular packing arrangement. It is an intrinsic property concerning the specific crystal structure of the substance, and is not affected by the method of determination. The crystal density can be determined either by calculation or by simple measurement.

A. The calculated crystal density is obtained using:

1) For example, the crystallographic data (volume and composition of the unit cell) obtained by indexing the perfect crystal X-ray diffraction data from single crystal or the powder X-ray diffraction data.

2) Molecular mass of the substance.

B. The measured crystal density is obtained as the mass to volume ratio after measuring the single crystal mass and volume.

Particle Density

The particle density takes account both the crystal density and the intraparticulate porosity (sealed and/or experimentally non-accessible open pores) as a part of the particle volume. The particle density depends on the value of the volume determined, and the volume in turn depends on the method of measurement. Particle density can be determined either by gas displacement pycnometry or mercury porosimetry, but Japanese Pharmacopoeia XIV specifies the pycnometry as the ''Powder Particle Density Determination''.

A. The pycnometric density is obtained by assuming that the volume of the gas displaced, which is measured with the gas displacement pycnometer, is equivalent to that of a known mass of the powder. In pycnometric density measurements, any volume with the open pores accessible to the gas is not included as a part of volume of the powder, but the sealed pores or pores inaccessible to the gas is included as a part of the volume of the powder. Due to the high diffusivity of helium which can penetrate to most open pores, it is recommendable as the measurement gas of particle density. Therefore, the pycnometric particle density of a finely milled powder is generally not very different from the crystal density. Hence, the particle density by this method is the best estimate of the true density of an amorphous or partially crystalline sample, and can be widely used for manufacturing control of the processed pharmaceutical powder samples.

B. The mercury porosimetric density is also called granular density. This method also includes the sealed pores as a part of the volumes of the solid or the powder, but excludes the volume only from the open pores larger 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 pressure, the mercury does not penetrate the finenest pores accessible to helium. Since this method is capable of measuring the density which corresponds to the pore size limit at each mercury intrusion pressure, the various granular densities can be obtained from one sample.

Bulk Density and Tapped Density

The bulk density of a powder includes the contribution of interparticulate void volume as a part of the volume of the powder. Therefore, the bulk density depends on both the powder particle density and the space arrangement of particles in the power bed. Further, since the slightest disturbance of the bed may result in variation of the space arrangement, it is often very difficult to determine the bulk density with good reproducibility. Therefore, it is essential to specify how the determination was made upon reporting the bulk density.

Japanese Pharmacopoeia XIV specifies ''Determination of Bulk and Tapped Densities''.

A. The bulk density is determined by measuring the apparent volume of a known mass of powder sample that has been passed through a screen in a graduated cylinder (constant mass method). Separately, Japanese Pharmacopoeia XIV specifies the method of determining bulk density by measuring the mass of powder in a vessel having a known volume (constant volume method).

B. The tapped density is obtained by mechanically tapping a measuring cylinder containing a powder sample. After determining the initial bulk volume, carry out tapping under a fixed measurement condition (tapping rate and drop height), and the measurement is carried out repeatedly until the bulk volume variation obtained at consecutive two measurements is within an acceptable range (constant mass method). Separately, Japanese Pharmacopoeia XIV specifies the method of determining the tapped density by measuring the mass of a fixed volume of the tapped powder (constant volume method).

Add the following:

26. Total Protein Assay

The following procedures are provided as illustrations of the determination of total protein content in pharmacopoeial preparations. Other techniques, such as HPLC, are also acceptable if total protein recovery is demonstrated. Many of the total protein assay methods described below can be performed successfully using kits from commercial sources.

Note: Where water is required, use distilled water.

Method 1

Protein in solution absorbs UV light at a wavelength of 280 nm, due to the presence of aromatic amino acids, mainly tyrosine and tryptophan. This property is the basis of this method. Protein determination at 280 nm is mainly a function of the tyrosine and tryptophan content of the protein. If the buffer used to dissolve the protein has a high absorbance relative to that of water, there is an interfering substance in the buffer. This interference can be compensated for when the spectrophotometer is adjusted to zero buffer absorbance. If the interference results in a large absorbance that challenges the limit of sensitivity of the spectrophotometer, the results may be compromised. Furthermore, at low concentrations protein can be absorbed onto the cuvette, thereby reducing the content in solution. This can be prevented by preparing samples at higher concentrations or by using a nonionic detergent in the preparation.

Note: Keep the Test Solution, the Standard Solution, and the buffer at the same temperature during testing.

Standard Solution Unless otherwise specified in the individual monograph, prepare a solution of the reference standard or reference material for the protein under test in

the same buffer and at the same concentration as the Test Solution.

Test Solution Dissolve a suitable quantity of the protein under test in the appropriate buffer to obtain a solution having a concentration of 0.2 to 2 mg per mL.

Procedure Concomitantly determine the absorbances of the Standard Solution and the Test Solution in quartz cells at a wavelength of 280 nm, with a suitable spectrophotometer, using the buffer as the blank. To obtain accurate results, the response should be linear in the range of protein concentrations to be assayed.

Light-Scattering The accuracy of the UV spectroscopic determination of protein can be decreased by the scattering of light by the test specimen. If the proteins in solution exist as particles comparable in size to the wavelength of the measuring light (250 to 300 nm), scattering of the light beam results in an apparent increase in absorbance of the test specimen. To calculate the absorbance at 280 nm due to light-scattering, determine the absorbances of the Test Solution at wavelengths of 320, 325, 330, 335, 340, 345, and 350 nm. Using the linear regression method, plot the log of the observed absorbance versus the log of the wavelength, and determine the standard curve best fitting the plotted points. From the graph so obtained, extrapolate the absorbance value due to light-scattering at 280 nm. Subtract the absorbance from light-scattering from the total absorbance at 280 nm to obtain the absorbance value of the protein in solution. Filtration with a filter having a 0.2-μm porosity or clarification by centrifugation may be performed to reduce the effect of light-scattering, especially if the solution is noticeably turbid.

Calculations Calculate the concentration, C_U, of protein in the test specimen by the formula:

$$C_U = C_S\ (A_U/A_S),$$

in which C_S is the concentration of the Standard Solution; and A_U and A_S are the corrected absorbances of the Test Solution and the Standard Solution, respectively.

Method 2

This method, commonly referred to as the Lowry assay, is based on the reduction by protein of the phosphomolybdictungstic mixed acid chromogen in the Folin-Ciocalteu's phenol reagent, resulting in an absorbance maximum at 750 nm. The Folin-Ciocalteu's phenol reagent (Folin's TS) reacts primarily with tyrosine residues in the protein, which can lead to variation in the response of the assay to different proteins. Because the method is sensitive to interfering substances, a procedure for precipitation of the protein from the test specimen may be used. Where separation of interfering substances from the protein in the test specimen is necessary, proceed as directed below for Interfering Substances prior to preparation of the Test Solution. The effect of interfering substances can be minimized by dilution provided the concentration of the protein under test remains sufficient for accurate measurement. Variations of the Lowry test that are indicated in national regulatory documents[1)] can be substituted for the method described below.

Standard Solutions Unless otherwise specified in the individual monograph, dissolve the reference standard or reference material for the protein under test in the buffer used to prepare the Test Solution. Dilute portions of this solution with the same buffer to obtain not fewer than five Standard Solutions having concentrations between 5 and 100 μg of protein per mL, the concentrations being evenly spaced.

Test Solution Dissolve a suitable quantity of the protein under test in the appropriate buffer to obtain a solution having a concentration within the range of the concentrations of the Standard Solutions. An appropriate buffer will produce a pH in the range of 10 to 10.5.

Blank Use the buffer used for the Test Solution and the Standard Solutions.

Reagents and Solutions—

Copper Sulfate Reagent Dissolve 100 mg of copper (II) sulfate pentahydrate and 200 mg of sodium tartrate dihydrate in water, dilute with water to 50 mL, and mix. Dissolve 10 g of anhydrous sodium carbonate in water to a final volume of 50 mL, and mix. Slowly pour the sodium carbonate solution into the copper sulfate solution with mixing. Prepare this solution fresh daily.

5% SDS TS Dissolve 5 g of sodium dodecyl sulfate in water, and dilute with water to 100 mL.

Alkaline Copper Reagent Prepare a mixture of 5% SDS TS, Copper Sulfate Reagent, and Sodium Hydroxide Solution (4 in 125) (2:1:1). This reagent may be stored at room temperature for up to 2 weeks.

Diluted Folin's TS Mix 10 mL of Folin's TS with 50 mL of water. Store in an amber bottle, at room temperature.

Procedure To 1 mL of each Standard Solution, the Test Solution, and the Blank, add 1 mL of Alkaline Copper Reagent, and mix. Allow to stand at room temperature for 10 minutes. Add 0.5 mL of the Diluted Folin's TS to each solution, and mix each tube immediately after the addition, and allow to stand at room temperature for 30 minutes. Determine the absorbances of the solutions from the Standard Solutions and the Test Solution at the wavelength of maximum absorbance at 750 nm, with a suitable spectrophotometer, using the solution from the Blank to set the instrument to zero.

Calculations [Note: The relationship of absorbance to protein concentration is nonlinear; however, if the standard curve concentration range is sufficiently small, it will approach linearity.] Using the linear regression method, plot the absorbances of the solutions from the Standard Solutions versus the protein concentrations, and determine the standard curve best fitting the plotted points. From the standard curve so obtained 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 specimen to

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1) Example: the Minimum Requirements for Biological Products and individual monograph of JP

remove interfering substances by precipitation of proteins before testing. This technique also can be used to concentrate proteins from a dilute solution.

Sodium Deoxycholate Reagent Prepare a solution of sodium deoxycholate in water having a concentration of 150 mg in 100 mL.

Trichloroacetic Acid Reagent Prepare a solution of trichloroacetic acid in water having a concentration of 72 g in 100 mL.

Procedure Add 0.1 mL of Sodium Deoxycholate Reagent to 1 mL of a solution of the protein under test. Mix on a vortex mixer, and allow to stand at room temperature for 10 minutes. Add 0.1 mL of Trichloroacetic Acid Reagent, and mix on a vortex mixer. Centrifuge at 3000 × g for 30 minutes, decant the liquid, and remove any residual liquid with a pipet. Redissolve the protein pellet in 1 mL of Alkaline Copper Reagent. Proceed as directed for Test Solution. [Note: Color development reaches a maximum in 20 to 30 minutes during incubation at room temperature, after which there is a gradual loss of color. Most interfering substances cause a lower color yield; however, some detergents cause a slight increase in color. A high salt concentration may cause a precipitate to form. Because different protein species may give different color response intensities, the standard protein and test protein should be the same.]

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 brilliant blue G-250 dye binds to protein. The Coomassie Brilliant Blue G-250 dye binds most readily to arginyl and lysyl residues in the protein, which can lead to variation in the response of the assay to different proteins.

Standard Solutions Unless otherwise specified in the individual monograph, dissolve the reference standard or the reference material for the protein under test in the buffer used to prepare the Test Solution. Dilute portions of this solution with the same buffer to obtain not fewer than five Standard Solutions having concentrations between 100 μg and 1 mg of protein per mL, the concentrations being evenly spaced.

Test Solution Dissolve a suitable quantity of the protein under test in the appropriate buffer to obtain a solution having a concentration within the range of the concentrations of the Standard Solutions.

Blank Use the buffer used to prepare the Test Solution and the Standard Solutions.

Coomassie Reagent Dissolve 100 mg of brilliant blue G-250[2] in 50 mL of ethanol (95). [Note: Not all dyes have the same brilliant blue G content, and different products may give different results.] Add 100 mL of phosphoric acid, dilute with water to 1000 mL, and mix. Filter the solution through filter paper (Whatman No.1 or equivalent), and store the filtered reagent in an amber bottle at room temperature. [Note: Slow precipitation of the dye will occur during storage of the reagent. Filter the reagent before use.]

Procedure Add 5 mL of the Coomassie Reagent to 100 μL of each Standard Solution, the Test Solution, and the Blank, and mix by inversion. Avoid foaming, which will lead to poor reproducibility. Determine the absorbances of the solutions from the Standard Solutions and the Test Solution at 595 nm, with a suitable spectrophotometer, using the Blank to set the instrument to zero.

[Note: Do not use quartz (silica) spectrophotometer cells: the dye binds to this material. Because different protein species may give different color response intensities, the standard protein and test protein should be the same.] There are relatively few interfering substances, but detergents and ampholytes in the test specimen should be avoided. Highly alkaline specimens may interfere with the acidic reagent.

Calculations [Note: The relationship of absorbance to protein concentration is nonlinear; however, if the standard curve concentration range is sufficiently small, it will approach linearity.] Using the linear regression method, plot the absorbances of the solutions from the Standard Solutions versus the protein concentrations, and determine the standard curve best fitting the plotted points. From the standard curve so obtained 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^{+}) ion by protein. The bicinchoninic acid reagent is used to detect the cuprous ion. The method has few interfering substances. When interfering substances are present, their effect may be minimized by dilution, provided that the concentration of the protein under test remains sufficient for accurate measurement.

Standard Solutions Unless otherwise specified in the individual monograph, dissolve the reference standard or the reference material for the protein under test in the buffer used to prepare the Test Solution. Dilute portions of this solution with the same buffer to obtain not fewer than five Standard Solutions having concentrations between 10 and 1200 μg of protein per mL, the concentrations being evenly spaced.

Test Solution Dissolve a suitable quantity of the protein under test in the appropriate buffer to obtain a solution having a concentration within the range of the concentrations of the Standard Solutions.

Blank Use the buffer used to prepare the Test Solution and the Standard Solutions.

Reagents and Solutions—

BCA Reagent Dissolve about 10 g of bicinchoninic acid, 20 g of sodium carbonate monohydrate, 1.6 g of sodium tartrate dihydrate, 4 g of sodium hydroxide, and 9.5 g of sodium hydrogen carbonate in water. Adjust, if necessary, with sodium hydroxide or sodium hydrogen carbonate to a pH of 11.25. Dilute with water to 1000 mL, and mix.

Copper Sulfate Reagent Dissolve about 2 g of copper (II) sulfate pentahydrate in water to a final volume of 50 mL.

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[2] Dye purity is important in the reagent preparation.

Copper-BCA Reagent Mix 1 mL of Copper Sulfate Reagent and 50 mL of BCA Reagent.

Procedure Mix 0.1 mL of each Standard Solution, the Test Solution, and the Blank with 2 mL of the Copper-BCA Reagent. Incubate the solutions at 37°C for 30 minutes, note the time, and allow to come to room temperature. Within 60 minutes following the incubation time, determine the absorbances of the solutions from the Standard Solutions and the Test Solution in quartz cells at 562 nm, with a suitable spectrophotometer, using the Blank to set the instrument to zero. After the solutions are cooled to room temperature, the color intensity continues to increase gradually. If substances that will cause interference in the test are present, proceed as directed for Interfering Substances under Method 2. Because different protein species may give different color response intensities, the standard protein and test protein should be the same.

Calculations [Note: The relationship of absorbance to protein concentration is nonlinear; however, if the standard curve concentration range is sufficiently small, it will approach linearity.] Using the linear regression method, plot the absorbances of the solutions from the Standard Solutions versus the protein concentrations, and determine the standard curve best fitting the plotted points. From the standard curve so obtained 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 an alkaline solution and the resultant development of absorbance at 545 nm.

Standard Solutions Unless otherwise specified in the individual monograph, prepare a solution of Albumin Human for which the protein content has been previously determined by nitrogen analysis (using the nitrogen-to-protein conversion factor of 6.25) or of the reference standard or reference material for the protein under test in sodium chloride solution (9 in 1000). Dilute portions of this solution with sodium chloride solution (9 in 1000) to obtain not fewer than three Standard Solutions having concentrations between 0.5 and 10 mg per mL, the concentrations being evenly spaced. [Note: Low responses may be observed if the sample under test has significantly different level of proline than that of Albumin Human. A different standard protein may be employed in such cases.]

Test Solution Prepare a solution of the test protein in sodium chloride solution (9 in 1000) having a concentration within the range of the concentrations of the Standard Solutions.

Blank Use sodium chloride solution (9 in 1000).

Biuret Reagent Dissolve about 3.46 g of copper (II) sulfate pentahydrate in 10 mL of water, with heating if necessary, and allow to cool (Solution A). Dissolve about 34.6 g of sodium citrate dihydrate and 20.0 g of anhydrous sodium carbonate in 80 mL of water, with heating if necessary, and allow to cool (Solution B). Mix Solutions A and B, and dilute with water to 200 mL. This Biuret Reagent is stable at room temperature for 6 months. Do not use the reagent if it develops turbidity or contains any precipitate.

Procedure To one volume of the Standard Solutions and a solution of the Test Solution add an equal volume of sodium hydroxide solution (6 in 100), and mix. Immediately add a volume of Biuret Reagent equivalent to 0.4 volume of the Test Solution, and mix. Allow to stand at a temperature between 15°C and 25°C for not less than 15 minutes. Within 90 minutes after the addition of the Biuret Reagent, determine the absorbances of the Standard Solutions and the solution from the Test Solution at the wavelength of maximum absorbance at 545 nm, with a suitable spectrophotometer, using the Blank to set the instrument to zero. [Note: Any solution that develops turbidity or a precipitate is not acceptable for calculation of protein concentration.]

Calculations Using the least-squares linear regression method, plot the absorbances of the Standard Solutions versus the protein concentrations, and determine the standard curve best fitting the plotted points, and calculate the correlation coefficient for the line. [Note: Within the given range of the standards, the relationship of absorbance to protein concentration is approximately linear.] A suitable system is one that yields a line having a correlation coefficient of not less than 0.99. From the standard curve and the absorbance of the Test Solution, determine the concentration of protein in the test specimen, making any necessary correction.

Interfering Substances To minimize the effect of interfering substances, the protein can be precipitated from the initial test specimen as follows. Add 0.1 volume of 50% trichloroacetic acid to 1 volume of a solution of the test specimen, withdraw the supernatant layer, and dissolve the precipitate in a small volume of 0.5 mol/L sodium hydroxide TS. Use the solution so obtained to prepare the Test Solution.

Comments 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 minimize the effects of interfering substances also can be used to determine the protein content in test specimens at concentrations below 500 μg per mL.

Method 6

This fluorometric method is based on the derivatization of the protein with o-phthalaldehyde (OPA), which reacts with the primary amines of the protein (i.e., NH_2-terminal amino acid and the ε-amino group of the lysine residues). The sensitivity of the test can be increased by hydrolyzing the protein before testing. Hydrolysis makes the α-amino group of the constituent amino acids of the protein available for reaction with the OPA reagent. The method requires very small quantities of the protein.

Primary amines, such as tris(hydroxymethyl)aminomethane and amino acid buffers, react with OPA and must be avoided or removed. Ammonia at high concentrations will react with OPA as well. The fluorescence obtained when amine reacts with OPA can be unstable. The use of automated procedures to standardize this procedure may improve the accuracy and precision of the test.

Standard Solutions Unless otherwise specified in the individual monograph, dissolve the reference standard or the reference material for the protein under test in the buffer used to prepare the Test Solution. Dilute portions of this solution with the same buffer to obtain not fewer than five Standard Solutions having concentrations between 10 and 200 μg of protein per mL, the concentrations being evenly spaced.

Test Solution Dissolve a suitable quantity of the protein under test in the appropriate buffer to obtain a solution having a concentration within the range of the concentrations of the Standard Solutions.

Blank Use the buffer used to prepare the Test Solution and the Standard Solutions.

Reagents and Solutions—

Borate Buffer Dissolve about 61.83 g of boric acid in water, and adjust with potassium hydroxide to a pH of 10.4. Dilute with water to 1000 mL, and mix.

Stock OPA Reagent Dissolve about 120 mg of *o*-phthalaldehyde in 1.5 mL of methanol, add 100 mL of Borate Buffer, and mix. Add 0.6 mL of polyoxyethylene (23) lauryl ether, and mix. This solution is stable at room temperature for at least 3 weeks.

OPA Reagent To 5 mL of Stock OPA Reagent add 15 μL of 2-mercaptoethanol. Prepare at least 30 minutes prior to use. This reagent is stable for one day.

Procedure Adjust each of the Standard Solutions and the Test Solution to a pH between 8.0 and 10.5. Mix 10 μL of the Test Solution and each of the Standard Solutions with 100 μL of OPA Reagent, and allow to stand at room temperature for 15 minutes. Add 3 mL of 0.5 mol/L sodium hydroxide TS, and mix. Using a suitable fluorometer, determine the fluorescent intensities of solutions from the Standard Solutions and the Test Solution at an excitation wavelength of 340 nm and an emission wavelength between 440 and 455 nm. [Note: The fluorescence of an individual specimen is read only once because irradiation decreases the fluorescent intensity.]

Calculations The relationship of fluorescence to protein concentration is linear. Using the linear regression method, plot the fluorescent intensities of the solutions from the Standard Solutions versus the protein concentrations, and determine the standard curve best fitting the plotted points. From the standard curve so obtained and the fluorescent intensity of the Test Solution, determine the concentration of protein in the test specimen.

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 protein can affect the determination of protein by this method. Nitrogen analysis techniques destroy the protein under test but are not limited to protein presentation in an aqueous environment.

Procedure A Determine the nitrogen content of the protein under test as directed elsewhere in the Pharmacopoeia. Commercial instrumentation is available for the 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 test protein. Some instruments convert the nitric oxides to nitrogen gas, which is quantified with 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 standard or reference material that is relatively pure and is similar in composition to the test proteins is used to optimize 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 the nitrogen content of the appropriate reference standard or reference material.

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